Edited by
Ashish S. Verma
Anchal Singh

Animal Biotechnology

Second Edition

Models in Discovery and Translation

ANIMAL BIOTECHNOLOGY

ANIMAL BIOTECHNOLOGY

Models in Discovery and Translation

SECOND EDITION

Edited by

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Kindest Mentor and Friend

Professor Ashish Swarup Verma
(1964 – 2019)

Thank you today
tomorrow and always...

Anchal Singh

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Preface

In the year 2016 Professor Ashish S. Verma received a call from Academic press office with a request to come up with the second edition of our book *Animal Biotechnology: Models in Discovery and Translation*. Soon he shared this information with me and both of us were thrilled and super excited as it had been just 3 years since the first edition was launched and we had never thought that we would be working on the second edition so soon. The challenge was to add, update, and revise the first edition because by that time both of us had moved to different places, I had joined Banaras Hindu University, Varanasi, India, and Prof. Verma became the acting Vice-Chancellor of Jadavpur University, Kolkata, India. Even though his unending administrative responsibilities kept him busy day and night still he had solutions to every problem that I could fathom in editing the next edition at that time. Ultimately we started working together on this edition by reviewing all the inputs and criticism we had received for the first edition, from students, instructors, teachers, and scientists.

We always felt that the field of Animal Biotechnology as such is well explored and is continuously evolving to come up as a full discipline very soon. Nevertheless, it is still difficult to find dedicated books on the subject, and at the same time books that can cater the needs of students, instructors, and teachers alike. The first edition of *Animal Biotechnology: Models in Discovery and Translation* was developed as a Resource Book as it would provide sufficient information and literature for instructors to teach the subjects while students will find ample information to gain a better insight of the topic. While working on the second edition, we have maintained the same pattern as before, in fact, the second edition includes substantial revision and update of the content and information while continuing with the easy-to-follow language and uniformity of the style and presentations which the first edition had. Considering the recent advancements in nanotechnology, enzyme technology, and ayurveda we strongly felt to have chapters that will help in having a view of Animal Biotechnology from these perspectives. As a result the second edition has entirely new chapters comprising of various aspects of nanotechnology, ayurveda, enzyme technology, euthanasia, CAM assays, and also some chapters dealing with newer models to study different human diseases.

Almost all the chapters from the first edition have been retained but they have been rigorously updated with the most recent information available with the experts of the field. Moreover each chapter contains interesting information that is added as a Turning point, it's something which we hope that our readers will cherish while reading the topic of their interest. We have also added a sub-topic on Clinical Co-relation in each chapter so that readers who have an interest in the clinical aspect of different topics can find the relevant information in one place while developing the subject. Many sections have been rewritten for better clarity, understanding, fluency of style/presentation, and language. We have tried to develop each chapter in an individual manner so that readers who prefer to purchase individual chapters can find all the details related to the topic in the same chapter itself. We have tried our best for improvement of the figures and illustrations, some of these have been revised, few new figures have been added in each chapter and the figures from the last edition have been updated. Very similar to the first edition, each chapter has a subsection on the available internet resources that are related to the topic and a list of review articles and textbooks as further reading is also provided for those readers whose jet-speed curiosities need still more jet fuel.

While we were working on the second edition, Prof. Verma was taken away by the cruel hands of destiny on May 11, 2019, and his sudden, untimely demise left me completely shocked. Me and Prof. Verma had been working together since 2008 and I was devastated as this entire project was mid-way and from then on I would be editing the book single-handedly. At this point I must appreciate the kind gesture of the entire Academic Press team especially Mr. Peter Linsley (Senior Acquisitions Editor) and Mr. Timothy Bennett (Editorial Project Manager) who gave me support and showed trust in my capabilities. They generously extended the book timelines due to which this book could be completed in its present form.

Finally, I would like to request the readers of this book to extend their full support as they had extended for the previous edition. We are always open to criticism, suggestions, and recommendations that can be helpful to improve the contents and presentation of the book. Your suggestions/criticism will allow us to explore other aspects of Animal Biotechnology and in our future ventures and endeavors.

Late Ashish S. Verma
and
Anchal Singh

Acknowledgments

As Editors, we would like to express our gratitude and thanks to all the contributing authors, whose expertise and experience is now with us in the form of book chapters in their respective fields. I understand that our contributors have worked hard to update, upgrade, and improve their chapters so that all chapters of the book could be uniform in style and presentation. This book could never have been possible without their valuable work and timely support.

Late Prof. Ashish S. Verma would have surely thanked his mother Ms. Sushma Saxena, for her great efforts to raise and groom him. His brother Mr. Saumya Swarup, sister in law Ms. Nimisha Swarup their kids Mr. Utkarsh and Ms. Shreeparna, his sisters, and their families also deserve a special thanks for their love and support.

I (Anchal) would like to thank my Ph.D. supervisor Prof. Sushma Rathaur, UGC-BSR fellow, Department of Biochemistry, Banaras Hindu University, India, who has always guided and helped me and has stood beside me as the biggest support till date. I wish to express my gratitude to my dad Mr. Kanhaiya Ji Singh, Mother Ms. Mohini Singh, brother Abhisar and his wife Meenakshi for their support, love, and help. My son Aviral and nephew Aarav were the "Stress breakers" and their frequent requests to indulge in games and activities were mostly ignored for the sake of meeting the timelines of book editing.

Anchal's research scholars namely Vipin Kumar and Ayushi Mishra greatly contributed their part for this book and are acknowledged for their help. At times they offered help and support to organize us better and at other times they didn't fail to criticize us, but whatever they have done toward this book is admired by us. We are indebted to Mr. Dinesh Kumar who has worked with us for the last 12 years and has been instrumental in providing his secretarial assistance.

Last but not least this book could never have been completed without Elsevier personnel working in this project. Mr. Peter Linsley (Senior Acquisitions Editor), Mr. Timothy Bennett (Editorial Project Manager), and Mr. Sreejith Viswanathan (Project Manager) who kept supporting, motivating, and pushing us throughout the project duration. We convey our heartfelt thanks to everyone who has contributed directly or indirectly to this book.

And all this would never have been possible without "The Almighty" GOD whom we owe our existence to. The one who gifted us (Human beings) a brain to hypothesize and analyze, courage to dream, and motivation to achieve, and hence we thank you for making our book instrumental in spreading knowledge and fueling creativity.

Late Ashish S. Verma
Anchal Singh

SECTION I

Human diseases: in vivo and in vitro models

CHAPTER

1

Drosophila: a model for biotechnologist

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Summary

Drosophila is a miniature yet versatile and manipulable model to address basic biological questions with potential implications plus applications to other metazoans. In this chapter, we emphasize the contributions of Drosophila to genetics and biotechnology and translational versatility of this model along with associated ethical issues and available resources.

Nobel love story of Drosophila

So far the outstanding contributions of fruit fly research have been recognized with six Nobel prizes in physiology or medicine

1933 Thomas Hunt Morgan—Chromosomes in heredity

1946 Hermann Joseph Muller—X-ray irradiation to increase mutation rates in fruit flies

1995 Edward B Lewis, Christiane Nüsslein-Volhard, and Eric F Wieschaus—Genetic control of embryonic development

2004 Richard Axel—Odor receptors and the organization of the olfactory system

2011 Jules A Hoffmann—Activation of innate immunity

2017 Jeffrey C Hall, Michael Rosbash and Michael W Young—Molecular mechanisms regulating circadian rhythms

What you can expect to know

Initially, this chapter facilitates learning of basic concepts of Drosophila, which evolved this organism as a model for genetics and biotechnology. The historical perspective helps the reader to assimilate the contributions of Drosophila research findings to biotechnology. The later part of the chapter acquaints the reader with a protocol to generate Drosophila transgenics and exemplifies the translational potential of the same toward understanding human diseases.

Introduction

Innovative genetic technologies have revolutionized the wisdom of science. For example, cloning and manipulation of gene sequences have helped in the generation of transgenics toward a substantial understanding of biological concepts and also for the betterment of life. However, the pivotal role played by model organisms for biotechnologists to achieve these challenges is indeed phenomenal. One such model organism that has helped biotechnologists to realize their dreams is Drosophila. With pinnacle contributions to genetics and development over 100 years, Drosophila continues to inspire the creativity of biotechnologists. Drosophila is so amenable to genetic manipulation that it is constrained only by the imagination of biotechnologists.

Drosophila is a tiny fly also known as vinegar-loving fly. The term *Drosophila*, meaning "dew-loving," is a modern scientific Latin adaptation from Greek words *drósos*, "dew," and *phílos*, "loving" with the Latin feminine suffix *-a*. It belongs to the Phylum Arthropoda, class Insecta and order Diptera and the famous family of Drosophilidae. *Drosophila* is a small fly, typically pale yellow to reddish-brown to black, with red eyes. The plumose (feathery) arista, bristling of the head and thorax, and wing venation are the characters used to diagnose the family. Most are small, about 2–4 mm long, but some, especially many of the Hawaiian species, are larger than a housefly. The genus *Drosophila* is found all around the world right from deserts to tropical rainforests to cities to alpine

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zones. Most species breed in various kinds of decaying plant and fungal materials including fruit, bark, slime fluxes, flowers, and mushrooms.

Of the various species of Drosophila, *Drosophila melanogaster* offers several advantages as a model for molecular studies. Being small, these flies are extremely simple to handle. The sexual dimorphism (males and females are different) permits easy differentiation of sexes. Further, these flies are nonpathogenic, have a shorter generation time (10–12 days), and can be cultured at a low cost in a limited space. In addition, Drosophila offers various molecular and genetic tools that a biotechnologist can dream of and the fully sequenced genome coupled with bioinformatics tools enhance the translational utility of this model.

In this chapter, we initially describe the classical aspects of Drosophila such as life cycle, cytology, and development. Subsequently, we provide a historical perspective of research hallmarks that led to the utility of Drosophila as a model for molecular studies. In addition, we discuss the translational significance of Drosophila by emphasizing Drosophila models available for human diseases. Finally, we discuss the ethical issues and concerns associated with this model.

Classical aspects of *Drosophila melanogaster*

Physical appearance

The body of Drosophila, like that of any other insect (and typical of Arthropoda), is segmented. The body plan typically consists of head, thorax, and abdomen. While multiple segments give rise to the head, three segments constitute the thorax and eight segments form the abdomen. Head consists of antennae whereas legs and wings arise from thoracic segments. *D. melanogaster* has transverse black rings across their abdomen. Males are easily distinguishable from females by the presence of a distinct black patch at the abdomen that is absent in females. Males also have sex combs, a row of dark bristles on the tarsus of the first leg, which are absent in females. Furthermore, males have a cluster of spiky hairs called claspers that surround the anus and genitals used to attach to the female during mating.

Life cycle

D. melanogaster is a popular experimental animal because it is easily cultured in mass out of the wild, has a short generation time, and mutant animals are readily obtainable. Typically, in a laboratory, *D. melanogaster* is grown on cornmeal–yeast–fruit juice mixture at 25°C. Life cycle of this organism consists of a number of stages: embryogenesis, three larval stages, a pupal stage, and the adult stage. The development period for

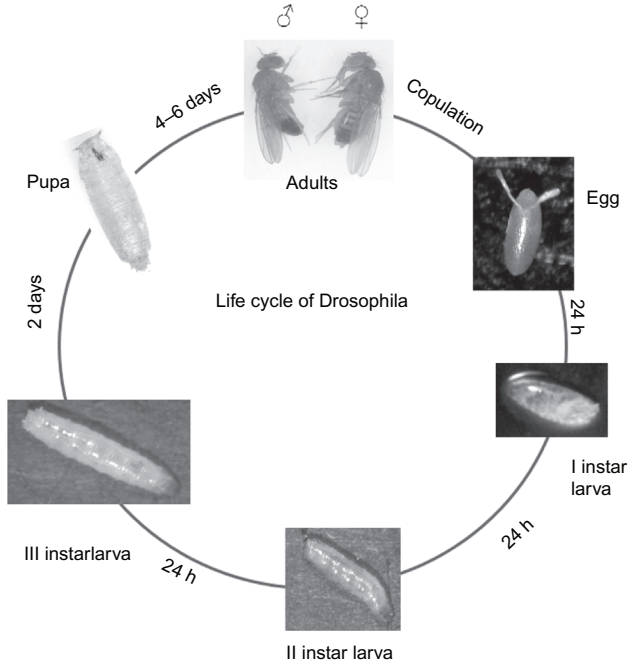


FIGURE 1.1 Life cycle stages of *Drosophila melanogaster*.

D. melanogaster varies with temperature. The time required for complete development at 25°C is 8–9 days. Females lay some 400 eggs, about five at a time on overripe fruit or other suitable materials. The eggs, which are about 0.5 mm long, hatch after 20–22 hours at 25°C. The resulting larvae grow for about 3 days while molting twice into second and third-instar larvae, at about 24 and 48 hours, respectively, after eclosion. The larva then encapsulates in the puparium that is immobile and undergoes a 4-day-long metamorphosis at 25°C. A fly finally emerges from a puparium after metamorphosis, a process referred to as eclosion. The life cycle of Drosophila is schematically depicted in Fig. 1.1.

Drosophila development

Drosophila has contributed to most of our existing knowledge on the mechanisms of the development of organisms. In Drosophila, the complex adult body plan is accomplished from the fertilized embryo through developmental processes. Development in Drosophila is holometabolous, which involves developing stages morphologically distinct from adults (Hartenstein, 1993). Early processes of development occur in the fertilized egg laid by the female to give rise to the larva. The larva subsequently gives rise to the puparium, after undergoing a series of modifications and two moltings, driven by the hormonal titers and molecular signals. During the pupal stage, many larval structures are broken down, and adult structures undergo rapid development. In this section,

we describe the key aspects of Drosophila development: embryogenesis, pattern formation, and homeotic genes.

Embryogenesis in Drosophila

The early development of Drosophila begins with the formation of oocytes through oogenesis in the ovary (Hartenstein, 1993). These oocytes are packed with maternal RNA, protein, ribosomes, and mitochondria that assist in the egg to embryo transition. Fertilization (union of these oocytes and sperm) triggers mitosis in the embryo. Several nuclear divisions without cytokinesis (division of cytoplasm) occur in the early embryo, resulting in a cell with many nuclei in the cytoplasm. At the 10th· nuclear division, these nuclei migrate toward the surface of the embryo, resulting in the formation of the syncytial blastoderm. At the 13th· nuclear division, membrane invaginations enclose the nuclei leading to cellularization and the formation of the cellular blastoderm. At the time of cellularization, the major body axes and segment boundaries are determined. After cellularization, the embryo proceeds through gastrulation: cells from the ventral surface invaginate to create the ventral furrow. Ventral furrow is critical for the formation of the mesoderm. Subsequently, at the anterior and posterior ends of the ventral furrow, the invagination of prospective endoderm occurs. Gastrulation is followed by the convergence of certain ectodermal cells on the surface with the mesoderm and their migration toward the ventral midline to form the germ band, a collection of cells that will form the trunk of the embryo. The germ band extends posterior and wraps around the dorsal surface of the embryo. At this extended state, several morphogenetic processes occur: organogenesis, segmentation, and segregation of imaginal discs that will unfold during metamorphosis to form adult fly structures such as antennae, legs, and wings (Campos-Ortega and Hartenstein, 1985; Martinez Arias, 1993). Interestingly, at all the stages of development, the general body plan remains the same. The generalized body plan consists of a segmented region sandwiched between a distinct head or anterior region and the tail or posterior region. Drosophila, with its versatile genetic tools, has led to the understanding of pattern formation and differentiation during early animal development. Three scientists, namely, Ed Lewis, Christiane Nusslein-Volhard, and Eric Wieschaus pioneered our understanding of pattern formation and differentiation. These scientists not only laid the platform for our understanding of development but also deciphered the underlying dynamics. Nusslein-Volhard and Wieschaus focused their studies on understanding early embryogenesis while Ed Lewis concentrated on late embryogenesis.

Pattern formation in Drosophila

The metamorphosis of a simple egg into an adult with the complex body plans requires three classes of genes. These classes comprise the maternal genes, the segmentation genes, and the homeotic genes. Nusslein-Volhard and Wieschaus (1980) were the first to report the key contributions of each gene that regulates a particular pattern formation event, the segmentation of embryo. They looked for recessive embryonic lethal mutations, in a systematic genetic screen encompassing the whole genome to identify genes critical for embryonic development. Subsequently, they analyzed the phenotypes of dead embryos and classified these genes according to their phenotype before death. Based on their phenotypic analyses, Nusslein-Volhard and Wieschaus (1980) identified three categories of mutations. The first category comprised mutations that result in the loss of multiple adjacent segments (called genes encoding the same as gap genes). The second category included mutations that cause missing of alternate segment size units (accordingly, named their genes as pair-rule genes). The third category of mutations triggered the loss of part of each segment and duplication of the remaining part of the segment (named as segment polarity genes). In view of their findings, Nusslein-Volhard and Wieschaus (1980) proposed that gap genes, pair-rule genes, and segment polarity genes (together called as segmentation genes) are critical for subdividing the embryo and segment formation. Another class of genes, including homeotic genes, defines the identity of the segment. However, to put these genetic cascades into motion, maternal components are essential.

Maternal components play a critical role in determining the embryonic patterning (Hartenstein, 1993). As discussed above, even before fertilization, Drosophila eggs are loaded with regulatory molecules that determine the anteroposterior axis of the egg and development of the organism. Eggs are preloaded with bicoid and nanos mRNA, and these are translated upon fertilization (Hartenstein, 1993). Of these two, bicoid is essential for the formation of the head: females carrying mutant alleles of bicoid give rise to offspring with defects in head development. Prior to cellularization, these proteins form concentration gradients in the embryo. At the anterior end, bicoid is at a higher concentration (Fig. 1.2), whereas at the posterior end, nanos are abundant. These concentration gradients are critical for regulating segmentation genes (see above), which define the segmentation pattern. Temporal and spatial activation of gene cascades is the hallmark of development. During the initial phase of development, bicoid and nanos differentially regulate the gap genes. At the anterior end, bicoid triggers the transcription of a gap gene, *hunchback*. However, at

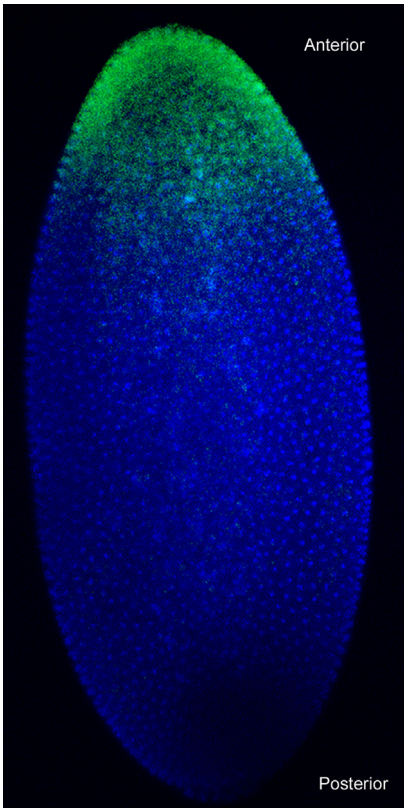


FIGURE 1.2 Localization of Bicoid in Drosophila embryo. Immunostaining of Drosophila embryos with anti-Bicoid antibody reveals the accumulation of Bicoid protein (green) in the anterior part of the embryo. Blue color represents the nuclear staining by DAPI.

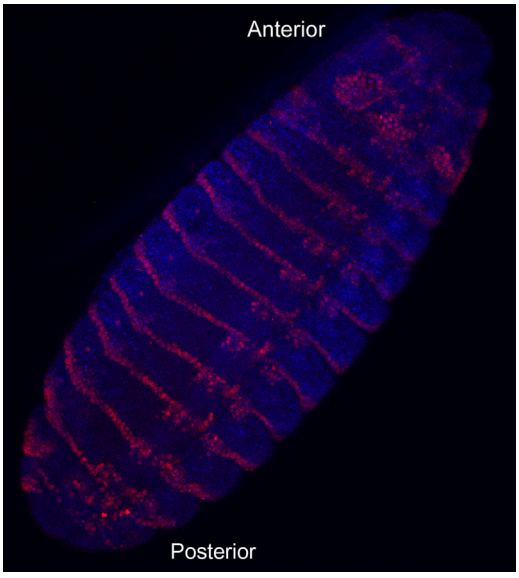


FIGURE 1.3 Localization of Engrailed in Drosophila embryo. Immunostaining of Drosophila embryos with anti-Engrailed antibody reveals Engrailed localization (red) within the posterior section of every segment within the embryo. Blue color represents the nuclear staining by DAPI.

the posterior end, nanos inhibit *hunchback* RNA from being translated, thereby forming a hunchback protein gradient in the embryo (Wreden et al., 1997). This hunchback protein triggers, in a concentration-dependent manner, the transcription of other gap genes such as *Kruppel*, etc. (Schulz and Tautz 1994; Zuo et al., 1991), which in turn defines large areas surrounding the anteroposterior axis (Gilbert et al., 2003). The gap genes encode transcription factors that regulate the expression of certain pair-rule genes, which in turn regulate other pair-rule genes. These pair-rule genes, which are expressed along the stripes of the embryo, divide the embryo into pairs of segments. Pair-rule genes encode transcription factors that regulate segment polarity genes. These segment polarity genes define the anteroposterior axis of each of the segment (Fig. 1.3). Once the pattern of segmentation is established, the segments achieve unique identities through homeotic genes.

Homeotic genes in Drosophila

The term homeosis represents the transformation of one structure of the body into the homologous structure of another body segment. In Drosophila, as mentioned earlier, in adults, structures such as legs, wings develop from thoracic segments whereas antennae appear on the head. The segment-specific development of these structures requires the action of homeotic genes. Homeotic genes are a group of genes that regulate the pattern formation. These genes, although do not specify the elements of the pattern, indeed assign identities to these elements. Mutations in these genes result in the development of the elements of the specified pattern with inappropriate identities. The best example to describe these genes is Antennapedia (Antp). As the name suggests, a dominant mutation in this gene transforms the antennal structures on the head to an additional second leg. Generally, normal Antp is required in the second thoracic segment to initiate the cascade of events that lead to the development of leg. Perhaps, this involves the regulation of several genes. Hence, the homeotic genes are considered as master controllers of developmental programming (Abbott and Kaufman, 1986).

Most of our knowledge on homeotics is due to the pioneering works of Ed Lewis on bithorax complex (BX-C) (Lewis, 1978). The homeotic genes consist of 180 nucleotide consensus sequence called the homeobox. The homeobox corresponds to a 60 amino acid domain, namely, the homeodomain, which is involved in the DNA binding. These homeobox-containing (HOX) genes are not limited to Drosophila but later

have been found and studied in many other organisms ranging from invertebrates to vertebrates, including mammals (Ruddle et al., 1994; Santini et al., 2003). HOX genes occur in clusters, and interestingly, not only these genes but also the synteny (relative gene order) within the cluster is conserved (Ruddle et al., 1994). Moreover, the order of HOX genes on the chromosome is same as the order of the segments that they affect along the anterioposterior axis. Genetic analysis revealed the existence of posterior dominance: genes acting at the anterior are regulated by their posterior neighbors. For example, BX-C comprises regions encoding three homeodomain genes called Ultrabithorax (Ubx), Abdominal-A (AbdA), and Abdominal-B (AbdB) and a noncoding RNA, iab-8-ncRNA (Gummalla et al., 2012; Lewis, 1978). In this case, iab-8-ncRNA represses AbdA (Gummalla et al., 2012) and both AbdA and AbdB repress Ubx (Lewis, 1978). In addition to the intrahomeotic regulation, these homeotic genes are also regulated by segmentation genes. For example, hunchback (gap gene) is known to limit the Ubx expression (Wu et al., 2001) and mutations in pair-rule genes influence the expression of homeotic genes. At present, however, the knowledge of how segmentation genes regulate homeotic gene expression/repression is limited. Nevertheless, as discussed so far, studies on Drosophila have tremendously contributed to the understanding of development in metazoan animals. The events associated with pattern formation in Drosophila are schematically depicted in Fig. 1.4.

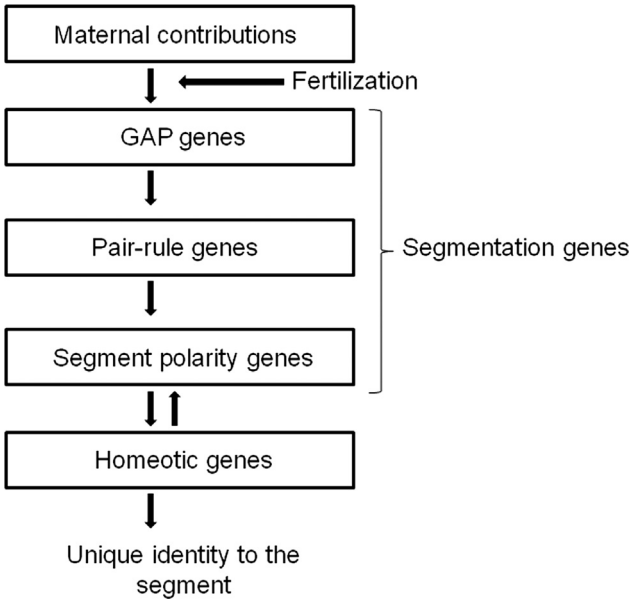


FIGURE 1.4 Schematic depiction of classes of genes associated with pattern formation in *Drosophila melanogaster*.

Drosophila genome

D. melanogaster has four pairs of chromosomes: an X/Y pair and three autosomes labeled 2, 3, and 4. The fourth chromosome is quite tiny. The size of the genome is 165 million base pairs and contains an estimated 14,000 genes (Adams et al., 2000) (by comparison, the human genome has 3400 million base pairs and may have about 25,000 genes, International human genome consortium, 2004). *Drosophila* genome contains a considerable amount of nonprotein-coding DNA sequences that are involved in the control of gene expression. The determination of sex in *Drosophila* occurs by the ratio of X chromosomes to autosomes.

History

Historical perspective of Drosophila contributions to biotechnology

The seeds for modern biotechnology research in Drosophila were sown by the 1970s. In 1974, random clones for Drosophila, the first for any organism, were generated in the D. S. Hogness laboratory at Stanford University. By early 1975, clone libraries representing the entire genome were generated and screens for clones carrying specific sequences with the newly developed method of colony hybridization were in place. In early 1979, cloning of a gene, ultrabiothorax, was achieved for the first time. By late 1980, many mutant alleles had been cloned and shown to be the consequence of chromosomal breakage or transposable element insertion. Subsequently, the availability of transposable element vectors only added to the growth of Drosophila as a model in the field of biotechnology. The use of transposable elements for generating transgenic flies has revolutionized gene manipulation in Drosophila and pioneered the development of a powerful array of techniques in Drosophila, many of which were ultimately adapted to other metazoans. These methods range from enhancer traps (1987), large-scale insertional mutagenesis (1988), site-specific recombination for generating chromosomal rearrangements (1989) to the highly popular binary systems for controlling ectopic gene expression (1993). By 1999, over 1300 genes were cloned, sequenced and functions were characterized using the loss of function phenotypes. These enormous tools were so meaningful that most researchers did not even consider the whole-genome sequencing of Drosophila. Ultimately, when the Drosophila genome was sequenced in 2000, it gave another value addition to the field of biotechnology, the whole-genome shotgun approach, for genome sequencing. Subsequently, the vast resources made available in recent years by the research community

for genome wise ectopic expression and knockdown (RNAi), both in vitro and in vivo, have enhanced the utility of the Drosophila model. Recent efforts by fly researchers have deciphered the stage as well as tissue-specific expression as well as localization of the majority of the genes in Drosophila. The fully sequenced genome coupled with these various genetic and molecular tools led to the upsurge of Drosophila as a model for basic as well as translational research.

Principle

Drosophila is a well-studied and highly tractable genetic model system to decipher the molecular mechanisms underlying various biological processes. The completion of genome sequencing and annotation discovered the high degree of conservation of fundamental biological processes between Drosophila and mammals. This has prompted the biotechnologists to utilize Drosophila to understand the molecular basis of human diseases. The ease at which Drosophila transgenics can be created was also instrumental in the success of this model for understanding human diseases. Using a plethora of molecular tools available for Drosophila, biotechnologists genetically manipulated Drosophila by either inserting the human genes in the fly genome or modifying the function of human disease orthologs in Drosophila.

Methodology

Given the focus of this chapter, here we describe only those Drosophila-based methods essential for the germ-line transformation of Drosophila to generate transgenics.

Culturing of Drosophila

D. melanogaster is reared on standard Drosophila food medium at 22°C ± 1°C. Stocks are usually maintained in vials (up to 20–30 flies/vial), and experimental cultures are maintained in bottles (as they permit the growth of flies in large numbers).

Preparation of Drosophila food medium

Materials required

Glass or plastic vials (70–90 mm height with 25–30 mm outer diameter)
Round flat bottom half-pint glass or plastic bottles
Agar-agar, maize powder, sugar, yeast, methylparaben, and propionic acid

The recipe to prepare 1 L of fly food is as follows:

Agar-agar	8 g
Maize powder	15 g
Sucrose	100 g
Bakery dry yeast	100 g
10% Benzoic acid	5 mL
Propionic acid	8 mL
Water	1000 mL

One liter of water is added to a 2-L (glass or stainless steel) beaker, and the same is to be kept on a hot plate. Sugar is added slowly to the water, the beaker is covered with a glass plate and the water is heated. In the meantime, the solid ingredients (agar-agar, maize powder, and dry yeast) should be mixed and to be added to water once it starts to boil, with constant stirring. The contents are boiled for 15–20 minutes. Subsequently, measured quantities (as above) of 10% benzoic acid and propionic acid are added with thorough stirring. The heater is turned off and food is brought to the table and can be poured (3–5 mL) into the vial depending upon the requirement. The food should be allowed to cool and solidify before plugging the vials with cotton. A couple of yeast granules should be added to these vials and need to leave them overnight. Now these food vials are ready for usage. The same is the case with bottles except that the quantity of food will be proportionally higher.

Handling of flies

Drosophila, which belongs to a class of insects, tends to fly. Therefore these flies need to be put to sleep for sexing of males and females and to setup/carry out the experiment, depending upon the requirement. Several methods are available to put flies to sleep. These include exposure of flies to ether, chilling (or cooling), CO_2, or nitrogen, the latter three being least harmful. Of these three choices, cooling is little bit messy but is the simplest without the requirement for any sophisticated equipment and needs merely ice and petri dishes. In addition, it is the only method which will not affect fly neurology. The remaining two methods require commercially available gas cylinders and controllers to provide a regulated supply of the gas to incapacitate the flies.

Fly disposal

This is a very essential step when using flies. A bottle or beaker with (new/used) oil (whose density is heavier than water, such as mineral oil), referred to

as fly morgue, is generally used. The anesthetized but unused flies should be dumped directly into the fly morgue. Generally, they drown to the bottom but need to be ensured (especially in the case of old morgue). Discarding of flies in the morgue is aimed at minimization of stock contamination and to keep the lab environment fly free. The old vials and bottles containing flies should be autoclaved to kill the flies, prior to discarding.

Egg collection

Abundant batches of eggs synchronized in age are required for experiments. In general, 200–300 adults from fresh cultures should be transferred into bottles or collection chambers containing tiny petri plates containing fly food (or grape juice-agar food). To optimize egg collections, flies are starved for 4–6 hours under light in empty bottles. Subsequently, flies are transferred to collection chambers and kept in dark. The first hour's collection should be discarded, to avoid those eggs retained by females in anticipation of fresh food. Thereafter, egg collection plates can be removed and replaced with new ones at 30-minute intervals.

Dechorination of eggs

To prepare the eggs for microinjection, the outer covering of egg, namely chorion, should be removed. For chemical dechorination, eggs are washed from the egg collection plate to a net well using egg wash buffer (0.03% Triton X-100, 0.4% NaCl) and the net well containing the eggs is placed into 2% sodium hypochlorite (bleach) for 2 minutes. Thereafter, the eggs are thoroughly washed with egg wash buffer and collected onto an agar bed or onto a petri plate. Using a fine brush, the eggs are arranged in a vertically linear fashion and transferred to a slide using a double-stick tape (Scotch 665). After optimal dehydration, the eggs are covered with a layer of halocarbon oil. Later, DNA is injected (through a fine glass needle) into these eggs under the inverted phase contrast microscope using micromanipulator.

Preparation of DNA for injection

To generate the transgenic, the gene of interest should first be cloned into a vector used for germ-line transformation. For Drosophila, P-element-based vectors (e.g., pPUAST and pPCASPER) that can integrate the gene of interest into the fly genome are quite commonly used. Depending upon the requirement, the complete gene or the coding region can be cloned into the P-element vectors. Once the gene is cloned, the plasmid DNA is extracted. The successful transformation requires DNA at an optimal concentration of 1 μg/μL and should be endotoxin-free. Thereafter, the plasmid DNA is mixed with a helper plasmid DNA at a ratio of 3:1: helper encodes for transposase required for the transposition of the transgene into the fly genome. Subsequently, the mixed DNA is precipitated using ethanol and resuspended in the injection buffer (5 mM KCl; 0.1 mM $NaPO_4$ buffer pH 7. 5).

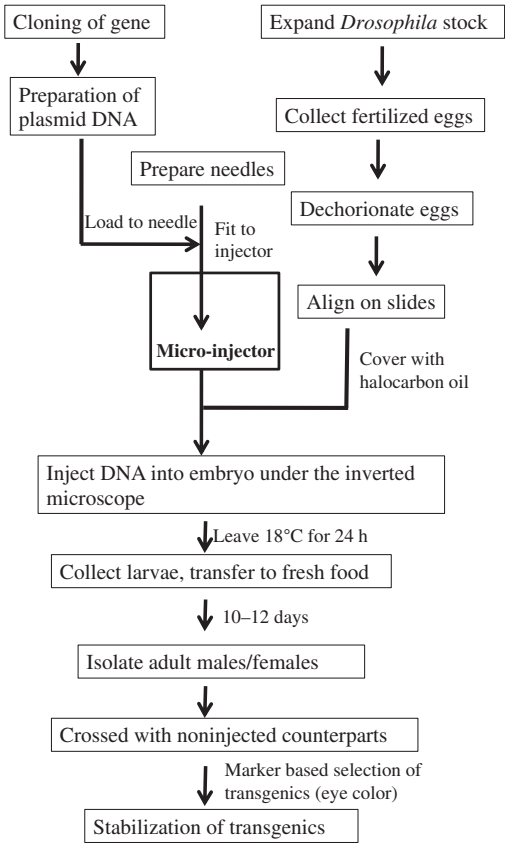
Protocols

Protocol for germ-line transformation in Drosophila

The protocol given below is adapted from those of Prof. John Belote, University of Syracuse, United States, and Prof. Heifetz, University of Jerusalem, Israel. This is schematically represented in Flow Chart 1.1.

Materials required

The required materials are as follows: plasmid purification kit; helper plasmid (generally, Δ 2–3); injection needle: glass with diameter of opening of the tip approximately 0.5 μm; fly strain: white eye of



FLOW CHART 1.1 STEPS FROM CLONING TO GERM LINE TRANSFORMATION.

D. melanogaster (w^{1118}); petri dishes, grape juice, agar, potassium chloride, sodium phosphate (monobasic and dibasic), double-stick tape (Scotch 665), tweezers (fine needle, sharp; Sigma), fine needles, halocarbon oil, permitted food color (red or green); and net wells.

Procedure

A. Preparation of DNA
- **i.** Prepare the endotoxin-free DNA of the gene clone using a plasmid maxi-prep kit. Wide varieties of kits are commercially available and follow the manufacturer's instructions to prepare the DNA.
- **ii.** In a 0.6-mL eppendorf tube, mix 15 μg of plasmid of interest with 5 μg of helper plasmid, ethanol-precipitate the mixture using 1/10 volume of 3 M NAOAC and two times volume of 100% ethanol. Leave tubes overnight at −20°C. Next morning, centrifuge the tubes at 14,000 rpm for 30 minutes, wash thrice with 70% ethanol (each for 3 minutes at 13,000 rpm), and ultimately resuspend the pellet in 50 μL injection buffer (5 mM KCl; 0.1 mM $NaPO_4$ buffer pH 7. 5). This can be stored at −20°C till further use.
- **iii.** A recent study (Sonane et al., 2013) has shown that the use of nanoparticles (NPs) to deliver DNA through microinjection can lead to the reduction of required DNA quantity by 10-folds without compromising on the efficiency of germ-line transformation.

B. Collection of fly embryos
- **i.** Use 4−6 days old adult flies from fresh cultures for embryo collection.
- **ii.** Place 200−300 w^{1118} adults in bottles or collection chambers containing tiny petri dishes with grape juice-agar medium.
- **iii.** Place the bottle in dark.
- **iv.** Discard the first hour's collection plate and place a fresh one.
- **v.** Replace egg collection plates with new ones at 30 minute intervals and use the embryos for injection.

C. Removal of chorion from embryos
- **i.** Wash the embryos from the egg collection plate to a net well using egg wash buffer (0.03% Triton X-100, 0.4% NaCl)
- **ii.** Place the net well containing the embryos in 2% sodium hypochlorite (bleach) for 2 minutes.
- **iii.** Wash the embryos thoroughly with egg wash buffer and collect onto an agar bed or onto a petri dish.

D. Preparation of embryos for injection
- **i.** Cut a small strip of double-stick tape (Scotch 665) and stick in the center of a slide.
- **ii.** Using a fine brush, arrange the embryos in a vertically linear fashion on agar bed.
- **iii.** Transfer the embryos to the slide by reversing the agar bed on to the edge of the double-stick tape. Final orientation of the embryos should be such that the posterior end of the embryo shall hang off the tape.
- **iv.** Observe the quality and developmental stage of the embryo. Discard those embryos with poor quality or aged embryos (visualization of syncytial blastoderm).
- **v.** Place the slide in a petri dish containing the desiccant for 4−5 minutes (optimal dehydration times are to be determined depending upon the relative humidity of the injection room).
- **xvi.** After optimal dehydration, cover the embryos with a layer of halocarbon oil using a glass/plastic pipette.

E. Preparation of needle for injection
- **i.** Depending upon the microinjector being used, either pull glass needles using a needle puller or buy commercially available glass needles meant for Drosophila injections. In both cases, diameter of the opening of the tip is expected to be approximately 0.5 μm.
- **ii.** Mix 15 μL of DNA prepared for injection with 4 μL of food color (green or red, depending upon availability).
- **iii.** Load the needle with 2−5 μL of DNA either by using a pipette or through capillary action (particularly in case of pulled needles).
- **iv.** Connect the loaded needle to the holder connected to a micromanipulator or a syringe. In case of pulled needles, ensure to break the tip of the needle by rubbing the same against the corner of the cover glass, placed on a slide.
- **v.** Place a slide containing a drop of halocarbon oil and ensure that the needle is in the same vertical focal plane as embryos.

F. Injection of embryos
- **i.** Lift the needle vertically and replace the slide containing the halocarbon oil with the slide containing embryos.
- **ii.** Position the needle slightly away from the middle of the posterior tip of the embryo and penetrate the embryo. After penetration, draw the needle as back as possible, without leaving the embryo, and deposit the DNA in the posterior-most region of the embryo cytoplasm.
- **iii.** Inject all the embryos on the slide in a similar manner.

G. Postinjection care of embryos
- **i.** Remove the noninjected embryos to avoid false negatives.

ii. Place the slide with injected embryos in a petri plate containing the moist tissue paper.
iii. After 24–28 hours, transfer hatched larvae using a fine needle to a vial containing fresh food.
iv. Monitor the development of these larvae and cross each eclosed adult to the opposite sex partner from w^{1118} stock.
v. After 10–12 days, screen for the red/orange/yellow-eyed flies, which are transformants.

H. Generation of stable transgenic flies
i. Collect unmated transformants and cross to the opposite sex partner from w^{1118} stock.
ii. Collect unmated heterozygous males and females resulting from this cross and place the same in fresh vials.
iii. In the resultant progeny, isolate unmated homozygous males and females (will have dark eye color) from heterozygous flies (eye color lighter than homozygotes) and set up a cross of homozygotes.
iv. Cross-check the resultant progeny for similar eye color in all flies to ensure that the line is stable.

Ethical issues

Biological research involving organisms requires ethical clearance. The rationale of introducing ethical issues lies majorly on the judicious use of the animals and their humane treatment before, during, and after the experiments. Therefore it is now mandatory in a research institution or in a university, where biological research is pursued, to have animal ethics and human ethics committee in place. These committees look into the details of the program that will be undertaken by the researchers for the possible use of laboratory animals (essentially mammals such as mice, rats, guinea pigs) and for the human samples. No research data on animals/humans are published nowadays in journals if the respective researchers do not provide the details of the ethical clearance data/number.

While in toxicology and other related fields, researchers majorly depend on the data generated on laboratory animals trying to extrapolate the data to humans. Several factors that remain as hurdles for the genuine extrapolation is the intraspecies variation and compounding effects resulting from one experiment to another.

Smaller animals have played pivotal roles toward our present-day understanding of fundamental facets of biology. In parallel, information generated in these organisms due to ease of handling, their isogenic conditions negating intraspecies variation, simple yet functionally homologous tissue architecture to higher mammals and more importantly in the postgenomic era, the gene homology existing between the smaller organisms like Drosophila, *Caenorhabditis elegans*, etc., to mammals and humans have led to important discoveries that have relevance to humans. In this context, limited ethical concerns are raised for the use of smaller organisms especially invertebrates and lower vertebrates in biological experiments and testing. European Centre for the Validation of Alternative Methods has recommended several such organisms to promote the basic principles of 3Rs (refine, replacement, and reduce) in biological research and testing (Balls et al., 1995).

Translational significance

How does Drosophila research over the years have made significant inroads in the area of biotechnology? This is a pertinent question that can be asked. Over the years, several significant information pertaining to genetics and development have been generated using Drosophila which has advanced our knowledge. Depending upon the type of plasmid used, the germ-line transformation can lead to the generation of transgenics where transgenes are randomly integrated into the genome or into specific sites (site-specific integration) within the genome. The latter would help to overcome the variations between transgenics arising due to insertion at random sites within the genome. By exploiting the same, researchers have generated a genome-wide collection of transgenics to knockdown or ectopically express genes in a stage- and/or tissue-specific manner. In addition, in the last couple of years, researchers have developed Drosophila reagents useful for generating Drosophila gene knockouts by employing CRISPR/CAS9 technology, which has been widely used with mammalian models. By manipulating the genome through germline transformation, during the last decade, number of Drosophila models useful for understanding several human diseases have been developed (see Clinical significance section for details). In addition, transgenic Drosophila are also being used in the field of toxicology. Transgenic Drosophila containing LacZ under the control of hsp70 promoter has extensively contributed to the understanding of stress response mechanism underlying heavy metal and/or pesticide toxicity (reviewed in Gupta et al., 2010). Further, transgenic Drosophila models have been utilized to assess the chemical-mediated reproductive toxicity (Misra et al., 2014a,b) and innate immune response (Pragya et al., 2015).

Clinical significance

Model organisms made invaluable contributions for studies of human disease mechanisms due to the high

degree of conservation of fundamental biological processes throughout the animal kingdom. In this context, it is very important to remember that human organization is much more complex than that of Drosophila. Despite sharing the fundamental processes, Drosophila and humans might still differ in mechanisms through which these processes are regulated/implemented. Consequently, researchers sensibly developed Drosophila-based models for human diseases associated with mutations in one or two genes or for those diseases where the essential pathways are conserved. These models are excellent for understanding the disease progression, pathogenesis, and underlying mechanisms. Further, these models are also useful in drug discovery, although caution should be exercised before extrapolating the dose data and/or delivery route to human use. Nevertheless, Drosophila-based models for human disease present a quick and inexpensive approach for first-tier screening of a large number of molecules during the drug discovery process. Below, we have provided a few examples of Drosophila-based models for human diseases.

Drosophila-based models for understanding human neurodegenerative diseases

Neurodegenerative diseases (NDs), as the name suggests, arise due to progressive loss of specific neuronal populations. Human genetic studies led to the identification of genes associated with certain NDs but mechanistic understanding of pathways and processes underlying the disease remained incomplete due to ethical and/or technical constraints. In this context, Drosophila, with its complex behavior, including learning and memory, driven by a sophisticated brain and nervous system, provided excellent models for deciphering signaling pathways and understanding the cellular processes defining human ND (reviewed in Hirth, 2010; Lu and Vogel, 2009). For example, Drosophila-based models are available for Alzheimer's disease (AD), the most common ND, characterized by age-dependent gradual impairment in memory and cognitive abilities. In humans, selective atrophy of the hippocampus and the frontal cerebral cortex, amyloid β (Aβ) plaque deposition, aggregation of the microtubule-associated protein tau are the hallmarks of AD pathology. The Aβ plaque is mainly composed of Aβ-40 and Aβ-42 peptides, the products of endoproteolysis of the amyloid precursor protein (APP) through secretases. Autosomal-dominant mutations and defective trafficking of APP affect the onset and/or progression of AD; both circumstances promote the generation of amyloid Aβ peptides. Drosophila genome contains homologs for human AD-related genes: APP, presenilin, and tau. Therefore researchers employed Drosophila as a model system for AD research. Drosophila models of APP-mediated AD do simulate certain characteristics of human AD pathology, including Aβ plaque deposition. In addition to providing insights to the pathology, these models are also useful to identify/target the modulators of AD pathology through genetic or pharmacological interference. Likewise, Drosophila models are available for Parkinson's disease (PD) too. PD is the second most common neurodegenerative disorder characterized by impaired motor skills: uncontrollable tremor, imbalance, slow movement, and muscle rigidity. PD pathology is associated with progressive loss of dopamine neurons in the substantia nigra pars compacta of the ventral midbrain. Formation of Lewy bodies, mainly composed of synuclein and ubiquitin, among other proteins is the pathological hallmarks of PD. Several genes have been associated with PD in humans: alpha-synuclein, parkin, ubiquitin carboxy-terminal hydrolase L1 (UCHL1), phosphatase and tensin homologue (PTEN)-induced kinase 1, (PINK-1), DJ-1, leucine-rich-repeat kinase 2 (LRRK2), high-temperature requirement protein A2 (HTRA2), glucocerebrosidase, polymerase gamma and tau. Mutations in alpha-synuclein and Parkin have been associated with PD in human. Except for alpha-synuclein, Drosophila carries the homologs of the remaining PD-associated genes. Accordingly, analysis of Drosophila mutations/transgenes of PD-associated gene homologs provided valuable insights to PD pathogenic mechanisms and targets of PD-related genes. Lack of alpha-synuclein homolog did not deter researchers from using Drosophila to study mechanism underlying alpha-synuclein-related PD and its targets. By utilizing the powerful genetic tools available with Drosophila, researchers expressed human alpha-synuclein in Drosophila to investigate its functional properties in PD and to elucidate its targets. Interestingly, metabolomic hallmarks of Drosophila with Parkinson-like symptoms are comparable to those of PD patients (Shukla et al., 2016). These findings highlight the utility of Drosophila as a model to understand the pathological intricacies underlying NDs.

Drosophila as a model for understanding human metabolic disorders

Diabetes and obesity are the most common metabolic disorders encountered in the present-day lifestyle. To date, obesity concerns are limited mainly to developed nations, although developing countries are catching up quickly. Further, obesity in the majority cases leads to diabetes. Diabetes, arising mainly from

errors in glucose metabolism, is the most common metabolic disorder with worldwide distribution. The World Health Organization (WHO) already declared diabetes as an epidemic because the number of diabetic patients or prevalence of diabetes has gone up dramatically over last few decades. Diabetes has multiple consequences, in that it causes two to four times more heart diseases and stroke, 60% of amputations, and 44% of kidney diseases. As per the latest report of International Diabetes Federation, at present 415 million people worldwide suffer from diabetes, and this number is projected to be 640 million by 2040. WHO predicted that developing countries will be at a higher risk of this epidemic in the 21st century. There are two types of diabetes, namely Type-1 and Type-2 depending upon the status of insulin, a peptide hormone, produced by the islets of Langerhans present in the pancreas. In humans, insulin plays a key role in glucose homeostasis along with glucagon, another peptide hormone, which is produced by the alpha cells of the pancreas. Insulin influences the cellular uptake of glucose from the blood whereas glucagon signals the release of glucose stored in liver cells into the blood, thereby maintaining healthy blood glucose levels. In Type-1 diabetes, previously named as juvenile-onset diabetes, insulin production is affected due to autoimmune destruction of pancreatic cells. Consequently, there is a rise in the circulating sugar levels due to the failure of insulin-dependent glucose transport, triggering a compensatory starvation response breakdown of glycogen and fat to produce energy since the body fails to generate energy from the ingested food resources. Type-2 diabetes, previously known as adult-onset diabetes, differs from Type-1 by having insulin but not being able to utilize the same efficiently, probably due to defects arising in insulin receptors or due to a complicated medical condition called "insulin resistance." The conservation of insulin/insulin-like growth factor-1 signaling (IIS) pathway between Drosophila and mammals (Brogiolo et al., 2001) and recent understanding of metabolic processes, glucose homeostasis, and endocrinology have led to Drosophila-based models relevant to human metabolic disorders and diabetes. Drosophila has seven insulin-like peptides, and five of these show significant homology to human insulin. These peptides are produced by neurosecretory cells in the pars intercerebralis (PI) region of the protocerebrum in both larvae and adults. Drosophila insulin-like peptides (dilps) affect the growth of the organism and energy homeostasis. In addition to dilps, Drosophila also contains the adipokinetic hormone (AKH), further illustrating the conservation of endocrine mechanisms regulating circulating glucose levels. In Drosophila, the ablation of AKH (synthesized and secreted by cells in the corpus cardiacum) results in decreased circulating carbohydrate levels. This antagonistic relationship between dilps and AKH is reminiscent of that between insulin and glucagon in mammals (Bharucha et al., 2008). To model the loss of insulin as observed in Type-1 diabetes, Zhang et al. (2009) used genetic approaches and deleted dilps 1−5 in Drosophila. Drosophila lacking dilps 1−5 recapitulated symptoms that appear similar to human diabetes: knockout flies had elevated sugar levels in their circulation and despite feeding at normal levels, they were generating energy from fat, which is generally a hallmark of starvation. Type-2 or insulin-resistant diabetes is caused by multiple factors. These include genetic factors, diet, and most obviously, obesity. Further, Type-2 diabetes due to a single mutation is rare in humans, and therefore models based on single-gene manipulations would limit the understanding of mechanism underlying Type-2 diabetes. To model the Type-2 diabetes resulting from diet/obesity, Musselman et al. (2011) fed Drosophila with high-sugar diet and induced characteristics representing Type-2 diabetes: hyperglycemia, insulin resistance, and increased levels of triglycerides and free fatty acids. Though these flies are yet to be exploited for drug discovery, the generated models help to understand not only the mechanism underlying human diabetes but also the downstream signaling components regulating glycogenolysis (Haselton and Fridell, 2010). In addition, the power of Drosophila genetic screens would help to unravel candidates critical for carbohydrate metabolism and energy homeostasis, thereby providing a window for approaches leading to pharmacological intervention.

Drosophila as a model for understanding nephrolithiasis (kidney stones)

An efficient kidney is critical for the filtration and elimination of waste from the blood. Given these roles, analysis of the renal system has the potential to provide indications of immune, toxic, and other insults to the body. Stone formation is the second most medical complication in the kidney. The cause of stone formation is multifactorial, and attempts for the prevention of the same have been complicated due to difficulties in finding a "model" system. Vertebrate kidneys are glomerular and structurally complex. On the contrary, the Drosophila renal system has aglomerular tubules and is simple. However, there exist certain structural similarities in the renal systems of insects and vertebrates. Malpighian tubules in Drosophila are the recognizable counterparts of the vertebrate renal tubules. Primarily, these tubules generate urine and may

selectively reabsorb some solutes. In addition to tubules, Drosophila, like other insects, also possess nephrocytes having roles in detoxification by filtration and endocytosis, followed by metabolism. Interestingly, feeding Drosophila with certain dietary or lithogenic agents such as ethylene glycol, hydroxyl-L−proline, or sodium oxalate results in the formation of calcium oxalate crystals within the lumen of the tubule in a dose-dependent manner (Dow and Romero, 2010; Chen et al., 2011). Drosophila is quite amenable to study kidney stones because these stones are hardly lethal to the simple tubule architecture of the insect. Indeed, two classes of stone can be constitutively produced in insects without being harmful to the organism. In the tubule lumen, calcium phosphate is stored as concentric spherites. The accumulation of uric acid crystals in the tubule lumen can potentially provide a natural model for urate nephrolithiasis. However, the extent to which the formation of stones observed in Drosophila mimics that of humans remains to be understood. Apart from this, Drosophila nephrocytes, which are analogous to podocytes in higher organisms, display hallmarks of diabetic nephropathy when flies were fed chronic high-sucrose diet (Na et al., 2015). Further studies on these models have the potential to provide insights to the pathophysiology underlying kidney stones and/or diabetic nephropathy that would be useful to develop new therapeutic approaches.

Drosophila-based model for understanding the human immunodeficiency virus pathology

Acquired immunodeficiency syndrome (AIDS) is a disease of the immune system caused by infection with the human immunodeficiency virus (HIV). The pathophysiology of AIDS is quite complex. After gaining entry to the body, HIV replicates to the level of several millions per milliliter of blood. This replication is associated with the depletion of $CD4^+$ T cells, thereby weakening the immune system and leading to secondary infections. To facilitate the replication of the viral genome, HIV encodes a Trans-activator of transcription (Tat), which functions as an activator of transcription by interacting with components from the transcription complex. Recent evidence points to the involvement of host-cytoskeleton in HIV pathogenesis. To test if Tat is involved in the cytoskeleton organization, Battaglia et al. (2001) generated Tat transgenic Drosophila lines and evaluated the effect of Tat by expressing it during oogenesis. These HIV transgenic flies helped to understand the action of viral gene products not just in a single cell but within a defined territory. The principle behind these studies is that any expansion or restriction of the territory in which the gene is expressed results in mutated phenotypes. Studies on these transgenic flies have shown that Battaglia and his coworkers have shown that Tat can interact with tubulin to alter the MT polymerization rate in HIV-infected cells, thereby furthering our understanding of the molecular mechanism underlying Tat-mediated pathogenesis.

Drosophila-based therapeutic peptide production

Drosophila is not only useful to understand the processes underlying diseases but also would be useful for producing peptides of therapeutic importance. By exploiting the conserved nature of peptide processing across Metazoa and the versatility as well as the scope of Drosophila genetics, Park et al. (2012) have successfully produced functional human insulin in vivo in Drosophila. This innovative metabolic engineering in Drosophila, however, requires several adjustments and improvements to make it cost-effective and commercially feasible. Nevertheless, coupling of this novel method with the power of Drosophila genetics would immensely help to meet the increasing demand for therapeutic bioactive peptides.

We discussed here only a few of the several human diseases. Drosophila is being used to study other human diseases, including oxidative stress-based disorders, tau neuropathies, Poly-Q-related neurodegeneration, cancers, epilepsy, and muscular dystrophy. Details pertaining to these and other diseases for which Drosophila can be a potential model are available in the Drosophila Genomic Resource Center (DGRC) website that lists the Drosophila counterparts of several human diseases. Given the power of Drosophila genetics/tools, we will be learning much more about our health using Drosophila in the future.

Turning point

The completion of Drosophila genome sequencing and subsequent Annotation (Adams et al., 2000) paved the way for the utilization of *D. melanogaster* as a model for studying the molecular basis of human disease. Comparison of human and Drosophila genomes suggested the presence of orthologs in Drosophila for about 75% of human disease-related genes. In view of these, researchers have exploited the versatile molecular tools available for Drosophila and modeled several human diseases in Drosophila (reviewed in Lu and Vogel, 2009).

World Wide Web resources

Resource availability is vital for the development of any community. Thanks to the cooperative work culture among Drosophila researchers both in the pre- and postgenome sequencing era. Drosophila research community is very well equipped with versatile physical as well as network resources for the knowledge generation and dissemination of the same. The physical resources ranged from stock centers that provide the required wild type stocks/mutants/transgenic flies to the specialized centers that cater to the biochemical reagent needs of Drosophila researchers. Virtual resources literally assisted the quick assembly/annotation of genomes across various species of Drosophila and also assisted in functional genomics/proteomics. Here, we listed a few commonly used network resources and the resources for stocks and reagents along with their utility (Tables 1.1 and 1.2). More details regarding the listed resources can be obtained

TABLE 1.1 Network resources available for Drosophila researchers and their utility.

Name of the resource	Web address	Purpose/utility
FlyBase	http://flybase.org/	Gateway to *Drosophila melanogaste*r genome
Berkely Drosophila genome project (BDGP)	http://www.fruitfly.org/	
Drosophila Genomics Resource Center (DGRC)	https://dgrc.bio.indiana.edu/Home	
FlyRNAi	http://fgr.hms.harvard.edu/	DRSC/TRiP (Transgenic RNAi project) Functional Genomics Resources
flyCRISPR	http://flycrispr.molbio.wisc.edu/	A resource for CRISPR RNA/Cas9 system
FlyAtlas	http://flyatlas.org/atlas.cgi	Road map to Drosophila gene expression
FlyPrimerBank	http://fgr.hms.harvard.edu/flyprimerbank	FlyPrimerBank provides a resource of precomputed primers appropriate for qPCR
Flymine	www.flymine.org/	An integrated database of gene expression and protein data for Drosophila and Anopheles
Flymove	http://flymove.uni-muenster.de/	Resource for university students and teachers studying Drosophila developmental biology
FlyEx	https://www.hsls.pitt.edu/obrc/index.php?page = URL1233940446	Database of Segmentation Gene Expression in Drosophila
FlySNP	http://flysnp.imp.ac.at/	High-density genome-wide map of single nucleotide polymorphisms (SNPs) and SNP genotyping
Flybrain	http://fruitflybrain.org/	Database of Drosophila nervous system
BioGrid	http://thebiogrid.org/	Protein and genetic interaction database
Drosophila proteome atlas	http://www.drosophila.jp/jdd/proteome_atlas/index.html	Proteomes of adult Brain, eye and reproductive system
DIOPT-DIST	http://www.flyrnai.org/diopt-dist	Map genes to putative orthologs in the human genome, and human disease genes
TargetScanFly	http://www.targetscan.org/fly_72/	To predict miRNA targets in Drosophila sequence
FlyTF	http://flytf.gen.cam.ac.uk/	Drosophila transcription factor database
TaxoDros	https://www.taxodros.uzh.ch/	Database on taxonomy of Drosophilidae
FlyTrap	http://www.fly-trap.org/	GFP protein trap database
DrosDel	http://www.drosdel.org.uk/	Isogenic deficiency kit for Drosophila *melanogaster*
FlyPNS	http://www.normalesup.org/~vorgogoz/FlyPNS/page1.html	Drosophila *melanogaster* embryonic and larval peripheral nervous system
flyDIVaS	http://www.flydivas.info/	A comparative genomics resource for drosophila divergence and selection

TABLE 1.2 Physical resources available for Drosophila researchers and their utility stocks and reagents.

Name of the resource	Web address	Utility
Bloomington Stock Center	https://bdsc.indiana.edu/	Various fly stocks (including mutants and transgenics) and also provides resources/recipes for fly work
NIG-FLY, Japan	http://www.shigen.nig.ac.jp/fly/nigfly/index.jsp	Mutants and RNAi stocks of Drosophila
Drosophila Genomics Resource Center (DGRC), United States/Japan	https://dgrc.bio.indiana.edu/Home	Stocks and reagents for Drosophila research
	http://kyotofly.kit.jp/cgi-bin/stocks/index.cgi	
VDRC Stock Center, Austria	http://stockcenter.vdrc.at/control/main	Drosophila RNAi lines
Drosophila Species Stock Center, United States	http://blogs.cornell.edu/drosophila/	Source for Drosophila species stocks

by visiting the sites using the web address given. There are much more than those listed here and they can be accessed at http://flybase.org/wiki/FlyBase:External_Resources

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Glossary

Allele An alternate form of a gene. A gene may have many different alleles that differ from each other by as little as a single base or by the complete absence of a sequence.

Centimorgan (cM) The metric used to describe the distance between two genes, which is determined by using the frequency of recombination between these genes. For example, a recombination frequency of 10 amounts to 10 cM. This term is named in honor of Thomas Hunt Morgan, who first conceptualized linkage while working with Drosophila.

Genotype The set of alleles for a given character. A genotype can be either homozygous (with two identical alleles) or heterozygous (with two different alleles) or hemizygous (in case of sex-linked alleles).

Homolog A gene whose sequence is similar to a greater extent to a gene from another species and has commonalities in origin and function.

Inbred Organisms that result from the process of brother–sister matings for multiple generations. This process is called inbreeding.

Gene linkage Presence of loci so close to each other on a chromosome that they tend to be inherited together and such that recombination between them is reduced to a level significantly less than 50%.

Locus Any genomic site mapped to a chromosome through formal genetic analysis.

Mutation A heritable variation in the sequence of a gene that alters the amino acid sequence of its protein. These mutations can influence the production, structure, and function of proteins.

Phenotype The physical manifestation of a genotype within an organism. For example, hair color in human is a phenotype.

Transgene A fragment of foreign DNA incorporated into the genome through the manipulation of embryos. For example, insertion of human insulin gene in Drosophila by manipulating their embryos.

Transgenic Refers to organisms containing a transgene or genes that are foreign. For example, cotton seeds developed by Monsanto are transgenic.

Isogenic Characterized by essentially identical genes. For example, identical twins are isogenic.

Eclosion The emergence of an adult insect from its pupal case. For example, the emergence of silk moth from its coccoon.

Transposable element A genetic element capable of moving from one chromosome to the other or within the chromosomes. These elements can potentially disrupt the function of other genes

Homeobox A regulatory DNA sequence present in the genes that control pattern formation in organisms during development

Ortholog Genes in different species with a similarity to each other due to their common ancestral origin.

Balancer chromosome Chromosome comprising inversions that facilitate stable maintenance of lethal mutations as heterozygotes in a manner that does not require selection.

Neurodegeneration This refers to progressive loss and/or death of structure and/or function of neurons. For example, Parkinson's disease is a result of neurodegeneration

Neurodegenerative disorder A disease condition that involves or causes neurodegeneration.

Abbreviations

AbdA	Abdominal-A
AbdB	Abdominal-B
AD	Alzheimer's disease
AIDS	Acquired immunodeficiency syndrome
AKH	Adipokinetic hormone
AntP	Antennapedia
APP	Amyloid precursor protein
Aβ	Amyloid β
BX-C	Bithorax Ccomplex
CAS	CRISPR-associated proteins
CRISPR	Clustered regularly interspaced short palindromic repeats
DILP	Drosophila insulin-like peptides
HIV	Human immunodeficiency virus
Hox	Homeobox containing
Hsp	Heat Heat-shock protein
HTRA2	High-temperature requirement protein A2
LRRK2	Leucine-rich-repeat kinase 2
MT	Microtubule
ND	Neurodegenerative diseases
NPs	Nanoparticles
PD	Parkinson's disease
PI	Pars intercerebralis
PINK-1	PTEN-induced kinase 1
PTEN	Phosphatase and tensin homologue
RNA	Ribonucleic aAcid
RNAi	RNA interference
Tat	Trans-activator of transcription
Ubx	Ultrabithorax
UCHL1	Ubiquitin carboxy-terminal hydrolase L1
WHO	World Health Organization

Long answer questions

1. Describe the molecular events underlying the development of metazoans.
2. Describe germ-line transformation in Drosophila.
3. What is the translational significance of Drosophila biology?
4. Explain the contributions of Drosophila to the understanding of pathophysiology of neurodegenerative diseases.
5. Explain how Drosophila can be considered as Cinderella of biotechnologists.

Short answer questions

1. State the different stages in the life cycle of Drosophila.
2. What is holometabolous development?
3. Provide two examples of Drosophila models for human diseases.
4. How conserved are the genomes between Drosophila and humans?
5. What is germ-line transformation?
6. Can Drosophila be a model for therapeutic peptide production?

Answers to short answer questions

1. Egg, larva (three instars), pupa, and imago (adult).
2. A specific type of insect development involving complete metamorphosis involving different stages of development to give rise an imago that appears entirely different from the developmental stages.
3. Diabetes and Parkinson's diseases.
4. As many as 75% of human disease genes are conserved in *Drosophila*.
5. Germ-line transformation is a method through which DNA is incorporated into the germ line of the individual for its faithful inheritance to subsequent generations of transgenic organisms.
6. By exploiting the conserved nature of peptide processing across Metazoa and the versatility as well as the scope of Drosophila genetics, Park et al. (2012) have successfully produced functional human insulin in vivo in Drosophila. Coupling of this novel method with the power of Drosophila genetics would immensely help to meet the increasing demand for therapeutic bioactive peptides.

Yes/no type questions

1. Whether Drosophila is a mammal?
2. Whether Drosophila undergoes holometabolous development?
3. Whether Drosophila genome has been completely sequenced?
4. Are the genomes between Drosophila and humans largely conserved?
5. Whether incubation of Drosophila with DNA leads to transgenics?
6. Can germ-line transformation be used to manipulate Drosophila genome?
7. Is there translational significance for Drosophila transgenics?
8. Are the Drosophila models for human diseases clinically relevant?
9. Can Drosophila be a model for studying neuropathy or kidney stones?
10. Are there web resources available to exploit Drosophila genome?

Answers to yes/no type questions

1. No—Drosophila is an insect having sequence homology to mammals.

2. Yes.
3. Yes—The complete genome sequence of Drosophila was published in 2000.
4. Yes—As many as 75% of human disease genes are conserved in Drosophila.
5. No.
6. Yes—This method can be used to genetically manipulate the Drosophila genome and that depends on the kind of plasmid being injected.
7. Yes—Transgenic Drosophila are being extensively used to understand human diseases and also in drug/toxicity screens.
8. Yes—Drosophila models have led to the mechanistic understanding of human diseases, including diabetes and Parkinson's diseases.
9. Yes.
10. Yes—Flybase.org is the best resource to gain insights to Drosophila genome.

CHAPTER

2

Animal models of tuberculosis

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Summary

Animal models for tuberculosis (TB) research provide valuable and specific information about the nature of the disease (pathology and the immune response). Refinement of animal models may pave the way to new information of great importance. The choice of model is mainly dependent on cost, availability, space, as well as biosafety requirements.

There are no naturally occurring animal reservoirs for *Mycobacterium tuberculosis*, and many different animal species are susceptible to infection with this organism. It has been observed that there is extreme variation in the pattern of pathological reactions between different species. Though different animal models are in use for long for tuberculosis research providing valuable information about the nature of the disease including specific information about the disease pathology and the immune response to the infection, none completely mimic the human model. However, refinement of animal models may pave the way to new information of great importance. No single model is good enough for evaluation. The choice of model is mainly dependent on cost, availability, space, as well as biosafety requirements. The most commonly used experimental animal models of TB include the mouse, rabbit, and guinea pig. There are established protocols for infecting animals with TB and further analysis. These protocols are summarized at the end of the chapter and provide valuable information regarding the course of infection, the basic immune response, and the extent of lung pathology of experimental pulmonary tuberculosis.

Introduction

Tuberculosis still remains to be focal public health priorities for many of the developing countries of the world. According to WHO, globally, around 8.87 million new incidents of TB are being diagnosed as new cases in 2011. Most of the cases occurred in the South-East Asia (55%) and the African (30%) regions. The five countries with the largest numbers of cases include India, China, South Africa, Nigeria, and Indonesia. Of the 8.87 million new TB cases in 2011, about 15% were HIV positive, 78% of these HIV-positive cases were in the Africans, and 13% were in the South-East Asia regions. Identification of multidrug-resistant (MDR) strains, defined as mycobacteria resistance to at least rifampicin and isoniazid (two first-line anti-TB drugs), and extensively drug-resistant (XDR) strains, defined as MDR mycobacteria with additional resistance to fluoroquinolones and at least one of the injectable second-line antituberculosis drugs, has worsened the condition. Notably MDR- and XDR-TB have been recognized by WHO as a major challenge to be addressed in the fight against TB (Dube et al., 2012). Although delamanid and bedaquiline are newly approved drugs after a gap of 40 years for the treatment of multidrug-resistant TB, new molecules are still needed to end TB by 2030 (Migliori et al., 2017, Singh and Gupta, 2018).

When humans are infected with *M. tuberculosis*, they may develop primary active TB, latent TB, chronic active TB, or reactivation disease; 10% of non-immunosuppressed individual progress from latent to reactivation of TB over their lifetimes, while HIV-infected individuals have a 10% annual risk of reactivating latent

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disease suggesting that not all above-mentioned manifestations are mutually exclusive. The individual outcome is determined by various factors such as immunosuppression, HIV infection, and nutritional status, intensity of exposure, BCG vaccination, and age. The re-exposure to TB and reinfection plays a role in the risk of developing disease, which is less commonly reported. However, it attracts an increased importance. Each of these stages of infection in humans can be approached by the use of one or more of the animal models which are discussed further in this chapter.

For more than a hundred years, animals have indeed taught humans a great deal about TB and promise to be of potentials as increasingly useful tool in studying immunologic, genetic, molecular, and pharmacologic characteristics of bioactives, infection, and related pathogenesis. Animal models have become standard tools for the study of a wide array of human infectious diseases. Many animal models of TB have been developed. Given the complexity of human TB, animal models of TB offer a vast resource to study a multitude of unresolved questions; the genetics of host defense, microbial virulence, latency, reactivation, reinfection, drug therapy, and immunization are just a few to name. Researchers are fortunate to have many well-developed experimental animal models from which exhaustive knowledge can be attained. The most commonly used experimental animal models of TB include the mouse, rabbit, and guinea pig. Although, substantial differences in TB susceptibility and disease manifestations exist between these species, they have contributed significantly to understanding various aspects of TB. Current concepts in TB pathogenesis have also been derived from animal studies involving experimentally induced infections with related mycobacteria (e.g., *M. bovis*). The manifestations in select animal hosts may mimic the etiology of tuberculosis in human TB.

Comparative pathology of tuberculosis in humans and animals

Robert Koch recognized and reported the spectrum of pathology of TB in different animal species based on his seminal studies on TB. The examination of clinical specimens from infected humans, cattle, deer, badgers and possums confirmed the extreme variation in the pattern of pathological reactions between different species (Table 2.1). There is also an associated spectrum of resistance to infection. Guinea pigs, ferrets, possums, and badgers are innately susceptible to TB, while humans, rabbits, mice, cattle, and deer express varying levels of resistance, depending on their genotype.

Studies in laboratory animals such as guinea pigs and rabbits have significantly enhanced the understanding of the etiology and pathogenesis of TB. Humans, cattle, deer, guinea pigs, and rabbits have similar pathology, however, differ in susceptibility to TB. As compared to humans and ruminants that are relatively resistant, fewer than five virulent organisms introduced by the aerosol route into guinea pigs consistently produce lung lesions, bacteremia, and fatal disease. Studies in guinea pigs and rabbits have made an important contribution in the understanding relating to the virulence and pathogenesis of TB, but they have limited use in the study of the protective immune response (Smith and Wiegeshaus, 1989).

Characteristics of a model for tuberculosis with respect to infection and pathogenesis

- Experimental infection mimics natural disease.
- Infection results from low-dose challenge.
- Route of exposure simulates natural exposure.
- Pathology present in relevant target organs.

TABLE 2.1 Pathogenesis found in different animal species infected with virulent *M. bovis*.

		Pathology				
Animal species	Predominant site for lesions	Caseation fibrosis	Langbans giant cells	Acid-fast bacilli	DTH	Antibody (IgG)
Guinea pig	Lung, liver, lymph node, spleen	++	–	+	++++	+
Ferret	Lung, liver, lymph node, spleen, kidney	–	–	+++	–	++
Rabbit	Lung, liver, lymph node, spleen, kidney	+	++	+	+	++
Mouse	Lung, liver, spleen, lymph node	–	–	++	+/–	+/–
Possum	Lung, liver, spleen, kidney, lymph node	–	–	+++	+/–	+/–
Badger	Lung, kidney, lymph node	–	–	+++	–	+/–
Cattle	Lung, lymph node	++	+++	+	++	+/–
Deer	Lung, lymph node	++	+++	++	++	++
Human	Lung, lymph node	++	+++	++	+++	++

- Lesions analogous to those found in naturally infected host.
- Spectrum of disease equivalent to that in the naturally susceptible host.

Pathogen diversity: crossing species barriers

In 1865, Villemin proved that TB was an infectious disease by inoculating laboratory rabbits with mycobacterium isolates from infected humans and cattle, and in 1882, Robert Koch defined the etiology of TB using pure cultures in experimental animal models, particularly the highly susceptible guinea pigs. At that time, Koch regarded mycobacterial isolates from humans and cattle as interchangeable, but after following Theobald Smith's distinction of *M. bovis* from *M. tuberculosis* in 1896, Koch argued strongly that the bovine tubercle bacillus presented minimal health risk to humans. Emil von Behring, a junior colleague of Koch, took a different view and advocated vaccination with the human tubercle bacillus which he considered to present minimal health risk to cattle. The host-restriction of mycobacterial strains remains imprecise. "*M. tuberculosis*" is generally associated with human disease, but can be found in cattle; "*M. bovis*" is generally associated with animal disease, but can be found in humans as well. This contrasts with the increasing precision was elaborated and provided by genetic analysis (Hershberg et al., 2008). Now, we recognize seven major lineages as members of the "*M. tuberculosis* complex"; six *M. tuberculosis* lineages differentially distributed among different human populations and one *M. bovis* lineage that includes multiple "ecotypes" differentially distributed among different mammalian species (Smith et al., 2006). Some of the major questions which are still to be answered include-whether these distributions are simply the products of history and geography, or each of the variants uniquely adapted to different species or ethnic groups? Should we consider pathogen genotype when selecting host–pathogen combinations for experimental models? Major differences in disease progression were seen with different isolates in animal models. They certainly warrant further analysis and subsequently von Behring observed that repeated passage (in culture and in animals) has a significant and differential impact on the ability of isolates to cause a disease in different hosts.

Host diversity: fundamental processes and fine-tuning

There are distinct differences in the pathological manifestations of TB in different mammalian species in terms of the patterns of cellular aggregates (more commonly known as granulomas) surrounding infected foci (Basaraba, 2008). Although human TB is associated with a diverse range of lesion types, caseous necrosis—comprising a well-structured ring of lymphocytes surrounding the remnants of dead cells—is regarded as the hallmark of human pathology. Physiological roles that have been assigned to these lesions include a positive contribution to containment of the infection, as well as detrimental contributions associated with sequestration of the bacteria from drugs and provision of a hypoxic microenvironment that produces a non-replicating, drug-tolerant state of bacterial persistence (Via et al., 2008). Breakdown of caseous necrotic lesions results in cavities that are capable of supporting the extensive bacterial replication required for subsequent transmission. In contrast, TB in the lungs of mice is generally associated with more diffuse cellular infiltration, lacking the structural organization and the necrotic foci which are the characteristics of the human disease. Caseous necrosis is seen in guinea pigs and in rabbits, while TB in cynomolgus macaques produces the full repertoire of lesions similar to human beings (Lin et al., 2006). This difference in disease pathology is central to the argument that the mouse is not a good model of TB. Although mice provide a model to explore the general effect of drugs on bacterial growth during infection, they are inevitably unreliable in the case of drugs customized for activity against lesion-specific bacterial subpopulations.

Approaching the issue of host diversity in terms of immunology lends a different perspective. The extensive range of immune reagents and recombinant inbred strains has allowed very detailed analysis of the immune response to mycobacterial infection and vaccination in mice (North and Jung, 2004). In naïve mice, there is an initial period during which cells of the innate immune system—macrophages, neutrophils and NK cells—are engaged but largely fail to contain the infection. A few weeks after infection, recruitment of antigen-specific T cells and stimulation antimicrobial function of macrophages restricts further bacterial growth but fails to eliminate the existing infection. Chronic infection persists, leading to death a year or so later. By establishing a population of primed T cells, vaccination in the effect initiate and accelerate the involvement and engagement of the adaptive immune response, thus lowering the bacterial "set point" in the chronic phase and as a result delaying, but not preventing death. IFN-γ and TNF-α are crucial mediators in the containment of infection.

Although opportunities for detailed immunological analysis are limited in other species, the basic features of the mouse model would appear to hold true for all mammals, including humans. For control of human

TB, the critical role of T cells is evident from the dramatic increase in risk of disease for individuals co-infected with HIV; the role of TNF-α is shown by TB reactivation during anti-TNF-α therapy and the central role of IFN-γ was demonstrated by hypersusceptibility to mycobacterial infection in individuals with rare mutations which affect IFN-γ signaling. This last example is interesting in that the IFN-γ effect is generally not reproduced in the common tissue culture model using monocyte-derived macrophages from human peripheral blood.

A general conclusion is that mice provide a robust and predictive model for studying fundamental features of the immune response to TB, a conclusion that is integral to the triage system that has led to the selection of the front-line TB vaccine candidates currently moving into clinical trials. However, it is important to consider whether effective vaccination against reinfection/reactivation TB in adults will in fact require the modification of core immune mechanisms or rather the fine-tuning of immune regulation, perhaps manipulating species-specific processes that contribute to differences in lesion architecture discussed above. To address this, Apt and Kramnik (2009) put forward a compelling case that, rather than discarding the mouse and its immune opportunities, we should take advantage of inbred and recombinant genetics to select mice that recapitulate the relevant aspects of human pathology.

Animal models of tuberculosis: limits and lessons

Artificially infected guinea pigs, mice, and rabbits have served as indispensable tools through which transmission, immunopathogenesis, tuberculin response, vaccine and antimicrobial efficacy, genetic resistance, and many other important facets of TB have been studied. Results, however, are usually not entirely reflective of TB infection and disease in humans. Substantial differences in TB susceptibility, disease patterns, and temporal course exist among species (see Table 2.2). The extent of organ involvement, immune response to aerosol or parenteral infection, and histopathology also vary considerably from species to species. In addition, a variety of clinical and laboratory strains of *M. tuberculosis* exist to infect animals experimentally, and these mycobacterial strains often differ greatly in infectivity, virulence, and immunogenicity in different animal models (Gagneux and Small, 2007). Well-defined host and pathogen variability allows researchers to control these factors, selecting those combinations needed to create animal models suited to the purpose being solved. Although infection by inhalation is the most relevant model for human infection, animal infections are also produced by parenteral inoculation. Like humans, TB in animal models is also treated with antimicrobials given orally (by gavage) or by parenteral routes.

In addition to these considerations, animal species vary based on size, laboratory space requirements, rearing costs, and ability to approximate the disease process in humans. In subsequent section, the key features of the well-developed animal models of TB and briefly; less commonly used animal species will be discussed. Despite several important differences outlined in the sections that follow, the murine, rabbit, and guinea pig models have emerged at the forefront of TB research because

1. infection can occur with inhalation,
2. animals manifest an innate and acquired immune response,
3. animals often initially control bacillary growth in the lung, and
4. they ultimately succumb to the disease.

Animal models: contributions in tuberculosis vaccine testing

Animal model possesses remarkable similarities with human physiology and provide important information about human system. This serves as the basis for testing of different vaccine candidates. Initially, Robert Koch employed mouse as an experimental model and subsequently, a variety of animal models

TABLE 2.2 Common animal models of TB—A comparative study.

Model	Histopathology			Relative susceptibility to *M. tuberculosis*	Immunologic reagents available	Laboratory space and cost
	Necrosis	Caseation	Cavitation			
Mouse	Minimal	Usually not	No	Low	Extensive	Relatively small
Rabbit	Yes	Yes	Yes	Very low	Moderate	Relatively large
Guinea Pig	Yes	Yes	Infrequent	Very high	Relatively few	Moderate
Nonhuman primate	Yes	Yes	Yes	High	Extensive	Large

including monkey, guinea pigs, rabbit, monkey, etc., were also used for studying the infection of *M. tuberculi*. Out of these several models, the mouse and guinea pig are being extensively used and provide new information about the host response in the lungs, changes in immunopathology, and the protective effect of new vaccine candidates throughout the world (Flynn et al., 2005). Every model has its own advantage and disadvantage, and the conclusion obtained from them can be extrapolated to humans; these models resemble one or the other aspect of human disease. These animals can be infected by pulmonary route without any difficulty, and this is the prime benefit of using these models for studying tuberculosis. Few virulent tubercular bacilli get deposited in alveolar space similar to the manner human do. Further, different stages of progression of TB can be easily studied with the help of animal models (Dharmadhikari and Nardell, 2008). Disease symptoms including fever, loss in weight, abnormal X-rays, and respiratory distress can also be observed in these models. Like human, the animals in due course meet death because of pulmonary insufficiency if left untreated. For the reason that of these similarities in human and animal models in the vulnerability as well as resistance to TB, disease progression, and finally death, the animals are considered good models for evaluating new anti-TB treatment regimen including bioactive and vaccines. Upon infection, humans can develop any type of the TB including primary active TB, latent TB, chronic active TB, or reactivation disease. The progression of the disease also depends upon exposure intensity, nutritional status, BCG vaccination, immunosuppression, age, and prevalence of other disease like HIV infection. Each of these stages of infection in humans can be approached by the use of one or more of the aforementioned animal models.

Various animal models

Mouse model

Robert Koch discovered the very first time that experimental mice can be used as an animal model for TB infection. The inoculation with *M. tuberculosis*-induced lesions are more similar to those seen in the case of natural disease in humans. Subsequent work established the pattern of disease in the more resistant mouse model. The strong immune response of this model for TB infection is emerged slowly. Mice are generally more resistant model for TB infection as compared with rabbits, guinea pigs, and even humans, as evidenced by their ability to tolerate relatively large bacillary numbers within their lungs and other organs without signs of illness. They develop noncaseating granulomata in response to infection and generally manifest a chronic phase of disease that represents the immune-mediated tissue destruction on a background of slowly progressive bacterial growth, ultimately resulting in death. This persistent stage of infection might be due to the chronic exposure of TB antigens on T-cell function, signifying the role of CD4/CD8 T-cell responses in the mouse remains robust over time. Previous reports addressed that murine macrophages use Toll-like receptors on their cell surfaces to identify the mycobacterial antigens and ultimately trigger the cytokine production, which are responsible for granulomatous response (Ito et al., 2007). The mice model also possesses the T cell−independent (natural killer cell) production of IFN-γ (a cytokine), which are crucial to the host immunologic response against TB. Several available options such as inbred and genetically knockout strains of mice have contributed in the understanding of the role of many specific cytokines, cells, and cell surface markers in containing bacillary growth (Kerantzas and Jacobs, 2017)

The mice models were successfully infected through pulmonary route using aerosolized microorganisms by means of nose-only exposure chambers to easily attain an easy infection with relatively low doses [∼50 colony-forming units (CFUs)] of *M. tuberculosis*. The growth of *M. tuberculosis* is logarithmic and then plateaus around 10^6 organisms are arrived in the lungs while cell-mediated immunity (CMI) develops during first 4 weeks. The plateau phase is an indicator of the persistent stage of infection, in which TB antigen can be more metabolically quiescent within macrophages. As reported earlier, this model is not capable for replicating paucibacillary, latent human TB infection. TB-induced mice presented little necrosis before the last stages of the disease and the animals develop only weaker sensitivity to tuberculin. Mice, which are inhaling the virulent bacilli, die of a successive enlargement of pulmonary granulomatous tubercles. In addition, the virulence of a given strain of *M. tuberculosis* depends on several factors indicating the route of infection, the manner of preparation of the suspension, and the dispersion as well as the size of the suspension. Earlier studies reported that high-dose intravenous challenge model in mice, which have low protein levels, showed T-cell defects with a loss of control of virulent infection and impaired granuloma formation. But, it was also observed that mice model could recover from TB infection, if they are allowed feeding with an adequate diet. The loss of TB resistance in their model was mainly due to the decreased nitric oxide production by activated macrophages that occurred secondary to an IFN-γ defect especially in malnourished animals (Chan et al., 1996).

The course of disease can be also influenced by the genetic variation among various inbred strains of mice.

The number of viable H37Rv bacilli in the lungs of BALB/c mice was 2 log lesser than their number in DBA/2 mice during the stationary phase after intravenous injection. The mice of BALB/c and C57BL/6 strains survived two times longer than DBA/2 and C3H/HeJ strains. The knockout mice (lacking genes for acquired immune response) also showed a higher number of bacillary titers during the stationary phase.

In Webster-Swiss male mice, the TB was induced by H37Rv strain of *M. tuberculosis* administered intravenously (McCune et al., 1966). The treatment was accomplished by using oral INH and pyrazinamide (PZA) for 12 weeks after bacterial inoculation. The drug-based therapy might be successfully employed for reducing the number of bacilli in mice tissues for up to 3 months after the termination of INH/PZA, to the extent that mycobacteria could not be cultured or otherwise isolated from lung or other tissue homogenates (e.g., essentially "sterilized"). If one carefully observes for longer periods of time, almost one-third of similarly treated animals spontaneously develop reactivation of TB, which is characterized by a recrudescence of the bacterial burden in their tissues. To understand the all "apparently sterile" animals those harbor dormant or latent organisms could turn to be reactivate at or after 3 months. The study concluded that appropriately timed steroids led to reactivation TB in most of these presumably "sterile" mice owing to residual viable organisms. However, this latent TB model although imperfect it could be an important adjunct to the conventional chronic TB disease model. The other researchers have manipulated a number of factors, such as the dose/duration of antibiotics, the time interval between antibiotic use and immunosuppression, and type of immunosuppression, to map specific cellular and cytokine mechanisms operative in the reactivation process.

Mouse is very cost-effective model for the evaluation of drugs, and it was more accurate as a measure of drug efficacy than other models such as the guinea pig. The antituberculous drug before entering into clinical phase have to undergo in vitro testing to measure their growth inhibitory potential, followed by the pharmacological activity for sterilizing activity in animals. The experimental studies investigated the sterilizing activity of effective drugs, as well as the efficacy of shorter treatment durations using combinations of new and existing drugs. Recent studies concluded that various effective regimens can substitute the existing moiety such as PA-824 for INH and rifampin, or rifapentine as an alternate of RIF. Rifapentine presented longer half-life and finally greater area under the curve (AUC), especially when administered daily or thrice weekly. Lenaerts et al. (2008) evaluated newer moiety within a subcategory of quinolones, the 2-pyridones. This compound was found to be more effective and better; however, it was INH but not superior than moxifloxacin.

Mice are particularly the favorable option for studying the immunology of mycobacterial infections and have contributed significantly for understanding of the roles of various immunological mechanisms of resistance. If concern with experimental aspect, mice is generally easier to maintain in BSL3 facilities and suggest an affordable, high-yield means to study vaccines, antitubercular drugs, immune mechanisms, host genetics and the contribution of host and pathogen strain differences leading to infection. The major disadvantage associated with mice model is the development of resistance to classic TB disease; as a result, they tend to be non-respondents to the therapy.

Guinea pig model

Guinea pigs models have been used to create models of TB transmission due to their exceptional susceptibility to infection with a few inhaled mycobacteria. Guinea pig is used as models in various forms of TB infection such as childhood TB and TB in immunosuppressed hosts, followed by the granulomata formation, primary and hematogenous pulmonary lesions, dissemination, and caseation necrosis (McMurray, 1994). In guinea pig, the lesion was developed after low-dose aerosol treatment with *M. tuberculosis* has similarities to natural infections in humans and all this make them attractive animal model to study the bacterial persistence. Similar to humans, naive guinea pigs first develop primary lesions that differ in their morphology as compared to secondary lesions. Primary lesions were originated after initial exposure, while secondary lesions were from hematogenous dissemination after the activation of acquired immunity (Lenaerts et al., 2007). It was previously reported that TB is an airborne infection; hence, this model can be easily experimentally induced by living air samples for airborne tubercle bacilli generated by TB patients. The infection was confirmed by both mycobacterial culture and histologic examination of various tissues namely lungs, spleens, and lymph nodes. To induce TB infection in guinea pigs, a limited number of animals were required as compared to mice as they have larger minute ventilation (Nardell, 1999). The course of infection first enters the logarithmic phase of bacillary multiplication in the lungs over 2−4 weeks. After that, it enters a stationary phase in lungs, whereas other organs showed the hematogenous dissemination.

The guinea pig model, however, at the back in position among the above-mentioned animal models in the context of the availability of immunologic reagents for studying guinea pig host immune responses. Moreover, a number of reagents are available to study cytokines and other inflammatory cells involved in pathogen recognition. Recent research has explored the new avenue

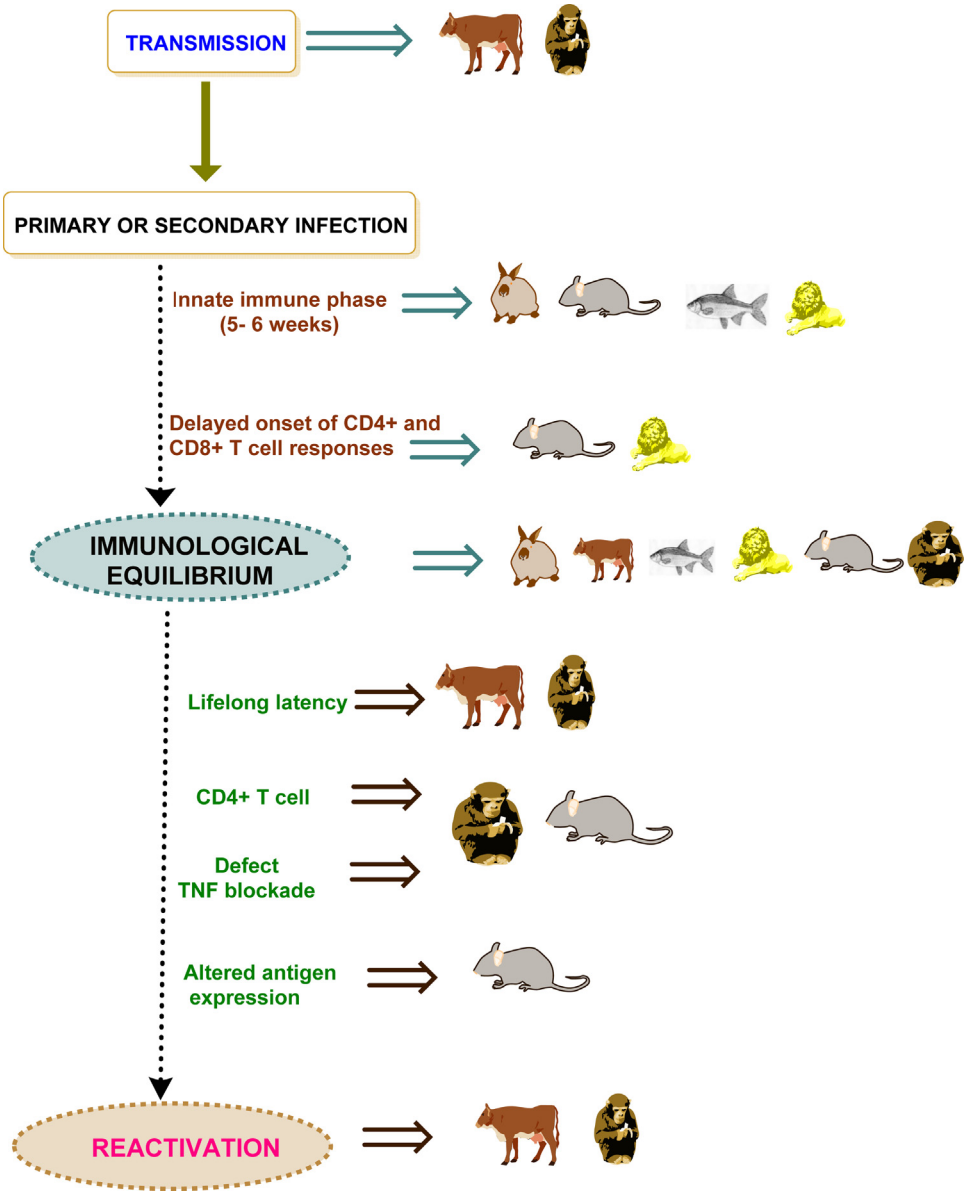


FIGURE 2.1 Stages of the immunological life cycle of human tuberculosis that can currently be modeled in experimental animals.

in the cloning of guinea pig cytokine and chemokine genes and their expression in recombinant guinea pigs as well as reagents which are used in the study of the response of guinea pigs to infection with virulent *M. tuberculosis* (Jeevan et al., 2006; Ly et al., 2007) (Fig. 2.1).

Rabbit model

Rabbits showed a higher resistance to *M. tuberculosis* infection and occupy an important position, as humans are also relatively resistant. Only 10% infected individuals allow progression of their TB infection. The rabbit-based model enabled us to understand the pathology of TB infection by *M. bovis* infection. However, previous experiments demonstrated that rabbits are generally resistant to infection with airborne *M. tuberculosis*. The pulmonary infections with *M. tuberculosis* have formed the cavity, which ultimately may regress and heal. The rabbits are infected with bovine mycobacterial infection, that is, *M. bovis*; their pulmonary pathology to inhaled bovine tubercle infection is more similar to human *M. tuberculosis* infection than those recorded in the case of other models such as mice and guinea pigs. In rabbits, the pulmonary cavity is first developed followed by bronchial spread of microorganisms. Subsequently, it was presented that

delayed-type hypersensitivity (DTH) and CMI are major contributory factor for developing the cavity in rabbit-based model. The cavity formation is mainly due to pulmonary cavities which possess large populations of bacilli reaching to the bronchial tree and also due to degree of sputum culture positivity, showing high bacillary burden. Rabbits, which are known to produce cavitation, have major perspectives in the study of disease transmission as compared to other animal models. Rabbits are also employed as the model for studying the latent or paucibacillary TB states in humans. Rabbits easily get the paucibacillary state through their own immune systems involved in the control of infection. Moreover, the experimentally immunosuppressed animals achieve the latent stage of infection easily. However, the rabbit model lacks the immunological reagents. Rabbits are more costly as compared to mice and guinea pigs and require large laboratory space.

Nonhuman primate model

The nonhuman primates are also successfully employed as model for latent TB infection. This latent state is metastable and can get reactivated easily when the animals are stressed or possible even without any exogenous immunosuppression administration. The main reason may be that they are closely related to humans, but owing to the difficulty such as higher cost, they have not been utilized to a larger level. The other animal models, however, fail to mimic the human disease entirely; therefore, research has been shifted and focused on the nonhuman primate model for the TB infection. The Philippine cynomolgus monkey (*Macaca fasicularis)* developed acute infection, and they have been rapidly progressive for highly fatal multilobar pneumonia, when challenged intratracheally with higher dose of *M. tuberculosis* (10^5 or 10^4 CFU). On the other hand, a lower dose of *M. tuberculosis* (10^3 CFU) causes a chronic, slowly progressive, and localized form of pulmonary TB, while the significant proportion of monkeys might be challenged with 10^2 or 10^1 CFU to induce the infection in subclinical state. In nonhuman primates, the cynomolgus macaque have been effectively used to simulate the TB infection and also primarily infected with low-dose aerosol concentrations (~25 CFUs). They manifested dormant infection and reactivation as they appear in humans. Capuano et al. (2003) concluded that cohort of macaques might be challenged with low-dose aerosol. Though indications for TB infection were provided by tuberculin skin test or lymphocyte proliferation assays to PPD, however, only 60% subsequently developed active TB. This model may be an attractive option to study the interaction between simian viral immunodeficiency (SIV) and TB as a model for human HIV/TB co-infection. Moreover, their antigens cross-react with immunologic reagents developed for human cells and tissue as well as with macaque-specific reagents and allow for immunohistochemical examination to understand the mechanisms of disease, although nonhuman primates are restricted owing to higher cost, handling difficulties and space requirements with BSL3 facilities as well as higher susceptibility to TB infection and capable of horizontally transmitting the disease.

Cattle model

In cattle model, the *M. bovis*-based infections have been successfully established and used to understand the molecular mechanisms of the TB infection. In context with the pathology, bovine TB appears to be similar to human TB in regard to granulomatous reactions and CMI; however, it differs in regard to cavitation. Cattle are a natural host for TB and the disease induces comparable pathological and immune responses to those which are seen in humans. The infection in cattle is mainly localized to the respiratory tract and clinical disease may take years to develop. Several immunological agents are also available to study infection in this species. The human *M. tuberculosis*-based infection was diagnosed by new IFN-γ release assays; originally, it was developed to diagnose TB in cattle (Vordermeier et al., 1999). Moreover, the cattle model is an eminent option for the secondary screening of TB vaccines as there is more of similarity between the disease in cattle and humans, and also, outbred animals could be used (Table 2.3).

Protocols

This section of the chapter deals with describing the established protocols for infected animal models of TB, subsequent processing for the analysis and for studying various aspects of biosafety that must be observed, in all the set of conditions. All the protocols are illustrated by means of flow charts.

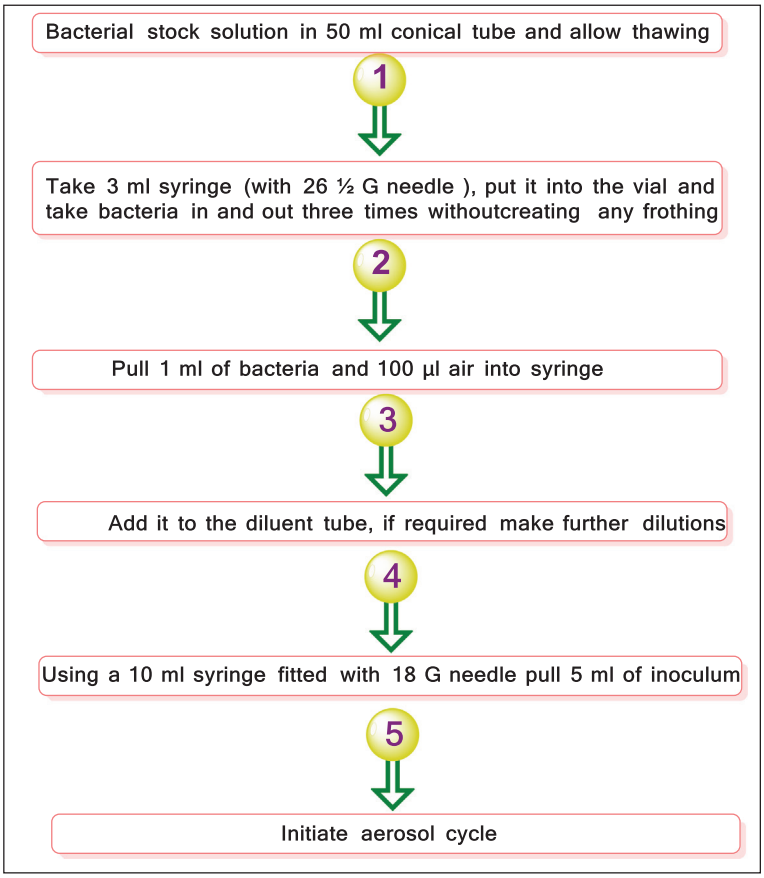
Preparing M. *tuberculosis* inoculum for aerosol exposure

From the known concentration of *M. tuberculosis* stock solution, dilutions are prepared to infect mice or pigs by aerosols. To verify the amount of inoculums used in the nebulizer, diluted aliquot of bacterial suspension is plated on 7H11 agar. Care should be taken during serial dilutions to prevent the generation of aerosol. All pipette tips, spills, or drops if any should be disinfected with 5% lysol (Flow Chart 2.1).

TABLE 2.3 Summary of current experimental models of TB and the particular aspects associated with these models.

Model	Pathology	Immunology	Genetics	Drugs	Vaccines
Zebra fish	Excellent for imaging of early pathology	Good for innate immunity	Good for making mutants		
Mice	Loosely organized granulomas	Extensive range of immunology reagents and recombinant animals	Diverse genetic backgrounds, wide range of mutants	Routinely used model, works for current drugs	Routinely used model
Guinea pigs	Well-structured granulomas, caseous necrosis	Limited range of immunology reagents		Routinely used model	Routinely used model, large ''window'' for BCG
Rabbits	Well-structured granulomas, caseous necrosis			Useful for assessing lesion–specific activities	
Cattle		Moderate range of immunology reagents	Well-defined lineages (Bostaurus, Bosindicus), cross-breeds and inbred herds		Experimental challenge and natural transmission models
Nonhuman primates	Range of lesions parallel those in humans	Most human immunology reagents can be used		Expensive; use for proof-of-concept	Mimics human response (highly diverse); use for proof-of-concept

FLOW CHART 2.1



Aerosol infection of mice using the middlebrook apparatus

Middlebrook airborne infection apparatus is most widely used in aerosol generation device. It is used to establish an animal model of pulmonary tuberculosis to resemble normal route and the site of infection in humans. This instrument consists of aerosol chamber that contains basket or cage with five compartments that can accommodate 25 mice. Compressed air flows through the nebulizer and produces a fine mist of bacterial suspension carried into aerosol chamber. This is inhaled by the animals. Finally UV lamp is used to decontaminate the surface (Flow Chart 2.2; Fig. 2.2).

Aerosol infection of guinea pigs using a madison chamber

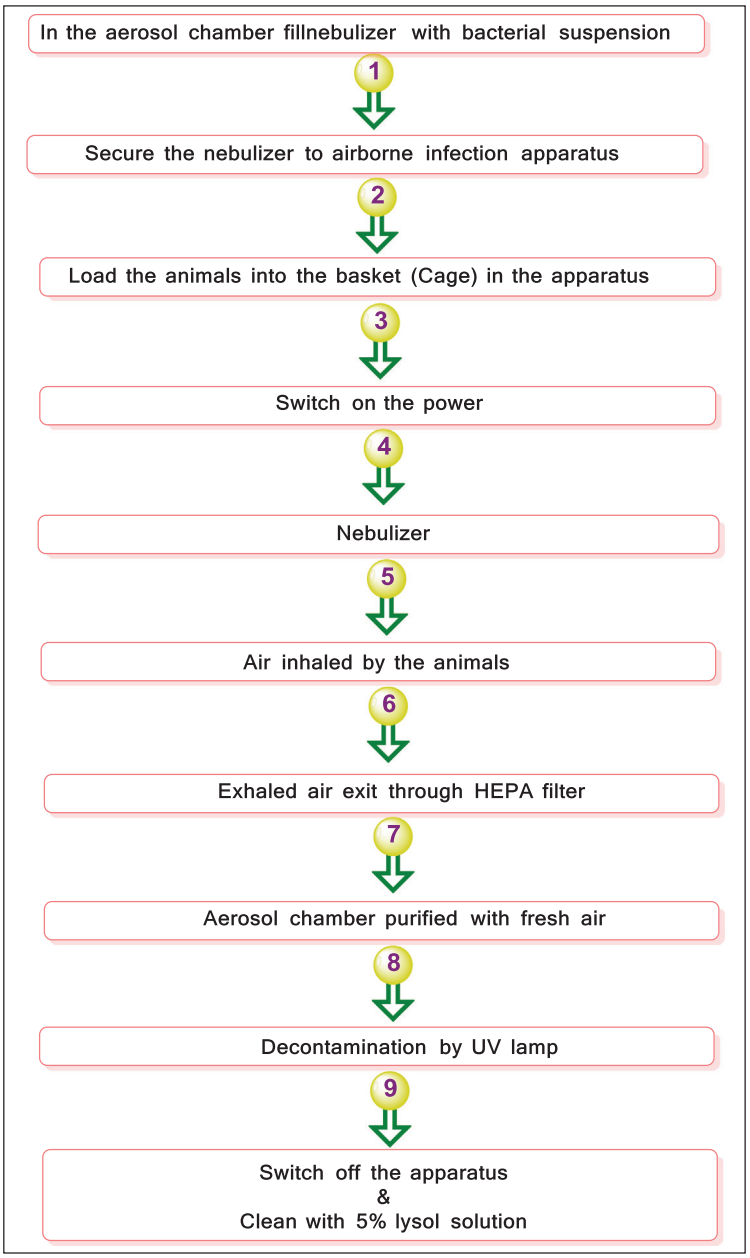
Madison chamber is aerosol generation device used for the guinea pigs because of their larger size. The surfaces of the Biosafety Class II Cabinet should be sterile with 5% Lysol and 70% ethanol

TEST RUN—to ensure adequate pressure driving (Flow Chart 2.3; Figs. 2.3 and 2.4).

Bacteria loading

Before the bacterial loading, each of the corresponding guinea pig cage should be labeled by writing aerosolized

FLOW CHART 2.2



with *M. tuberculosis* on date/initials. A stainless steel container filled with 5% lysol solution should also be placed next to the infection chamber (Flow Chart 2.4).

FIGURE 2.2 Glass-Col apparatus. This instrument is the advancement of earlier models developed by Middlebrook. Eighty to hundred mice can be exposed to aerosol at one time.

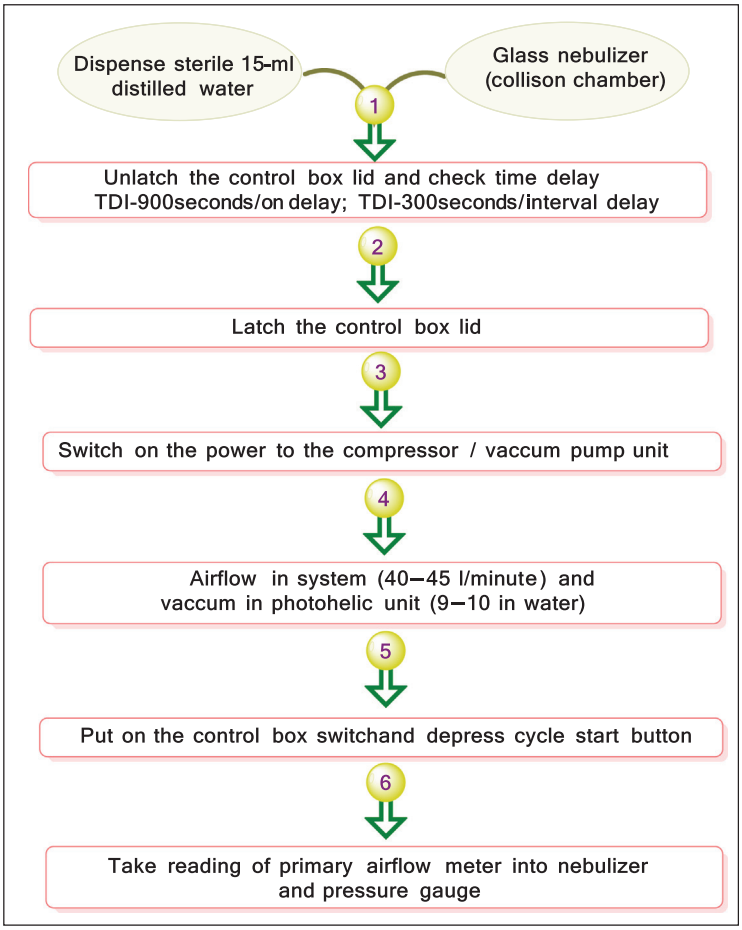
Intravenous infection of mice with *M. tuberculosis*

Intravenous infection requires absolute concentration and good eyesight with no distraction. Mouse is placed in a restraint device so that the tail can be immobilized, and then, the injectate is injected into the lateral vein (Flow Chart 2.5).

Isolation of samples for determining *M. tuberculosis* load by real-time-PCR

Expression of the multiple genes during the infection of *M. tuberculosis* can be measured by isolation of total RNA and then running polymerase chain reaction (PCR) assay. During PCR assay, contamination of test samples with RNAase and extraneous sources of DNA should be prevented. The samples containing RNA and cDNA should be dealt in separate working areas. The protocol describes sample collection from the infected tissues to be processed for the PCR analysis (Flow Chart 2.6).

FLOW CHART 2.3



Determination of bacterial loads in target organs

Processings of infected animal should be done in a biosafety cabinet. All the necessary items should be kept in the biosafety hood without restricting the airflow. Animals should be euthanized according to the guidelines provided by the IACUC. Due to the chronic nature of the infection, the design of the study depends on the time course to be studied. The number of animals per time point should also be considered which can be determined through statistical power calculation. For the bacterial load curve, a power of >0.8 can be achieved using four to five mice (Flow Charts 2.7 and 2.8).

The process includes the following:

1. preparation of agar plates;
2. bacterial count set up;
3. necropsy;
4. homogenization of tissues;
5. plating; and
6. bacterial count colonies.

Preparation of lungs or other tissues for histology

Useful information about the host response can be acquired by the histological examination of organs from infected animal. The size of granulomas, their make up in terms of lymphocytes and macrophages, the degree of lung tissue they are consolidating, the development of necrosis, and so forth can be invaluable information. It is important that the specimens are frozen as soon as they are collected to preserve their morphology and integrity of the antigens. Sections are stained with hematoxylin and eosin after fixation and paraffin embedding (Flow Chart 2.9).

Preparation of lung cell suspension

Cell suspensions from naïve and *M. tuberculosis*-infected mice are used for single cell analysis studies by flow cytometry by cell culture. Cells from the culture of infected animals provided the information such as cell activation, proliferation, and cytokines as well as chemokines production (Flow Chart 2.10).

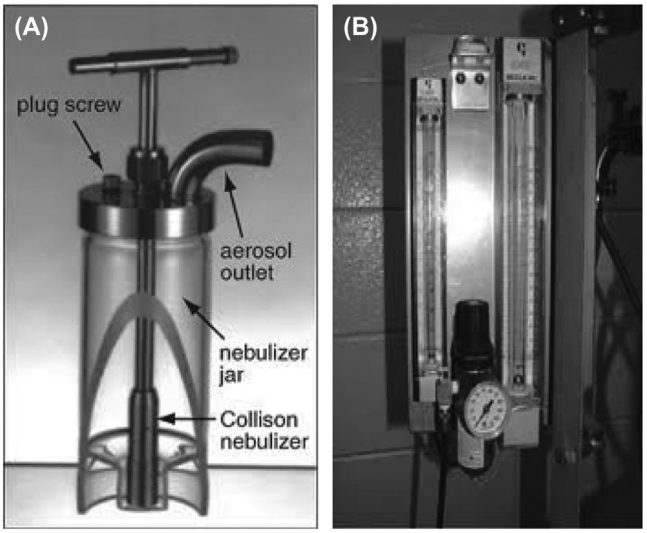


FIGURE 2.3 (A) Collison nebulizer unit, complete with the surrounding glass nebulizer jar, for the Madison guinea pig aerosol chamber, (B) Photohelic meter for Madison chamber.

Ethical issues

Infectious diseases such as TB continue to cause substantial morbidity and mortality. Continued research is critical to finding safe and effective ways to prevent and treat infectious diseases. The challenge experiment is an important method that is sometimes used to study the pathogenesis of infectious diseases and, especially, to evaluate the initial efficacy of vaccines before large-scale field tests are conducted.

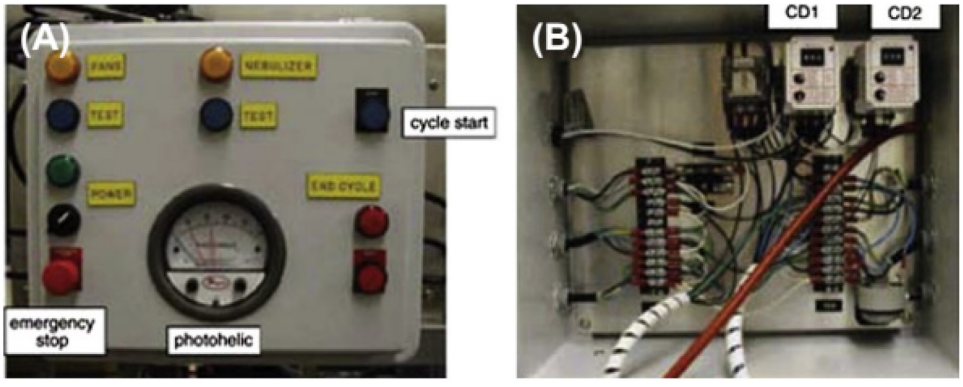
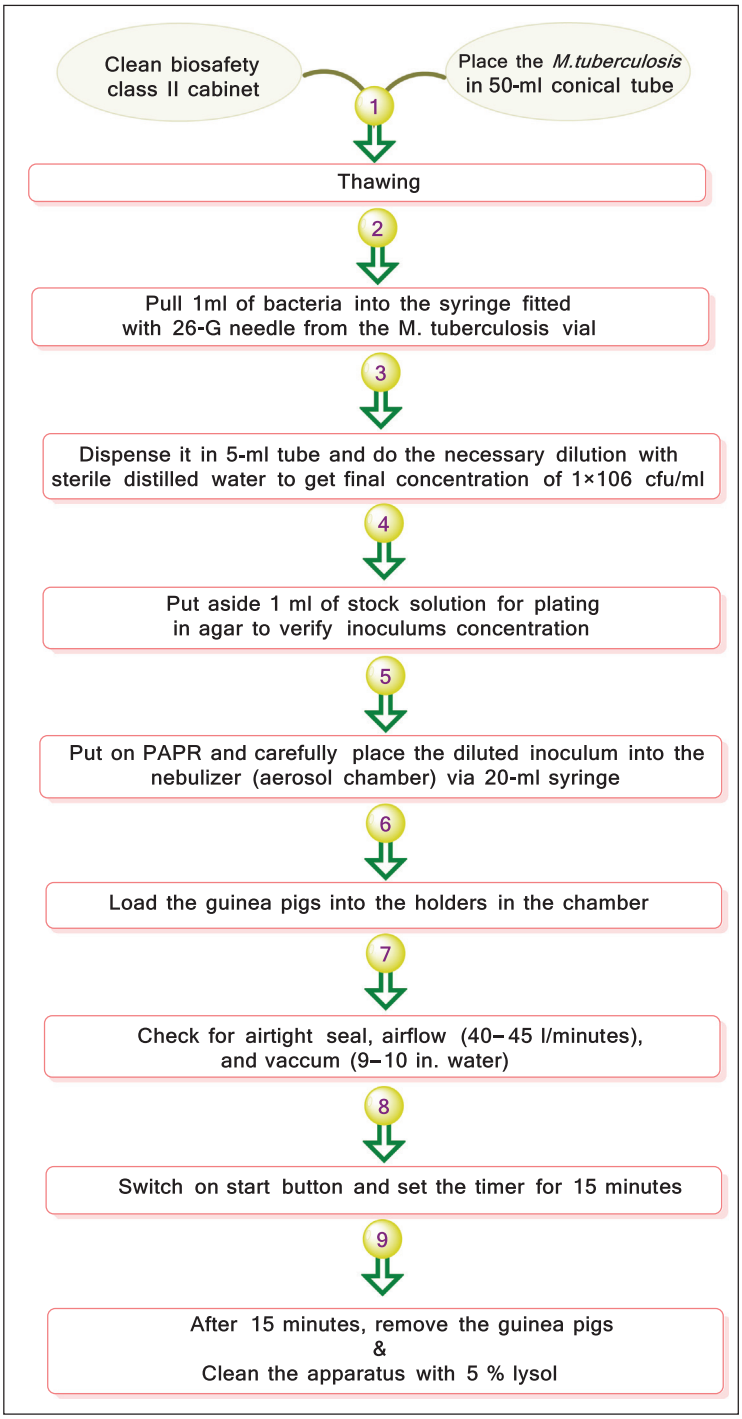


FIGURE 2.4 (A) The exterior and (B) interior of the Madison chamber control box.

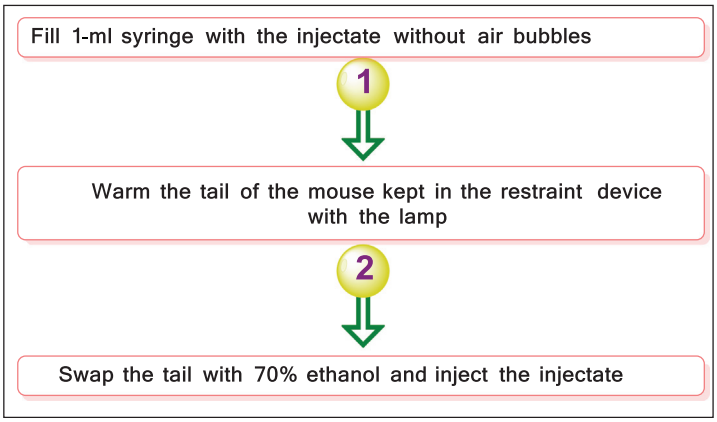
FLOW CHART 2.4



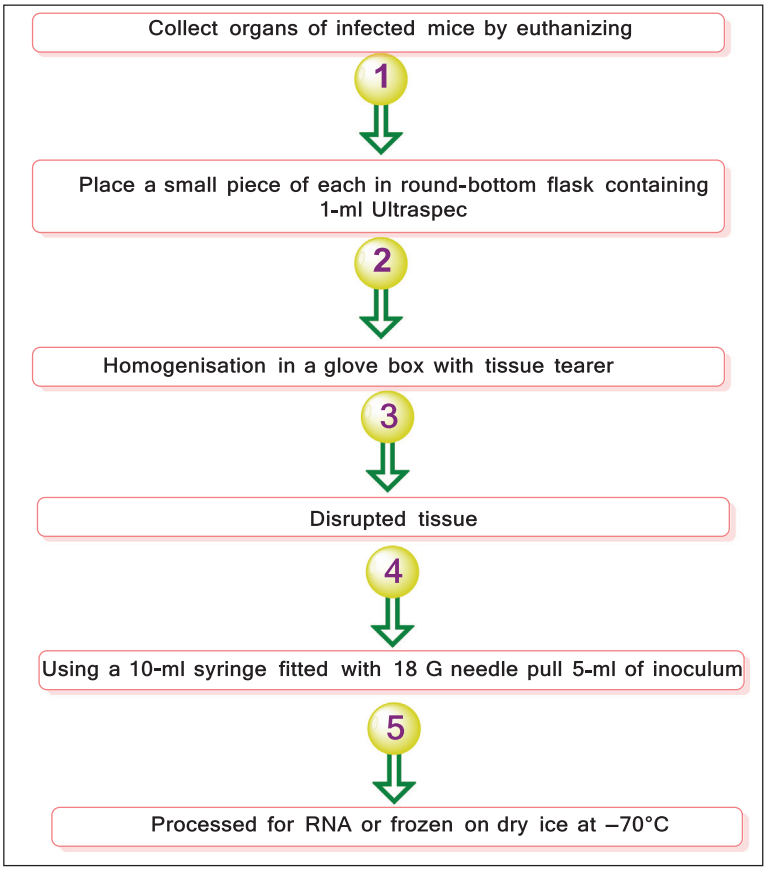
In challenge experiments, infections are deliberately induced under carefully controlled and monitored conditions, usually in inpatient settings. Research volunteers are exposed to bacteria, viruses, or parasites. Induced infections are usually either self-limiting or can be fully treated within a relatively short period of time.

Experiments conducted by physician investigators designed to cause infections that have uncomfortable symptoms in human subjects are likely to evoke serious moral concern. The Three R's principle (Replacement of animal experiments with alternative approaches, Reduction of animal numbers and Refinement to improve animal welfare) emerged as a way for scientists to ease this dilemma by developing research methods that decrease pain and distress. Nevertheless, the use of animals in research is still controversial, with recent voices also questioning the translational validity into humans.

FLOW CHART 2.5



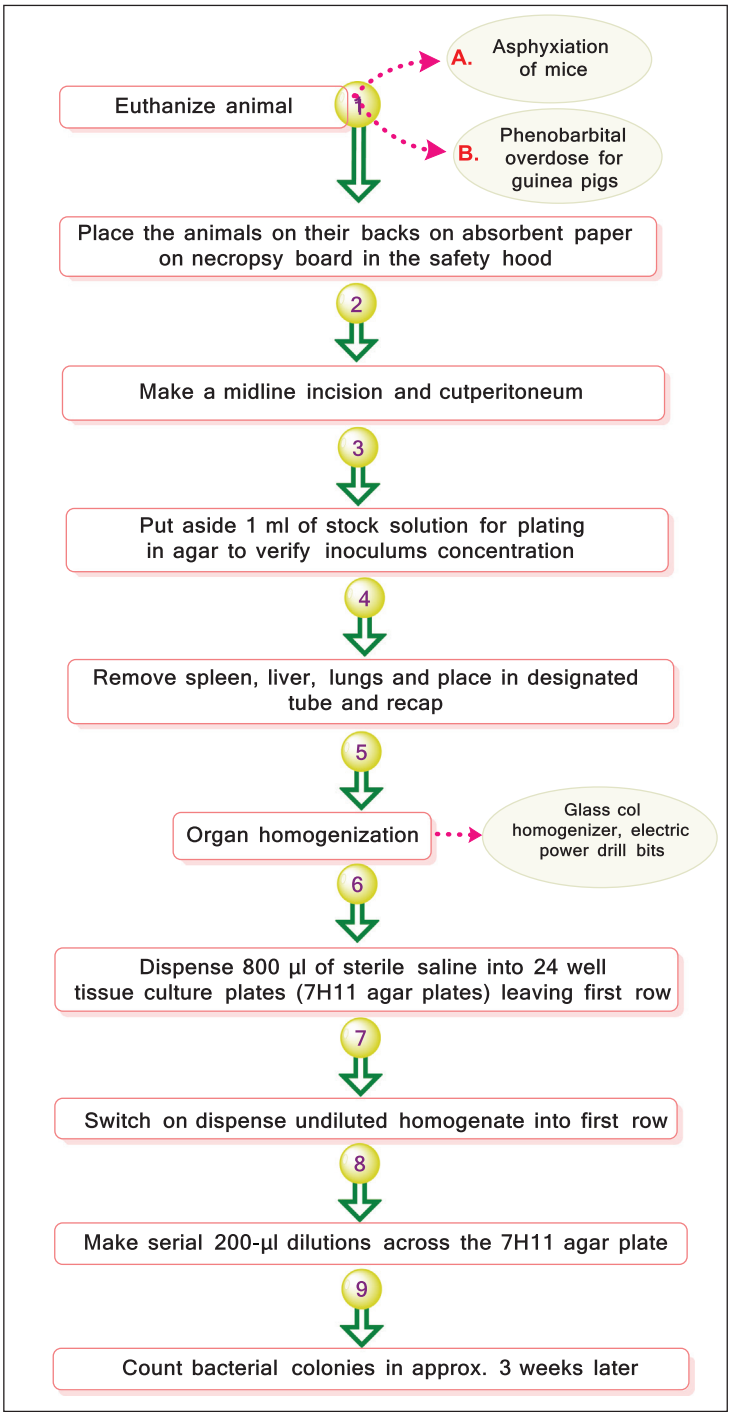
FLOW CHART 2.6



Franco et al. (2012) identified the main sources of animal distress and to assess the possible implementation of refinement measures in experimental infection research, using mouse models of TB as a case study. Table 2.4 briefly describe the most relevant experimental procedures with an impact on animal welfare, as well as the main welfare issues raised by the manifestation of active disease.

Literature published between 1997 and 2009 was analyzed, focusing on the welfare impact on the animals used and the implementation of refinement measures to reduce this impact. The number of articles per year increased almost fivefold between 1997 and 2009. Regarding genetic status of the animals, the majority of studies (71%, overall for all years) used non-genetically modified inbred strains. Information on the sex of animals used was not available in 34% of articles. The proportion of articles reporting to induce experimental *M. tuberculosis* infection through aerosol exposure rose significantly between 1997 and 2009.

FLOW CHART 2.7

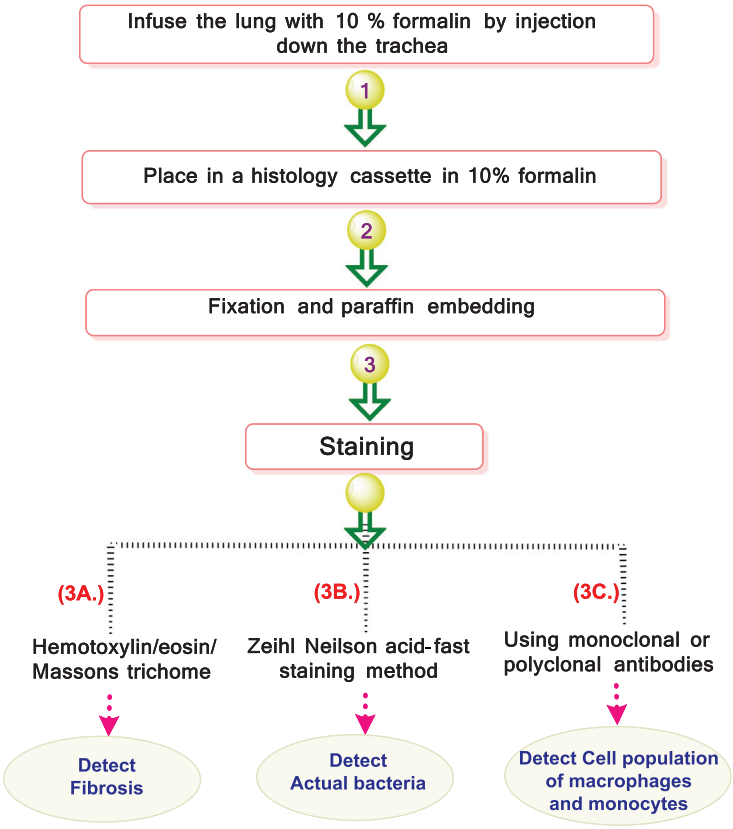


The use of the intravenous route—originally the most recurrent method—decreased, whereas the use of the intratracheal route remained relatively stable throughout the analyzed period. The intraperitoneal route was the least chosen.

Information on important research parameters, such as method for euthanasia or sex of the animals, was absent in a substantial number of papers. With no significant differences between years, 80% of the articles omitted this information. Moreover, when information on euthanasia was given, it was often incomplete and therefore difficult to interpret. For example, anesthetic overdose was often reported without indicating the route, compound, or dose, and exsanguination was frequently referred to with no indication of whether to be done under anesthesia or not.

In this 12-year period, it was observed that a rise in reports of ethical approval of experiments has taken

FLOW CHART 2.8



place. The proportion of studies classified into the most severe category did, however, not change significantly over the studied period. The majority of the studies analyzed were terminated before infected animals reached very severe morbidity, whereas the remaining allowed mice to reach terminal stages, with no significant variation in this proportion across years.

Overall, the study showed that a progress has been made in the application of humane endpoints in TB research, but that a considerable potential for improvement remains. Of course, such measures should not be taken to the expense of research quality and relevance. The best way to avoid a conflict between ethical constraints and scientific motivations is probably for scientists to be proactive and initiate a critical discussion within their own field, rather than awaiting limitations imposed from outside. In the field of experimental studies of important infections such as TB, a reassessment of the need for such a large proportion of studies to involve end-stages of the disease seems particularly pertinent.

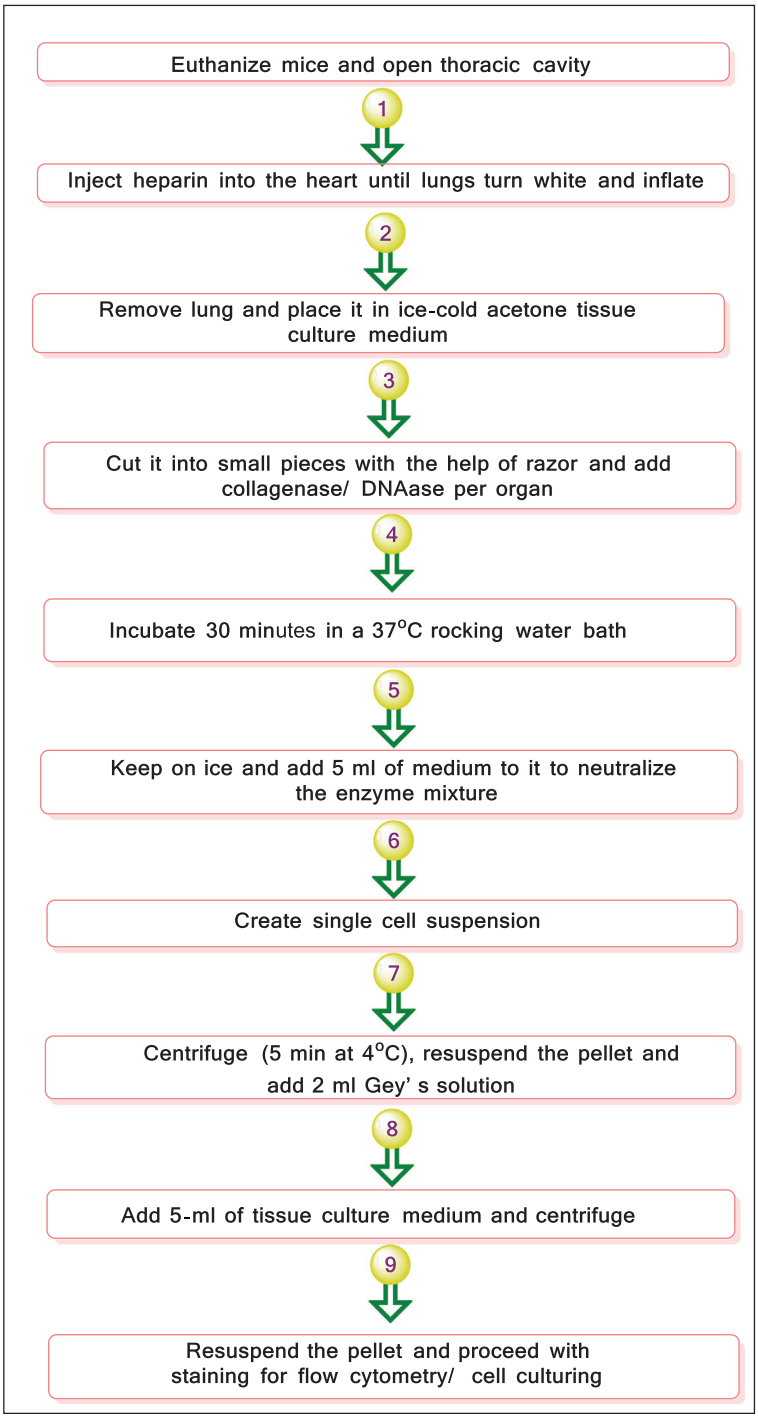
Translational significance

Animal models have and will continue to aid in early discovery as well as the pre-clinical testing phase of new drugs for efficacy and toxicity. The goal in modeling TB in animals is to mimic as closely as possible the pathology and clinical progression of the naturally occurring disease.

For practical or economic reasons, some species are more widely used for efficacy studies while others are preferred for pharmacokinetic and toxicity studies. For example, despite the documented differences in the immune response between mice and humans, mice are still the most widely used animal model for studying the immunological responses to *M. tuberculosis* infection and TB vaccines. However, due to species-specific differences in disease progression and lesion morphology, responses to drug therapy in mice may or may not reflect the desired effects in people.

The differences in lesion morphology among the different animal species infected with *M. tuberculosis* provide different levels of stringency for testing new drugs. Mouse strains that develop only solid lesions are best suited for discovery and early testing of drugs for in vivo effects and toxicity. Certain highly susceptible mouse strains have the added benefit of not only developing necrotic lesions but also having a more rapid disease progression, thus shortening the in vivo testing intervals. Species such as guinea pigs and cotton rats provide a wider variety of lesion types that include necrotic and mineralized lesions for a higher

FLOW CHART 2.9



level of in vivo testing stringency and to test adjunct therapies against novel therapeutic targets. The nonhuman primate and rabbit models develop an even wider variety of lesion types and are the most appropriate models to test drugs specially designed to treat cavitary lesions.

Since experimental *M. tuberculosis* infections are progressive in the majority of model species, all are suitable for testing the effects of drugs on extrapulmonary lesions.

World wide web resources

The sixteenth global report on tuberculosis (TB) published in 2011 by WHO is in a series that started in

FLOW CHART 2.10

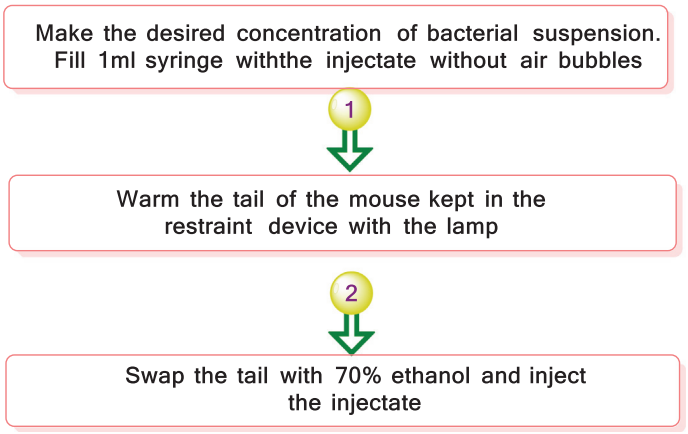


TABLE 2.4 Potential causes of pain and distress in studies on experimental infection with *M. tuberculosis*.

Infection route	
Intratracheal instillation	Surgical procedure under general anesthesia: Inoculum delivery through an incision in the trachea, that heals in 2–3 days
Intraperitoneal injection	This injection method offers no possibility to visually confirm correct delivery, and accidental penetration of the bladder, intestine, muscular or fatty tissue may occur.
Treatment administration	
intraperitoneal injection	This injection method offers no possibility to visually confirm correct delivery, and accidental penetration of the bladder, intestine, muscular, or fatty tissue may occur
Repeated oral gavage	Difficult procedure with the risk of fluid aspiration by the lungs or perforation of esophagic or gastric wall. Irritation, swelling, and ulceration of the esophagus from repeated dosing. Unexpected deaths as well as inappetence and weight loss reported in experimental infection studies. Reports of increased TB susceptibility due to gavage-induced stress
Immunization	
Footpad immunization	Immune reaction to antigen, causing swelling and inflammation in situ, potentially causing pain and lameness
Intramuscular immunization	Painful injection that may cause mechanical trauma and potential nerve damage; immune reaction may lead to painful swelling
Health status	
Signs of disease	Respiratory distress, hunched posture, lack of grooming; failure to eat or drink, fever, severe cachexia. Increasingly severe clinical signs, progressing to a hypokinetic irresponsive ("moribund") state, culminating in death.

1997. It provides a comprehensive and up-to-date assessment of the TB epidemic and progress in implementing and financing TB prevention, care, and control at global, regional, and country levels using data reported by 204 countries and territories that account for over 99% of the world's TB cases (Global Tuberculosis Control 2011: http://www.who.int/tb/publications/global_report/2011/gtbr11_full).

The official website of the Nobel Prize can be assessed for exploring the work of Emil von Behring who got Nobel prize in Physiology or Medicine in 1901 for his work on serum therapy, especially its application against diphtheria, by which he has opened a new road in the domain of medical science and thereby placed in the hands of the physician a victorious weapon against illness and deaths (http://nobelprize.org/nobel_prizes/medicine/laureates/1901/behring-lecture.html).

Laboratory Biosafety Manual published by WHO provide practical guidance on biosafety techniques for use in laboratories at all levels. The manual covers risk assessment and safe use of recombinant DNA technology, and provides guidelines for the commissioning and certification of laboratories. Laboratory biosecurity concepts are introduced in the manual, and the latest regulations for the transport of infectious substances are reflected. Material on safety in health-care laboratories, previously published elsewhere by WHO, has also been incorporated (http://www.who.int/csr/resources/publications/biosafety/WHO_CDS_CSR_LYO_2004_11/en/). Further, WHO also provides Tuberculosis Laboratory Biosafety Manual (http://apps.who.int/iris/bitstream/10665/77949/1/9789241504638_eng.pdf) where the recommendations are based on assessments of the risks associated with different technical procedures performed in different types of TB laboratories; the manual describes the basic requirements for facilities and practices, which can be adapted to follow local or national regulations or as the result of a risk assessment.

Safety considerations

The basis of safety in a laboratory is facility design, fail-safes, precise training, and an exposure control plan. Biosafety Level III laboratories are defined as the laboratories where defined air handling systems are installed that remove air under negative pressure many times per minute. These can be movable structure-like biobubble or solid structures and operate best as standalone buildings. At least tandem HEPA filter systems are required which are supposed to be regularly monitored. A high level of safety should also be maintained by the people working in the laboratory. For example, technician loading a nebulizer of an aerosol chamber should wear Occupational Safety and Health Administration (OSHA)-approved mask with a respirator on the top. It is also advisable that a technician may work with a colleague to check procedures and to ensure that there are no distractions. After the completion of every aerosol run, all the safety clothing should be autoclaved prior to next use. Only well-trained technicians should be allowed to work with *M. tuberculosis*. They should also be trained for actions needed in case anything go wrong while in the Biosafety Level III area. Biosafety officer must be appointed who should review all the procedures regularly and keep an eye on the agents going to be used. Clinical samples of *M. tuberculosis*, which may include drug-resistant isolates, should be listed by the Biosafety Officer, and the laboratory responsible should know their location (usually in a −70∘C freezer) and the number of vials present at any time. Official guidelines for the respective countries should be strictly followed by these laboratories.

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Glossary

Ecotype A population of a species that survives as a distinct group through environmental selection and isolation and that is comparable with a taxonomic subspecies
Caseous necrosis A form of cell death in which the tissue maintains a cheese-like appearance. The dead tissue appears as a soft and white proteinaceous dead cell mass.
Gavage Forced feeding by means of a tube inserted into the stomach through the mouth.
Cavitation The formation and then immediate implosion of cavities in a liquid—that is, small liquid-free zones ("bubbles")—that are the consequence of forces acting upon the liquid.
Paribacillary Having or made up of few bacilli.

Abbreviations

AUC	area under the curve
BCG	Bacillus–Calmette–Guérin
BSL	biosafety level
CFUs	colony-forming units
CMI	cell-mediated immunity
DTH	delayed-type hypersensitivity
HIV	human immunodeficiency virus
IFN-γ	interferon-gamma
INH	isoniazid
MDR	Multidrug resistant
NK cells	Natural killer cells
PPD	purified protein derivative
PZA	pyrazinamide
RIF	rifampicin
SIV	simian viral immunodeficiency
TB	tuberculosis
TNF-α	tumor necrosis factor-alpha
WHO	World Health Organization
XRD	extensively drug resistant

Long-answer questions

1. Discuss in detail the pathogenesis of tuberculosis and pathogenic diversity in different animal species?
2. What different animal models for tuberculosis teach us about the disease?
3. Write an exhaustive note of mouse model of tuberculosis?
4. Give a comparative outline of different animal models of tuberculosis?
5. Discuss in detail the established protocols of aerosolized infections of mouse and guinea pig using different apparatus?

Short-answer questions

1. With the help of flow chart discuss how mice can be infected with *M. tuberculosis* intravenously?
2. Write in brief the initial findings of Robert Koch?
3. Summarize the lessons learnt from animal models of tuberculosis?
4. Write in brief the importance of rabbit as model for tuberculosis?
5. What are the characteristics of models of tuberculosis with respect to infection and pathogenesis?

Answers to short-answer questions

1. Intravenous infection of the mouse is not by itself a difficult procedure, but requires investigators expertise. The procedure is as follows:
2. Robert Koch recognized and reported the spectrum of pathology of TB in different animal species based on his seminal studies on TB. Through experiments, he describe TB as an infectious disease caused by *M. tuberculosis*. He was one of the first people to envisage a vaccine for the control of TB. After 8 years of discovering the etiologic agent of tuberculosis, he announced the means of curing this disease. He suggested a vaccine for both the prevention and treatment of TB. Despite his first reports of a remedy for tuberculosis, studied in the guinea pig model, clinical trials soon demonstrated the ineffectiveness of his therapy.
3. Artificially infected guinea pigs, mice, and rabbits have served as indispensable tools through which important facets of TB have been studied. Results, however, are usually not entirely reflective of TB infection and disease in humans. Substantial differences in TB susceptibility, disease patterns, and temporal course exist among species. In addition to these considerations, animal species vary based on size, laboratory space requirements, rearing costs, and ability to approximate the disease process in humans. Despite several important differences, the murine, rabbit, and guinea pig models have emerged at the forefront of TB research.
4. Rabbits showed a higher resistance with *M. tuberculosis* infection. The rabbits are infected with

bovine mycobacterial infection, that is, *M. bovis*; their pulmonary pathology to inhaled bovine tubercle infection is more similar to human *M. tuberculosis* infection than those recorded in the case of other models such as mice and guinea pigs. In rabbits, the pulmonary cavity is first developed followed by bronchial spread of microorganisms. Rabbits are also employed as model for studying the latent or paucibacillary TB states in humans.

5. The characteristics of different models are summarized in tabular form as follows:

Model	Histopathology			Relative susceptibility to *M. tuberculosis*	Immunologic reagents available	Laboratory space and cost
	Necrosis	Caseation	Cavitation			
Mouse	Minimal	Usually not	No	Low	Extensive	Relatively small
Rabbit	Yes	Yes	Yes	Very low	Moderate	Relatively large
Guinea pig	Yes	Yes	Infrequent	Very high	Relatively few	Moderate
Nonhuman primate	Yes	Yes	Yes	High	Extensive	Large

Yes/no-type questions

1. Mice are generally more resistant models for TB infection as compared with rabbits, guinea pigs, and even humans. True/False
2. Who recognized and reported the spectrum of pathology of TB in different animal species?
3. Murine macrophages use which receptors on their cell surfaces to identify the mycobacterial antigens?
4. Nonhuman primates are also successfully employed as models for latent TB infection. True/False
5. Rabbits are also employed as models for studying the latent or paucibacillary TB states in humans. True/False
6. Guinea pigs models have been used to create models of TB transmission due to their exceptional susceptibility to infection with a few inhaled mycobacteria. True/False
7. Name the components of innate immune system?
8. Which strain of mice (C57BL/6 or BALB/c) are more resistant in terms of survival time post-infection with the decline in colony-forming unit (CFU) counts after the onset of adaptive immunity?
9. Guinea pigs may be useful as a model to study which type of TB infection?
10. Susceptibility of rabbits for infection varies with the strain of bacteria.

Answers for yes/no type questions

1. True
2. Robert Koch
3. Toll-like receptors (TLRs)
4. True
5. True
6. True
7. Neutrophils, dendritic cells, macrophages, mast cells, NK cells, NKT cells.
8. C57BL/6
9. Persistent TB *infection*
10. True

CHAPTER

3

Animal models for neurodegenerative disorders

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Summary

Recent advances in research on neurodegenerative disease have been made through animal models recapitulating human genetic mutations. This chapter aims to help readers for better understanding of basic researches on neurodegenerative diseases with a focus on motor neuron diseases, such as amyotrophic lateral sclerosis (ALS), spinal and bulbar muscular atrophy (SBMA), and spinal muscular atrophy (SMA). First, the clinical and genetic information for these diseases are intensively described. In addition, methodologies of engineering mouse models as well as characteristics of animal models of motor neuron diseases are provided. Moreover, the recent research progress of disease mechanisms using animal models is discussed.

What you can expect to know

In this chapter, you expect to know the clinical and genetic overview of neurodegenerative diseases, with a focus on motor neuron diseases, such as ALS, SMA, and SBMA, as a basis for generating animal models. For modeling motor neuron diseases in mice, we provide practical information including construction of transgenic mouse and Cre-loxP technology for conditional gene deletion or expression in mice. Finally, we review recent research progress through establishment and analysis of the animal models for neurodegenerative diseases.

History and methods

Introduction

Neurodegenerative diseases are progressive neurological disorders characterized by the death of specific nerve cells, excluding conditions such as ischemia, infection, intoxication, and malignant tumors. Representative examples include Alzheimer's disease (AD), which is the most common cause of dementia and compromises cognitive and memory functions of patients, and Parkinson's disease (PD), which is a progressive movement disorder exhibiting symptoms such as tremors, increased muscle tone, and slow movements. Many neurodegenerative diseases are genetically inherited. Through recent advances in genetics, many causative genes for neurodegenerative diseases have been identified. The breakthrough discovery of dominant mutations in amyloid precursor protein genes as causative for the familial AD in 1991 led researchers to study molecular mechanisms of neurodegenerative disease. Subsequently, the causative genes enable us to further investigate the pathomechanism of diseases by generating animal models recapitulating human neurodegenerative diseases. In this chapter, we first provide an overview of neurodegenerative diseases and then describe recent advances in neurodegenerative disease research, particularly focusing on motor neuron diseases through establishment and analysis of genetically modified mouse models.

Neurodegenerative diseases

Representative neurodegenerative diseases include AD, PD, frontotemporal lobar degeneration (FTLD), Huntington's disease (HD), spinocerebellar degeneration (SCD), and ALS. One of the characteristics of neurodegenerative diseases is death of specific type of neurons. In AD patients, the hippocampus, which is crucial for memory and learning, is affected at an early stage, and the disease spreads to other brain lesions as the disease progresses. In PD, FTLD, HD, SCD, or ALS patients, dopaminergic neurons in the midbrain,

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neurons in frontal and temporal lobes of cerebral cortex, neurons in basal ganglia, cerebellar neurons, or motor neurons are specifically affected, respectively. Here, the characteristics of well-known neurodegenerative diseases are summarized (Table 3.1). Some diseases (such as HD) are all genetically inherited, while others (such as AD, PD, and ALS) are not. Animal models have been generated for almost all diseases listed here based on the causative genes that have been identified.

The motor neuron diseases make up the group of neurodegenerative diseases that is characterized by selective death of the neurons controlling motor functions, without affecting other neural systems, such as sensory and cognition. The motor neuron diseases include ALS, SMA, SBMA, and other miscellaneous disease conditions. In the motor pathway, the signals from nerve cells in the primary motor cortex of the cerebrum (which are called as "upper motor" neurons) are transmitted to the nerve cells in the brain stem and the spinal cord (which are called as "lower motor" neurons). The signals from lower motor neurons then reach the muscles innervated by the particular motor neurons. Both upper and lower motor neurons are affected in ALS, while only lower neurons are affected in SMA or SBMA. Clinical information and overview of ALS, SMA, and SBMA are provided later as an introduction of this chapter.

Amyotrophic lateral sclerosis

ALS is the most common form of adult motor neuron disease. As first described by the French neurologist, Charcot, in 1869, the primary symptom of the disease is linked to the premature death of upper and lower motor neurons starting in adulthood. Neuronal death results in progressive paralysis of muscle in limbs and swallowing muscles, which is typically fatal in 2–5 years after the onset due to respiratory muscle paralysis. The disease prevalence is roughly same worldwide (6 in 100,000 population). The disease onset is usually middle to late in life (50–70 years old), but 10% of cases are affected before age 40. Males are slightly more affected than females (incidence of male patients is 1.5–1.8 times higher than female patients). Often the hands or feet (distal part of limbs) are affected first, while swallowing muscles are first affected in 10%–20% of cases. Sensation and bladder functions are spared. Among the motor functions, eye muscles are not usually affected, providing a clue to understand the mechanism of selective motor neurodegeneration. In many cases, intelligence is not affected; however, 10%–30% of ALS patients show cognitive decline.

Although most of the ALS cases are sporadic, 10% of ALS cases are inherited. To date, researchers have identified approximately 25 ALS causative genes, the investigation of which leads to many hypotheses to explain the pathomechanisms of ALS (Table 3.2).

TABLE 3.1 Representative neurodegenerative diseases.

Name of disease	Prevalence	Percent of familial cases	Main clinical symptoms	Affected brain area
Alzheimer's disease (AD)	16% of age 65 and older (United States)	1	Progressive disturbance of memory, learning, and cognition	Hippocampus, cerebral cortex
Parkinson's disease (PD)	1%–2% of age 60 and older	5–10	Involuntary movement (tremor), clumsiness, rigidity	Midbrain (dopaminergic neurons)
Frontotemporal lobar degeneration (FTLD)	15–20/100,000 of age 45–64	≈30	Progressive disturbance of cognition, abnormal behavior, or a loss of language	Frontal and temporal lobe of cerebral cortex, hippocampus
Spinocerebellar degeneration (SCD)	10–20/100,000 populations	30–40	Progressive loss of balance and coordination of walking, speech	Cerebellum
Huntington's disease (HD)	0.3 (Asia), 3–7 (United States, Europe)/100,000 populations	100 (autosomal dominant)	Progressive chorea, cognitive impairment, psychiatric problem	Striatum
Amyotrophic lateral sclerosis (ALS)	6/100,000 populations	≈10	Progressive motor weakness and paralysis	Spinal cord and cerebral cortex (motor neurons)
Spinal and bulbar muscular atrophy (SBMA)	1–2/100,000 males	100 (X-linked recessive)	Slowly progressive proximal motor weakness and atrophy, infertility	Spinal cord (motor neurons)

A list of adult-onset neurodegenerative diseases with prevalence, percent of familial cases, main clinical symptoms, and affected brain area is provided.

TABLE 3.2 ALS causative genes.

Notation	Inheritance	Chromosome	Gene (year of identification)	Clinical features and comments
Genes identified				
ALS1	AD	21q22	SOD1 (1993)	Typical ALS (adult), 20% of inherited ALS
ALS2	AR	2q33	ALS2 (Alsin) (2001)	Infantile onset, slowly progressive
ALS4	AD	9q34	Senataxin (SETX) (2004)	Juvenile onset, slowly progressive
ALS5	AR	15q15	Spatacsin (alleic to SPG11)	Juvenile onset, slowly progressive
ALS6	AD	16q12	FUS/translocated in liposarcoma protein (fused-in sarcoma) (2009)	Typical ALS
ALS8	AD	20q13.3	VAPB (2004)	Typical and slowly progressive, heterogeneous
ALS9	AD	14q11	Angiogenin (ANG) (2006)	Typical ALS (adult)
ALS10	AD	1p36.22	TDP-43 (TARDBP) (2008)	Typical ALS (adult)
ALS11	AR	6q21	FIG4 (2009)	
HMN7B	AD	2p13	Dynactin p150 subunit (2003)	Slowly progressive, lower motor neuron disease
ALS12	AR	10p14−15	Optineurin (OPTN) (2010)	Adult onset
ALS13	AD	12q24	Ataxin-2 (ATXN2) (2010)	Susceptible gene (27−39 CAG repeats)
ALS14	AD	9p13.3	VCP (2010)	
ALS15	X-linked	Xp11.21	Ubiquilin 2 (UBQLN2) (2011)	X-linked dominant, affecting male and female
ALS16	AR	9p13.3	Sigma-1 receptor (SIGMAR1) (2011)	Juvenile onset, slowly progressive
FTD-ALS1	AD	9q21−22	C9orf72 (2011)	Accompanied with FTD, 20%−40% of inherited ALS
ALS17	AD?	3p11.2	CHMP2B (2010)	Lower motor neurons are predominantly affected
ALS18	AD	17p13.2	Profilin 1 (PFN1) (2012)	
ALS19	AD	2q34	ErbB4 (2013)	
ALS20	AD	12q13	HNRNPA1 (2013)	
ALS21	AD	5q31	Matrin-3 (2014)	
ALS22	AD	2q35	TUBA4A (2014)	With or without FTD
ALS23	AD	10q.22.3	ANXA11 (2017)	
ALS24	AD	4q33	NEK1 (2016)	
ALS25	AD	12q13	KIF5A (2018)	KIF5A mutation is also causative for hereditary spastic paraplegia
FTD-ALS2	AD	22q11.23	CHCHD10(2014)	AD?
FTD-ALS3	AD	5q35.3	SQSTM1(p62, 2013)	AD?
FTD-ALS4	AD	12q14.2	TBK1(2015)	AD?, Variable phenotype
Loci identified				
ALS3	AD	18q21	Unknown	Typical ALS
ALS7	AD	20p13	Unknown	Typical ALS

Despite many studies, however, the causes of motor neuron death have not yet been clarified. There are no effective therapies for ALS, since the one prescribed drug (riluzole) approved in many countries prolongs life by only 2–3 months (Mitchell and Borasio, 2007).

Spinal muscular atrophy

SMA is an autosomal recessive neurological disorder mainly affecting children. The reduced amount of survival of motor neuron (SMN) protein due to the recessive mutations in the gene for *survival of motor neuron 1* (*SMN1*) is generally regarded as causative for SMA. SMA only affects lower motor neurons, producing muscle weakness in limbs and the body trunk. The incidence of SMA is about 1 case in 6,000–20,000 live births, but it varies among ethnicities. The male-to-female ratio is about 2:1. SMA in childhood is classified into three types by the age of onset and severity of disease. SMA type 1, also known as Werdnig–Hoffmann syndrome, is the most severe form of the disease and is evident at birth or before the age of 6 months. Proximal muscles are more affected than distal ones, with progressive weakness, resulting in complete paralysis of all limbs and respiratory failure, and 85% of children die before the age of 2 years. SMA type 2, the intermediate form, usually becomes symptomatic from the age of 6 months to 1.5 years. The patients may be able to sit but unable to walk without support. The rate of disease progression is variable; however, some patients show respiratory failure upon infection. SMA type 3, also known as Kugerberg–Welander disease, is a mild form of disease. Onset of the disease is usually in late childhood to adolescence (between 1.5 and 17 years old). Children often achieve an independent gait; however, they slowly exhibit progressive muscle weakness of proximal limbs. The disease course is mostly benign, and many children are quite intelligent and live a normal life span (Lunn and Wang, 2008).

As explained in the following section, the human is the only species that has two highly conserved *SMN* gene copies, *SMN1* and *SMN2*. Although the classification of SMA type 1, 2, and 3 is defined by clinical information, the earlier disease onset and disease severity among SMA patients is generally correlated with the lower expression level of SMN protein derived from *SMN2*, copy numbers of which have large variation.

Spinal and bulbar muscular atrophy

SBMA (also known as Kennedy–Alter–Sung disease) is an adult-onset slowly progressive motor neuron disease affecting lower motor neurons. SBMA is an X-linked recessive inheritance form of SMA, which mainly affects men, and is caused by the abnormal expansion of a CAG trinucleotide repeat in the exon 1 of *androgen receptor (AR)* gene, the mutation of which was identified by Spada and colleagues in 1991. Female heterozygous and homozygous carriers are usually asymptomatic although some have subclinical phenotypes only evident in the electrophysiological examination of muscle and blood tests. The disease prevalence is 1–2 in 100,000 population. SBMA usually affects after the age of 40 with slowly progressive weakness of limb and swallowing muscles; patients are dependent on wheelchairs and susceptible to pneumonia in 10–15 years. Due to the deficient AR, testicular atrophy and decreased fertility are observed. Patients with SBMA do not show cognitive impairments. Like ALS patients, the brain stem motor neurons to control eye movements are not affected in SBMA.

The recent studies indicate that SBMA is caused by a gain of toxicity from the abnormal AR protein with polyglutamine expansion to neurons. Very importantly, toxicity requires a male sex hormone, an androgen (a ligand for the AR), which explains why SBMA affects male patients (Adachi et al., 2007).

Principles

To engineer the animal models recapitulating genetic mutations observed in human neurodegenerative disease patients, it is very important to understand the genetics of neurodegenerative diseases. In this section, an overview of genetics in ALS, SMA, and SBMA is provided as a principle to design the animal models recapitulating motor neuron diseases.

Genetics of amyotrophic lateral sclerosis

To date, more than 20 genes have been identified as causative for familial ALS. The list of causative genes for ALS is provided in Table 3.2.

Superoxide dismutase 1-amyotrophic lateral sclerosis

Twenty percent of inherited ALS cases are caused by dominant mutations in the gene encoding for superoxide dismutase 1 (SOD1). SOD1, a ubiquitously expressed enzyme, consisting of 153 amino acids, catalytically converts reactive superoxide to oxygen and hydrogen peroxide. Since it was first identified as an *ALS* gene in 1993, more than 140 different mutations in *SOD1* genes have been identified to date

(see Glossary). Most of them are missense mutations, while some are the frameshift mutations that result in truncated protein products of mutant *SOD1*. Many *SOD1*-mutated ALS cases show a milder disease progression rate than that of sporadic ALS, with mean disease duration of SOD1-ALS being about 5−10 years. Studies from ALS patients with *SOD1* mutations revealed that there are no correlations between enzymatic activities of mutants and the clinical course. In addition, some disease-causing *SOD1* mutations retain full enzymatic activity. Therefore, it is now generally recognized that all different mutations of the *SOD1* gene (both enzymatically active and inactive mutants) uniformly cause neurodegeneration, not by a loss of enzymatic activity, but rather by a gain of toxicity. This gain of toxicity hypothesis is established by studies using SOD1-ALS mice as described in the later sections (Bruijn et al., 2004).

Amyotrophic lateral sclerosis: genes implicated in RNA metabolism

TAR-DNA-binding protein 43

About 1%−3% of each inherited ALS case is caused by dominant mutations in the gene encoding for TAR-DNA-binding protein 43 (TDP-43). TDP-43 is an RNA- and DNA-binding protein consisting of 414 amino acids with two RNA recognition motifs (RRMs) and a *C*-terminal glycine-rich region. More than 30 mutations have been found in familial and sporadic ALS cases to date, and all of them are found in the C-terminal glycine-rich region, except for one mutation. TDP-43 is predominantly localized in the nucleus, shuttles between nucleus and cytoplasm, and regulates RNA splicing and mRNA stability. Examples of the well-known functions of TDP-43 in RNA metabolism are the splicing regulation of *CFTR* (a gene mutated in the inherited disease, cystic fibrosis) to enhance an exon skipping through binding to the UG-repeat of intron, the splicing regulation of *SMN* and *ApoAII*, and regulating mRNA stability of TDP-43 itself via direct protein binding to 3′-untranslated region of its own mRNA.

Importantly, in sporadic ALS cases (that are approximately 90% of total ALS cases), TDP-43 is lost from the nucleus and forms abnormal protein aggregates in cytoplasm of the affected motor neurons of spinal cords. This abnormal localization of TDP-43 is also seen in familial ALS caused by TDP-43 mutations. To date, it remains unclear whether the neurodegeneration in ALS patients' motor neurons are provoked by loss of function of nuclear TDP-43 functions, gain-of-function of abnormal cytoplasmic aggregates, or both of them. Nevertheless, elucidating the mechanisms through which abnormal TDP-43 leads to neurodegeneration will reveal the pathomechanism of sporadic ALS in which abnormal TDP-43 is accumulated (Da Cruz and Cleveland, 2011).

Fused-in sarcoma/translocated in liposarcoma protein

Another 5% of inherited ALS cases are caused by dominant mutations in the gene that encode for fused-in sarcoma (FUS; also known as translocated in liposarcoma protein). FUS is also an RNA- and DNA-binding protein consisting of two arginine/glycine-rich (RGG) domains, a Zn-finger domain, RRM, a glycine-rich region, and Q/G/S/Y-rich region. Most of the disease-causing mutations are located in the C-terminal region, with a nuclear localization signal, but some mutations are in the glycine-rich region. Abnormal aggregates of FUS protein in the cytoplasm or nucleus are found in affected motor neurons of spinal cords. FUS also shuttles between nucleus and cytoplasm and regulates transcription and RNA splicing. Considering the similarity between TDP-43 and FUS function and the domain structure of the protein, defects in RNA metabolism could be one of the major pathways leading to neurodegeneration of ALS motor neurons (Da Cruz and Cleveland, 2011).

C9orf72

A large hexa-nucleotide GGGGCC repeat extension within intron or 5′UTR of *C9orf72* gene on chromosomal 9q21 also causes dominantly inherited ALS (DeJesus-Hernandez et al., 2011; Renton et al., 2011). In Finland, this repeat expansion within *C9orf72* accounts for approximately 46% of familial ALS. In North America and Europe (except Finland), abnormal repeat expansion of *C9orf72* is responsible for about 20%−40% of familial ALS; however, it has barely been found in Asian ALS patients so far. All patients with the repeat expansion had the founder haplotype, suggesting a one-off expansion event occurred about 1500 years ago (Majounie et al., 2012). Expansion of the nucleotide repeat in the non-coding region often results in a gain of toxicity derived from abnormal RNA. Moreover, abnormal nuclear aggregation containing RNA with the GGGGCC repeat was found in ALS patients, suggesting that a gain of toxicity of C9orf72 transcripts with the GGGGCC repeat may cause motor neuron degeneration of ALS patients. More recently, non-ATG−dependent protein translation from GGGGCC repeat was shown to be involved in the pathomechanism of this disease. Dipeptide repeat-carrying proteins were accumulated in the brain region of patients. Therefore, both the protein- and RNA-based toxicity hypothesis was proposed for C9orf72-mediated ALS (Taylor et al., 2016).

From the viewpoint of ALS causative genes, ALS is highly related to defects in RNA metabolism. Other

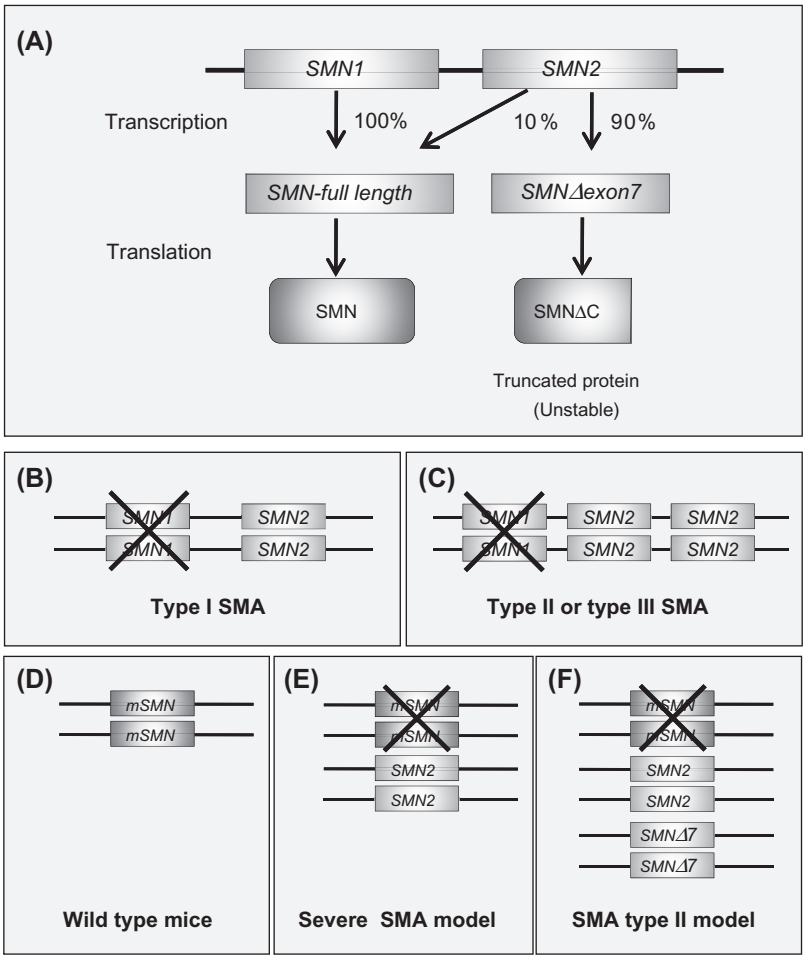


FIGURE 3.1 Genetic bases of SMA patients and SMA model mice. (A) Genomic organization of SMN locus in a human healthy individual. (B and C) Genomic organization of SMN locus in SMA patients. (D–F) Genomic organization of wild-type mice and SMA model mice.

ALS causative genes implicated in RNA metabolism include *senataxin* and *ataxin-2* (see Table 3.2). However, ALS causative genes for approximately 50% of inherited ALS cases remain unknown.

Genetics of spinal muscular atrophy

SMA is caused by a reduced amount of SMN proteins. This alteration is caused by the complex genetic molecular basis of SMA. Due to an interchromatin duplication on chromosome 5q13, humans possess two copies of the gene encoding SMN, *SMN1* and *SMN2*. Deletion or gene conversion events render SMA patients homozygous null for *SMN1* gene, whereas they maintain a variable copy number of *SMN2* gene. A critical C-to-T transition at position six of exon 7 causes aberrant splicing of 85%–90% of *SMN2* transcripts without exon 7, which encodes unstable truncated SMN protein. A small percentage of transcripts encodes full-length active SMN protein, and the SMN protein level correlates with disease severity (Fig. 3.1A–C) (Burghes and Beattie, 2009). Mouse studies demonstrated that complete loss of SMN protein causes embryonic lethality (Schrank et al., 1997).

SMN is widely and constitutively expressed and has been implicated in a wide range of cellular process, among which small nuclear ribonucleoprotein (snRNP) assembly is the best characterized. U-rich snRNP is the major component of the spliceosome, which carries out pre-mRNA splicing. SMN protein complex, consisting of several gemin family proteins, assists U-rich small nuclear RNA and seven Sm core proteins to form U snRNP. SMN is also implicated in mRNA transport in the axon of the nerve, the disturbance of which might explain the vulnerability of motor neurons in SMA (Burghes and Beattie, 2009). The gene encoding SMN is evolutionally conserved, and the effects of SMN protein reduction have been modeled in diverse organisms, including the invertebrates *Caenorhabditis elegans* and *Drosophila melanogaster* and the vertebrates *Danio rerio* and *Mus musculus*. Zebrafish and the invertebrate models are well studied to large-scale screening of drugs or genetic knockdown libraries prior to validation in mammals.

Genetics of spinal and bulbar muscular atrophy

Abnormal expansion of a CAG trinucleotide repeat in exon 1 of the *AR* gene localized in X chromosomes was identified as causative for SBMA. Since a CAG trinucleotide repeat encodes a stretch of glutamine (so-called polyglutamine), mutant AR protein encoded by the *AR* gene with longer CAG repeats contains an abnormal glutamine stretch. Normally, the number of CAG repeats in the *AR* gene ranges between 14 and 32, while it ranges between 40 and 62 in SMBA patients (Adachi et al., 2007). An inverse correlation has been reported between the age of disease onset and the number of CAG repeats, suggesting that the expansion of polyglutamine repeats in the AR exhibits a gain of toxicity in motor neurons. This inverse correlation is also seen in other polyglutamine diseases, such as HD, indicating that abnormal expansion of polyglutamine causes neurodegeneration through a gain of toxic mechanism.

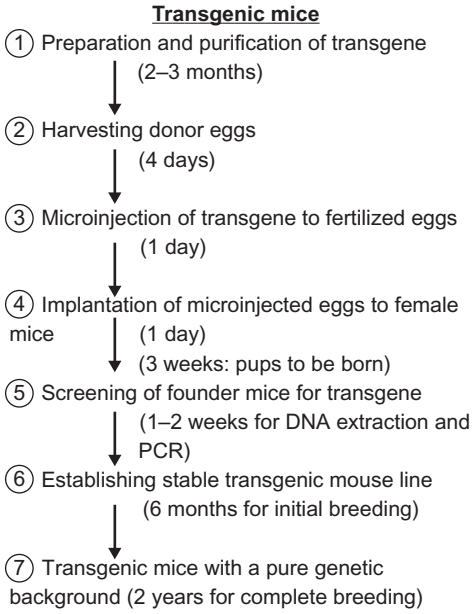
As revealed by the analysis of SBMA model mice, SBMA is not caused by a loss of AR function, but is rather caused by a gain of toxicity from abnormal AR proteins with polyglutamine expansion to neurons. The pathomechanism unique to SBMA is that the mutant AR proteins require androgen, a male sex hormone, to provoke toxicities to motor neurons, which is linked to androgen-dependent translocation of mutant AR to the nucleus.

Methodology

Genetically modified mice for modeling human diseases include transgenic mice, knockout mice, conditional knockout mice, and knock-in mice. In this section, a methodology for generating transgenic mice is provided. In addition, principle and technical details for Cre-loxP technology are discussed. These methods are used to generate the mouse models described in the "Examples and their applications" section. The list of the mice described here includes $SOD1^{G37R}$, $SOD1^{G93A}$, and $SOD1^{WT}$ transgenic mice as models for ALS, SMA model mice, and AR-97Q and AR-24Q transgenic mice as models for SBMA.

Generation of transgenic mice

The brief protocol for generating transgenic mice is provided in this section according to the one used in our institute. The protocol consists of six basic steps: (1) preparation and purification of transgenic construct, (2) harvesting donor eggs, (3) microinjection of transgene to the fertilized egg, (4) implantation of microinjected egg to the pseudopregnant female mice,



FLOW CHART 3.1 A BRIEF PROTOCOL AND TIMELINE FOR GENERATING TRANSGENIC MICE.

(5) screening of founder mice for transgene expression, and (6) establishing a stable transgenic line. The timeline is shown in Flow Chart 3.1. In order to generate transgenic mice, researchers should obtain approval from institutional committees for animal use and care, and biosafety for genetically modified organisms in advance (see Ethical issues section).

Preparation and purification of transgenic construct (step 1)

Designing the transgene construct is the most important step. First, fuse cDNA (coding region of the gene) with the appropriate promoter cassette for optimal expression in the cell types of interest. Alternatively, the genomic sequence of the gene of interest may be used. In this case, the gene will be transcribed under the control of its own promoter. The transgene DNA fragment, consisting of the promoter and the coding region of the gene, should be purified from the plasmid vector sequence. After digestion with restriction enzymes, the DNA fragment is separated with agarose gel electrophoresis and then purified. The method to purify DNA either uses a commercial kit or a sucrose gradient centrifugation. Here is a step-by-step protocol to purify DNA:

1. Run the digested DNA transgene on 0.8%−1.0% agarose gel.
2. Excise the transgene DNA under a long-wave UV light with a razor blade. (Minimize UV exposure to avoid the damage to the DNA.)

3. Purify DNA from the gel with QIAquick Gel Extraction kit (Qiagen, USA) according to the manufacturer's protocol.
4. Prepare DNA with 20–50 ng/μL in 50 μL TE buffer (10 mM Tris pH 8.0/0.1 mM EDTA).

Harvesting donor eggs (step 2)

The technical supports for step 2, 3, and 4 are usually offered by the animal facility of the research institute or university. Here, the outline for these steps is described. Egg donor female mice receive injection of pregnant mare's serum gonadotropin and human chorionic gonadotropin for superovulation. Approximately 400 fertilized eggs (zygotes) are obtained from 20 super-ovulated female mice.

Microinjection of transgene to fertilized egg (step 3)

Transgene DNA is microinjected to the pronucleus of a fertilized egg (zygote). Normally, DNA is diluted to 2 ng/μL, and approximately 2 pL of DNA solution is injected per zygote. Microinjected zygotes are subsequently incubated until 2-cell stage for 18 hours, and then 200 zygotes that are in good condition are used for implantation.

Implantation of microinjected egg to pseudopregnant female mice (step 4)

Typically 20–25 zygotes (per mice) are implanted to pseudopregnant female recipient mice.

Screening of founder mice for expression of transgene (step 5)

Most recipient female mice are pregnant and deliver the pups after 20 days of embryo transfer. Normally, around 40 pups are obtained for screening. At 2–3 weeks of age, we make a small puncture on the ear for identification and take 0.5 cm of tail to isolate genomic DNA for genotyping. A brief protocol for extraction of genomic DNA from a mouse tail is found in "Protocols" section.

1. The tail is incubated with 500 μL of lysis buffer (0.4 mg/mL proteinase K, 10 mM Tris, 100 mM NaCl, 10 mM EDTA, 0.5% sodium dodecyl sulfate (SDS)) at 55°C for 16 hours.
2. Add 500 μL of 1:1 phenol/chloroform solution and mix vigorously.
3. Centrifuge at 15,000 × *g* for 5 minutes at room temperature.
4. Carefully transfer aqueous supernatant to new Eppendorf tube.
5. Add 40 μL of 3 M sodium acetate and 1 mL 100% ethanol, mix gently, and centrifuge at 16,000 × *g*, 4°C to pellet genomic DNA. Remove supernatant.
6. Wash DNA with 1 mL of 70% ethanol, centrifuge at 16,000 × *g* for 1 minute, remove supernatant, repeat step 6, and air dry for 1 hour.
7. Dissolve DNA with 50–100 μL of TE buffer (pH 8). The DNA is ready to use for genotyping by polymerase chain reaction (PCR). Typically, 10%–25% of pups are positive for transgene. These pups are further screened for the transgene copy number by using quantitative PCR methods.

Establishing stable transgenic line (step 6)

A few transgene-positive pups (normally called "founders") with appropriate transgene copy number will be further bred with wild-type C57BL/6 mice to obtain the first generation (F1). Since transgene can be integrated to multiple sites of chromosomes, these mating steps (usually two to three generations) are necessary to establish a transgenic line with stable expression. In addition, to obtain the transgenic line with a pure genetic background (i.e., C57BL/6), it is necessary to breed mice for 7–10 generations with wild-type C57BL/6 mice.

Cre-loxP technology

The Cre-loxP system allows us to control the gene expression in a tissue-specific manner. Cre recombinase is a bacteria-derived enzyme that recognizes a portion of the specific DNA sequence (loxP) and deletes the DNA sequence between two loxP sites from the genome. LoxP sites are specific 34 base pair sequences (see Glossary). When two loxP sites are oriented in the same direction on the chromosome, Cre recombinase catalyzes the deletion of the DNA sequence between two loxP sites (Fig. 3.2A), while Cre mediates inversion of DNA sequence when two loxP sites are oriented in opposite directions (Fig. 3.2B). The most popular application of Cre-loxP technology is a conditional knockout strategy to eliminate the gene of interest in the cell-type or tissue-specific manner, allowing examination of the gene function in the specific cell-types in the mice (Fig. 3.2C). Another application is to express the gene of interest in the Cre-dependent, cell-type specific manner. For this purpose, you need to generate a transgenic or knock-in mouse carrying the conditional expression cassette, which consists of a promoter, an intervening stop sequence with two loxP sites at the each end, the coding sequence of interest, and a polyadenylation signal. The mouse expresses the transgene only when the intervening sequence is deleted by the action of Cre recombinase derived from the mating with a Cre transgenic mouse (Fig. 3.2D). For this purpose, many Cre transgenic mice with a cell type–specific promoter have been generated to date.

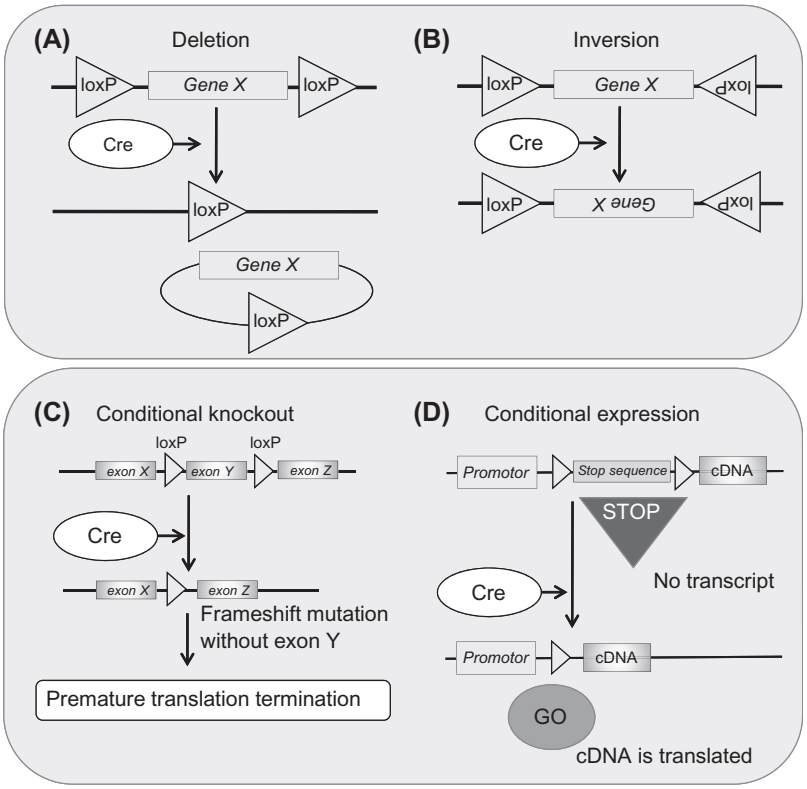


FIGURE 3.2 Principle and application of Cre-loxP system. (A and B) Cre recombinase mediates deletion (A) of the gene between two loxP sequences when loxP sites are oriented in the same direction, while it induces inversion (B) when two loxP sequences are oriented in the opposite directions.(C and D) Application of the Cre-loxP system to gene manipulation in the mouse models. (C) Conditional knockout strategy. The mouse strain designed for conditional knockout carrying the loxP-flanked exon critical for a target gene. When mated with Cre-expressing mice, the loxP-flanked exon is deleted from genome, resulting in the premature termination of translation due to the frameshift mutation caused by the deletion of exon Y. (D) Conditional expression of the transgene. The mouse strain carrying a transgene incorporated whose promoter elements are separated from the coding region with an intervening stop sequence (with loxP sites). The Cre-mediated recombination to excise the stop sequence occurs only in those cells expressing Cre. Therefore, the transgene can be transcribed in this specific cell type, whereas the transgene remains inactive in other Cre non-expressing cells.

Amyotrophic lateral sclerosis models

SOD1^{G37R} transgenic mice

To generate SOD1 transgenic mice, a 12-kb genomic DNA fragment encoding the human *SOD1* gene under an endogenous SOD1 promoter, with the mutation to convert codon 37 from glycine to arginine, was microinjected into hybrid (C57BL/6J × C3H/HeJ) F2 mouse embryos (Fig. 3.3A) (Wong et al., 1995). From 128 initial pups, 23 founders were identified with the G37R mutant. Protein levels were measured for all founders by immunoblotting of whole blood using an SOD1 antibody. Lines were established from eight founders expressing the highest levels of the G37R mutant. Quantitative immunoblotting coupled with SOD1 enzyme activity assays revealed that the mutant proteins accumulated between 5 and 12 times endogenous SOD1 in the spinal cord in the four G37R lines expressing the highest SOD1 level.

After backcrossing with C57/BL6 mice, two lines for SOD1^{G37R} transgenic mice were further characterized and frequently used for ALS research. SOD1^{G37R} (line 42) and SOD1$_{G37R}$ (line 29) developed clinical signs of motor neuron disease around 4 and 10 months of age and died around 6 and 13 months of age, respectively. SOD1^{G37R} (line 42) expresses a higher number of transgene copies than SOD1^{G37R} (line 29), indicating that the disease severity is correlated with an expression level of mutant SOD1 (Wong et al., 1995).

Subsequently, to test the role of individual central nervous system (CNS) cell types in ALS, loxSOD1^{G37R} mice expressing deletable SOD1^{G37R} transgene by using Cre-loxP technology were established. Addition of a pair of 34 base loxP sequences to each end of the SOD1 transgene allows Cre-mediated deletion of the transgene. While loxSOD1^{G37R} mice express mutant SOD1 in all the cell types under the authentic human SOD1 promoter, the mutant SOD1 transgene is excised in the specific cell type when the mice are mated with Cre-expressing mice under the cell type−specific promoter (Fig. 3.3D). The average life span of loxSOD1^{G37R} is 10−13 months (Boillee et al., 2006).

SOD1^{G93A} transgenic mice

A 12-kb human *SOD1* gene carrying a disease-causing mutation at codon 93 from glycine to alanine was injected into a hybrid (C57BL6 × SJL) F1 embryo (Fig. 3.3A) (Gurney et al., 1994). The transgenic mice with the highest copy number of SOD1 transgene with G93A mutation (24 copies) showed paralysis in limbs and died around 4.5 months of age. The SOD1^{G93A} line is most frequently used for ALS research. A mean survival time for the original line with mixed genetic background (C57BL6/SJL) or the line with pure C57BL/6 background is around 130 days or 155−160 days, respectively, indicating that the genetic background of ALS mice affects the disease onset and survival time.

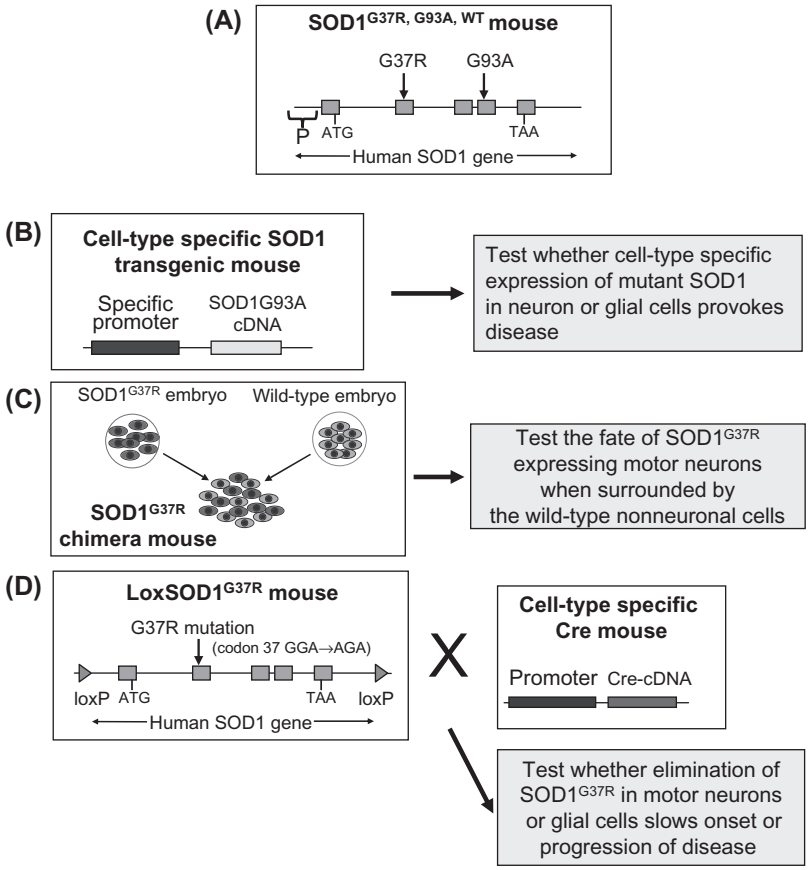


FIGURE 3.3 A schematic view of designing SOD1 transgenic mice. (A) A 12 kb human *SOD1* gene carrying either G37R (glycine to arginine at codon 37) or G93A (glycine to alanine at codon 93) mutation is microinjected to generate $SOD1^{G37R}$ or $SOD1^{G93A}$ mice. $SOD1^{WT}$ mice is generated from a wild-type human *SOD1* gene. "P" indicates endogenous SOD1 promoter region. (B–D) The design to construct SOD1-ALS model mice to test the involvement of each central nervous system cell type. (B) SOD1 cDNA was expressed under the cell-type specific promoter (neuron or astrocyte). (C) Chimeric mouse approach to test the fate of mutant SOD1-expressing motor neurons in the environment of wild-type nonneuronal cells. (D) Schematic view of $LoxSOD1^{G37R}$ transgene. To allow Cre-mediated gene excision, a pair of 34 base loxP sequence was added to each end of human $SOD1^{G37R}$ gene. Mating with Cre-expressing mouse will remove $SOD1^{G37R}$ transgene in a cell-type specific manner.

$SOD1^{WT}$ transgenic mice

$SOD1^{WT}$ transgenic mice were generated in parallel to $SOD1^{G93A}$ or $SOD1^{G37R}$ mice using the human *SOD1* gene (Fig. 3.3A). They express SOD1 proteins at the level similar to that of the mutant mice and therefore used as controls. In contrast to mutant SOD1 transgenic mice, the mice expressing high levels of wild-type SOD1 do not exhibit any clinical or pathological evidence of motor neuron disease (Wong et al., 1995; Gurney et al., 1994).

Spinal muscular atrophy models

Severe spinal muscular atrophy mice ($mSMN^{-/-}$; $SMN2^{+/+}$)

SMN knockout mice ($mSMN^{-/-}$ mice) were generated by the conventional gene targeting method to disrupt exon 2 of the mouse *SMN* gene by inserting the *Escherichia coli* LacZ gene (Schrank et al., 1997). SMN knockout mice ($mSMN^{-/-}$) die at the early embryonic stage, while heterozygote $mSMN^{+/-}$ mice do not show any detectable abnormalities. To rescue severe phenotype SMN knockout mice, the entire human *SMN2* gene including its promoter with a length of 35.5 kb was injected into embryos to generate SMN2 transgenic mice (Monani et al., 2000). Four founders were obtained. Two lines with 1 or 8 copies of *SMN2* genes were bred to C57BL/6J $mSMN^{+/-}$ mice to produce $mSMN^{+/-}$;*SMN2* progeny. The introduction of one copy of SMN2 gene rescued embryonic lethality from mSMN deficiency, resulting in severe SMA phenotype (Fig. 3.1E). The $mSMN^{-/-}$;$SMN2^{+/+}$ mice (called as severe SMA mice) can survive for 8 days, but most of them die between 4 and 6 days. SMN knockout mice carrying higher copy number SMN2 transgene did not show any obvious phenotypes, indicating that the introduction of eight copies of *SMN2* is sufficient to rescue the SMA phenotype.

Spinal muscular atrophy type II mice ($mSMN^{-/-}$; $SMN2^{+/+}$;$SMN\Delta7^{+/+}$)

To obtain SMNΔ7 transgenic mice, an SMN cDNA lacking exon 7 was introduced under the control of a 3.4-kb SMN promoter fragment (SMNΔ7) and microinjected into FVB/N mouse embryos (Le et al., 2005). Three founder lines carrying 2, 6, and 17 copies of SMNΔ7 transgenes were obtained. The integration of the transgene for all lines was a single tandem integration and followed Mendelian inheritance consistent with a single locus for genomic integration site of the transgene. The SMNΔ7 transgenic mice were crossed

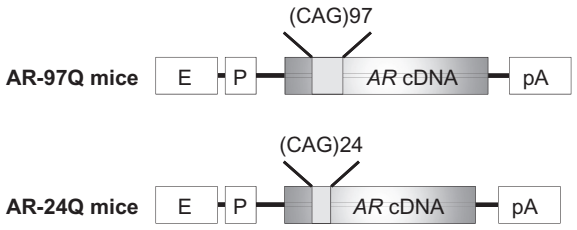


FIGURE 3.4 Schematic view of the AR transgenic mice for SBMA model. The microinjected fragment was composed of a cytomegarovirus enhancer (E), a chicken β-actin promoter (P), a human androgen receptor (AR) cDNA containing 24 or 79 CAG repeats with and a rabbit β-globin polyadenylation signal sequence (pA).

with mice expressing SMN2 and a mouse SMN knockout allele to obtain double transgenic mice $mSMN^{+/-}$;*SMN2*;*SMNΔ7* on a FVB/N genetic background. These double transgenic mice were interbred to obtain $mSMN^{-/-}$;*SMN2*;*SMNΔ7* mice. The SMNΔ7 transgenic mice with six copies of SMNΔ7 gave the highest levels of SMNΔ7 mRNA and protein and gave the most improvement in life span of severe SMA. The $mSMN^{-/-}$;$SMN2^{+/+}$;$SMNΔ7^{+/+}$ mice survived for a maximum 17 days with a mean of 13.3 days (Le et al., 2005). These mice are also called SMA type II model mice and have become the most widely used SMA models (Fig. 3.1F).

Spinal and bulbar muscular atrophy models

AR-97Q and AR-24Q transgenic mice

To generate SBMA model mice, the full-length human AR cDNA harboring 24 or 97 CAG repeats with a chicken β-actin promoter and a cytomegarovirus enhancer was microinjected into BDF1 mouse embryos (Fig. 3.4). Three founders with AR-24Q and five founders with AR-97Q were obtained, and these mice were backcrossed to C57BL/6J mice. Three lines with 1−5 copy numbers of AR-24Q were established, but none of them showed any manifested phenotypes. Three lines with 1−3 copy numbers of AR-97Q exhibited progressive motor impairment. All three lines of AR-97Q showed small body size, short life span, progressive muscle atrophy and weakness, as well as reduced cage activity, all of which were markedly pronounced and accelerated in the male AR-97Q mice, but not observed or far less severe in the female AR-97Q mice, regardless of the line (Katsuno et al., 2002).

Examples and their applications

The pathomechanisms of motor neuron diseases have been elucidated through the construction and analyses of mouse models recapitulating mutations of causative genes. Here, an example for evaluating the phenotypes and clinical course of SOD1-ALS model mouse is provided. Then, the representative examples of their applications, the research to understand the pathomechanisms for motor neuron diseases by using SOD1-linked ALS, other ALS, SMA, and SBMA model mice, are discussed.

Superoxide dismutase 1-linked amyotrophic lateral sclerosis

Gain of toxicity from mutant superoxide dismutase 1 established as pathomechanisms through engineering mutant superoxide dismutase 1 mice

The discovery of SOD1 mutations in familial ALS led researchers to develop animal models that recapitulate ALS-like disease. SOD1 is a metalloenzyme that coordinates one copper and one zinc in the protein and forms a dimer to exert full enzymatic activity to convert superoxide to oxygen and hydrogen peroxide, thus eliminating reactive oxygen species harmful to the cells. Initially, ALS researchers hypothesized that a loss of enzymatic activity of ALS-causing mutant SOD1 proteins is causative for motor neuron degeneration. However, SOD1 knockout mice do not show overt phenotypes, while the mice show increased vulnerability to motor neurons only after axonal injury. In contrast, the transgenic mice overexpressing human *SOD1* gene carrying a patient-derived mutation uniformly show a progressive neurodegenerative disease that closely resembles human pathology with a selective motor neuron death and gliosis accompanied by accumulation of misfolded proteins, while overexpressing wild-type SOD1 proteins in mice do not cause neurodegeneration (Wong et al., 1995; Gurney et al., 1994). These results led to the consensus that all different mutations of the *SOD1* gene (both enzymatically active and inactive mutants) uniformly cause toxicity in cells not by loss, but rather by gain, of function mechanisms. However, the exact mechanism and nature of toxicity are still unknown. Currently, numerous mechanisms of toxicity have been proposed that could mediate pathology in mutant *SOD1*-mediated ALS. The most important mechanisms are thought to be excitotoxicity from glutamate, endoplasmic reticulum stress, damage to mitochondria, neuroinflammation by secretion of superoxide and proinflammatory cytokines, axonal transport disruption, and spinal capillary microhemorrhages (Bruijn et al., 2004). All of these mechanisms are considered as the key processes for motor neurodegeneration, and convergence of all of these events contributes to the development of ALS pathology.

Evaluating phenotype and clinical course of mutant superoxide dismutase 1 transgenic mice

$SOD1^{G93A}$ mice, widely used model for ALS research (Gurney et al., 1994), show an observable phenotype of motor decline at around 90–100 days with a hind-limb tremor when hung by a tail. Subsequently, $SOD1^{G93A}$ mice show a walking abnormality, such as a waddling gait, due to the weakness of hind-limbs. A progressive loss of body weight is the objective measure of disease onset, which reflects a loss of muscle volume. As a measure of onset, researchers use a time for peak body weight (a time to start body weight decline) in combination with the neurological score. The hind-limb paralysis becomes clear at the age of 140–150 days (Fig. 3.5). The endpoint is normally determined as a time when the mice are unable to right themselves within 15–30 seconds if laid on either side. The mean survival time of $SOD1^{G93A}$ mice is 155–160 days in C57BL6 genetic background, while approximately 130 days in the mix genetic background (C57BL6/SJL) of the originally constructed mice.

Toxicity from misfolded mutant superoxide dismutase 1 protein

A molecular characteristic common to almost all mutant SOD1 proteins is protein misfolding: abnormally folded "misshaped" proteins that show altered biochemical properties, such as decreased solubility to detergents and increased aggregation propensities. Deposition of "misfolded proteins" is also common to neuropathology in many neurodegenerative diseases, including AD (amyloid beta and tau proteins) and PD (alpha-synuclein). Misfolding of mutant proteins is linked to the aforementioned mechanisms for motor neuron degeneration. One of the plausible hypotheses is mitochondrial dysfunction. Morphological abnormalities of mitochondria have been observed in the spinal cord lesion of mutant SOD1 mice (Wong et al., 1995). More importantly, the enzymatic activities of mitochondria are decreased rather selectively in the spinal cord, but not affected in other brain regions of SOD1 mice. Misfolded mutant SOD1 proteins are accumulated on the mitochondrial outer membrane and intermembrane space, which is revealed by the analysis of biochemically isolated mitochondria from the spinal cord of mutant SOD1 transgenic mouse or rats, suggesting the active role of misfolded mutant proteins in mitochondrial dysfunction (Liu et al., 2004). Recently, in vivo evidence of the pathogenic role of mutant SOD1 in mitochondria was demonstrated by mice expressing $SOD1^{G93A}$ mutant selectively in the intermembrane space of mitochondria of the CNS, which showed neurodegeneration, although it was not sufficient to recapitulate a full aspect of ALS phenotype (Igoudjil et al., 2011). More recently, the functional and structural deficit for mitochondria-associated membrane (MAM), which is an interface of mitochondria and endoplasmic reticulum, was also involved in mutant SOD1-mediated toxicity (Watanabe et al., 2016). Collapse of the MAM was also observed in the other causes of inherited ALS (Paillusson et al, 2016).

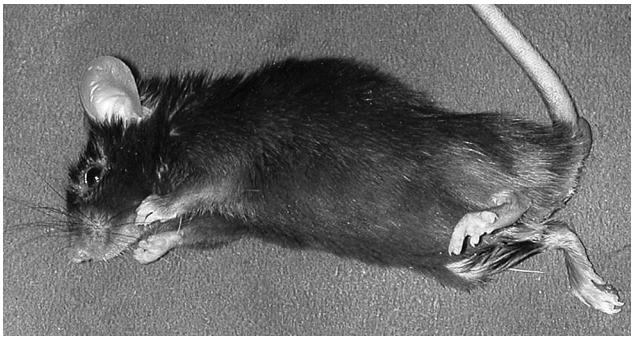


FIGURE 3.5 $SOD1^{G93A}$ mouse at end stage (around 160 days old) showing hind-limb paralysis.

Non-cell autonomous neurodegeneration demonstrated by superoxide dismutase 1 mouse models

The selective death of motor neurons initially led researchers to believe that cell-autonomous mechanisms were central to pathogenesis. However, genetic and chimeric mouse studies indicate that noncell autonomous processes might underlie motor neuron loss in these rodent models. Immunohistological studies show glial cell involvement in ALS pathology where extensive activation and proliferation of glial cells (also called gliosis), such as astrocytes and microglia, are observed in motor neuron diseases (Fig. 3.6). Astrocytes are glial cells that support the function of neurons by secreting neurotrophic factors and controlling neuronal functions by controlling ion balance, recycling the neurotransmitter glutamate, and vasculatures. Microglia are central to immune defense from scavenging pathogens and damaged neurons (see Glossary).

First, researchers tested the role of each cell type (motor neurons or glial cells) in mutant SOD1-mediated toxicities by generating transgenic mice to express mutant SOD1 in the specific cells in the CNS using a cell-type specific promoter (Fig. 3.3B). When expression of SOD1 mutations was restricted to either neurons or astrocytes, but not both simultaneously, it did not lead to the development of ALS. In an alternate approach, chimeric mice, which are a mixture of cells derived from mutant SOD1 transgenic mice and the wild-type cells, were constructed to test whether mutant SOD1-expressing motor neurons are damaged

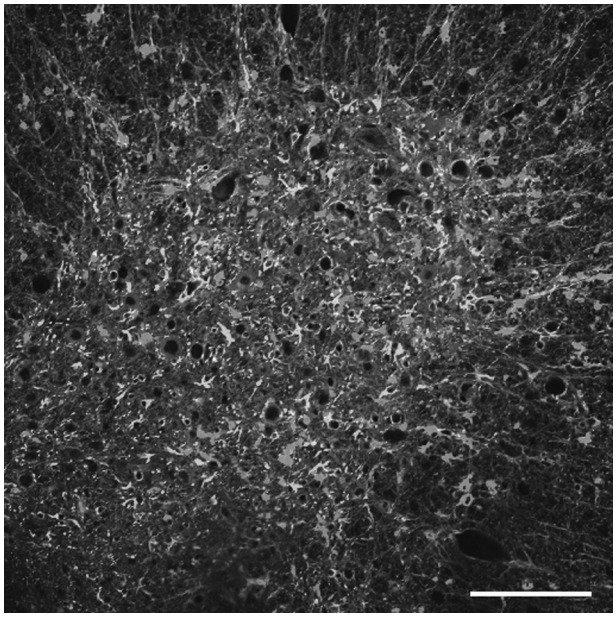


FIGURE 3.6 Activated microglia and astrocytes in lumbar spinal cord of symptomatic mutant SOD1 mice. microglia stained with anti-Mac2 antibody; astrocytes stained with anti-GFAP antibody, motor neurons stained with antineurofilament H antibody. Bar: 100 μm.

through a cell-autonomous fashion (Fig. 3.3C). In these experiments, mutant SOD1-expressing motor neurons, in chimeric mice with both wild-type and mutant SOD1-expressing cells, escaped from degeneration when surrounded by wild-type glial cells, indicating that mutant SOD1-mediated ALS is provoked in a non-cell autonomous fashion (Clement et al., 2003; Yamanaka et al., 2008a,b). Finally, to test the role of individual CNS cell types in ALS, researchers designed the loxSOD1^{G37R} transgenic mice expressing deletable mutant *SOD1* gene by using Cre-loxP technology (Fig. 3.3D). Reducing mutant SOD1 expression in either astrocytes or microglia using floxed *SOD1* gene excised by Cre recombinase slowed the disease progression and extended life span (Boillee et al., 2006; Yamanaka et al., 2008a,b). A series of these studies established the active role of glial cells in ALS pathogenesis through genetically engineering the ALS model mice (reviewed in Komine and Yamanaka, 2015).

Stem cell-derived motor neurons established from mutant superoxide dismutase 1 mice

These ALS model mice are also useful for modeling neurodegenerative diseases in vitro. Researchers established the embryonic stem (ES) cells from mutant SOD1 transgenic mice. The protocol to differentiate ES cell from motor neurons is available so that it is now feasible to model the motor neuron diseases in vitro to elucidate the detailed mechanisms underlying neurodegeneration (Wichterle and Peljto, 2008). Recent studies demonstrated that astrocytes expressing mutant SOD1 are toxic to ES cell-derived motor neurons, supporting the "noncell autonomous" neurodegeneration hypothesis of mutant SOD1-linked ALS (Nagai et al., 2007). Moreover, ES cell-derived motor neurons carrying ALS causative genes are also useful for drug screening, as well as neurons derived from human inducible pluripotent stem cells.

Other amyotrophic lateral sclerosis

Researchers have tried to develop ALS models recapitulating TDP-43 or FUS abnormalities, and many transgenic animals overexpressing human TDP-43 wild-type or ALS-linked mutants were generated; however, none of them represent the ALS-phenotype well. Both wild-type and ALS-linked mutant TDP-43 transgenic mice, when expressed at high level, showed neurological phenotypes, such as decline of motor performance and neurodegeneration. In contrast to SOD1-ALS models, cell-type specificity, and mutant specificity for neurodegeneration were not well achieved in TDP-43-ALS models. This may be because TDP-43 is essential to all cell types, and the amount of TDP-43 is tightly regulated by autoregulation of TDP-43 itself. Since TDP-43 downregulates its own mRNA level, a small amount of increase of TDP-43 does not affect the total amount of TDP-43. In contrast, the overexpression of a certain level of TDP-43 causes death in many types of cells, resulting in difficulty in recapitulating region-specific neurodegeneration. Currently, the mechanism underlying neurodegeneration in sporadic ALS is not well known, and it may be mediated by a combination of both loss of function of normal RNA-binding protein and gain of toxic function. The alternative approaches to develop ALS models have been in progress, including the Cre-loxP system to express the target protein in the specific cell types, virus-mediated expression of target proteins in the spinal cord of adult animals, and conditional deletion of target genes specific to motor neurons (Da Cruz and Cleveland, 2011).

Spinal muscular atrophy

Mice possess a single *SMN* gene, which has 82% amino acid identity with the human homolog; mouse and human *SMN* genes show a similar expression pattern. Homozygous deletion of the *SMN* gene results in massive embryonic cell death before implantation, as would be expected given the housekeeping functions of SMN (Schrank et al., 1997). Heterozygous SMN-null mice lack a marked clinical phenotype, and parental carriers of SMA-related mutations are phenotypically

normal, indicating that presumably only approximately 50% of the wild-type SMN level is required for normal function in cells. When the level of SMN is substantially reduced to lower than ~20% of the normal level, most or all cells die. Therefore, there seems to be a critical level of SMN at which many cell types are relatively unaffected, but a few cell types, such as motor neurons and possibly muscle cells, are compromised. The selective vulnerability of motor neurons for the low levels of SMN is not well understood.

Human SMN2 transgenic mice

Since more than 95% of SMA patients show homozygous deletion of *SMN1* and varied expression of *SMN2* (Fig. 3.1A–C), mice deficient for mouse *SMN* but expressing human *SMN2* seem to be an ideal model for SMA. Introduction of one or two copies of human *SMN2* genes to SMN knockout mice rescues the embryonic lethal phenotype, resulting in mice with severe SMA ($mSMN^{-/-}$;$SMN2^{+/+}$), which are indistinguishable from controls at birth, but die before postnatal day 7 (Monani et al., 2000; Hsieh-Li et al., 2000). Introduction of 8–16 copies of human *SMN2* gene completely rescued the phenotypes (Monani et al., 2000), indicating that modestly enhanced expression of full-length SMN protein from *SMN2* can prevent SMA, confirming the idea that enhancing *SMN2* transcription is a potential therapeutic strategy (Burghes and Beattie, 2009).

Since the phenotype of $mSMN^{-/-}$ mice carrying two copies of SMN2 is severe, researchers tried to reduce the severity in many ways. For example, introduction of a second transgene containing human *SMNΔ7* into severe SMA mice ($mSMN^{-/-}$;$SMN2^{+/+}$) extends the life span from 6 to 13 days (Le et al., 2005). This mouse ($mSMN^{-/-}$;$SMN2^{+/+}$;$SMNΔ7^{+/+}$), also called SMA type II model, has become the most widely used of SMA models. Introduction of other transgenes containing patient-derived point mutations in *SMN1* (A2G, A111G) have also resulted in increased life span. However, none of these mutants alone rescue the embryonic lethality caused by *SMN* depletion, indicating the importance of retaining at least some full-length SMN to function.

By using SMA type II models, tissue-specific abnormality of small nuclear RNA repertoires and profound pre-mRNA splicing defects were elucidated in vivo (Zhang et al., 2008). The work confirmed a key function of SMN complex in RNA metabolism and splicing regulation in vivo. The abnormality of small nuclear RNA repertories and splicing defects in SMA mice are not limited to the spinal cord, but are observed in many organs and remain an unsolved question regarding the vulnerability of motor neurons in SMA.

Neuron-specific deletion of survival of motor neuron in mice using Cre-loxP systems

The Cre-loxP system allows us to modify the gene expression in a tissue-specific manner. A mouse line carrying two loxP sequences flanking *SMN* exon 7 (*SmnF7*) has been established through homologous recombination. Crossing these mice with tissue-specific Cre mouse lines effectively produces complete deficiency of SMN in target tissues. Neuron-specific ablation of mSMN results in motor neuron loss and premature death of mice (Burghes and Beattie, 2009; Frugier et al., 2000). The complete absence of functional SMN, which never occurs in patients, suggests that these models provide limited insights into the pathogenesis of SMA.

Spinal and bulbar muscular atrophy

AR-97Q mice as spinal and bulbar muscular atrophy Model

After identification of expansion of polyglutamine in AR in SBMA patients, two hypotheses have been proposed for the role of polyglutamine-expanded mutant AR in the pathogenesis of SBMA: (1) mutant AR acquires a toxic property for motor neurons, or (2) a loss of normal AR function causes motor neuron degeneration. The loss-of-function hypothesis is not supported by the observation that patients with testicular feminization lacking AR function and *AR* gene knockout mice do show motor neuron diseases. In contrast, the transgenic mice expressing a full-length human *AR* gene with expanded CAG repeats under the control of an ubiquitous promoter (AR-97Q mice) recapitulated motor neuron disease, while the mice with normal CAG repeats (24 repeats) did not show any abnormality (Katsuno et al., 2002). More importantly, AR-97Q mice showed that motor neuron disease is dependent on gender. The disease phenotype was seen only in male AR-97Q mice, not female, recapitulating a key phenotype in SBMA. This gender effect and CAG repeat-dependent neurodegeneration are also observed in the other transgenic mice expressing full-length ARs with expansion of CAG repeats, establishing the successful SBMA model mice (Adachi et al., 2007).

Androgen hormone and mutant androgen receptor central to spinal and bulbar muscular atrophy pathogenesis

This significant gender difference in the phenotype of AR-97Q mice led researchers to test whether motor neuron degeneration requires the ligand of AR, testosterone. AR-97Q male mice that had been castrated (surgically removal of testes) or pharmacologically altered

to antagonize the effect of testosterone escaped from motor neuron disease and pathology; AR-97Q female mice that were administered testosterone did develop motor neuron disease (Katsuno et al., 2002). This ligand-dependent neurodegeneration is also observed in a fruit fly model of SBMA (Takeyama et al., 2002).

In contrast to other polyglutamine diseases such as HD, SBMA requires two elements (abnormal polyglutamine carrying mutant protein and its ligand, androgen) to develop motor neuron diseases. Based on the studies, it is now established that mutant AR proteins exhibit toxicity when translocated into a nucleus in the presence of androgens likely to provoke transcriptional dysregulation. Mutant AR proteins extracted from the affected tissue of male SBMA mice are misfolded and prone to form protein aggregates. Immunohistological studies revealed that extensive accumulation of AR proteins in the nucleus were observed in the male SMBA mice despite that there was no significant difference in the AR mRNA levels between the male and female SBMA mice. In support of the hypothesis in which nuclear translocation of mutant AR is a key process to provoke neurotoxicity, neuronal dysfunction was halted by genetic manipulation to prevent nuclear import of pathogenic polyglutamine protein in the mouse model of SBMA (Montie et al., 2009).

Clinical correlations

Mutant SOD1 transgenic mouse models recapitulate key pathology of ALS: motor neuron-specific degeneration and glial activation. Although they are excellent models and have been extensively used in ALS research, accumulation of misfolded SOD1 is not observed in sporadic ALS. Therefore, new rodent models recapitulating pathology of sporadic ALS are awaited.

Recent discovery of new genes, *TDP-43*, *FUS*, and *C9orf72*, responsible for ALS has provided new opportunity for the development of new animal models (Da Cruz and Cleveland, 2011). Although many animal models for TDP-43 and FUS are reported, there are no consensus for golden-standard models for ALS. Especially, C9orf72-expressing mice partially recapitulated pathological changes observed in patients, such as dipeptide and RNA accumulation in the lesions. However, their accumulation was not sufficient for neurodegeneration (reviewed in Taylor et al., 2016). Better rodent models useful for testing new candidate drugs are awaited.

In contrast, the translational efforts using SBMA animal models have been successful, partly because SBMA is a monogenic disease and the pathomechanisms are better understood. Leuprorelin, a potent luteinizing hormone-releasing hormone analog, is an approved drug for treating prostate cancer. Leuprorelin suppresses the release of the gonadotrophins, luteinizing hormone, and follicle-stimulating hormone. With subcutaneous administration of leuprorelin, male AR-97Q mice improved motor function and showed significant extension of their survival. Based on the results from SBMA mice, the clinical trial of leuprorelin for SBMA patients has been carried out. While the efficacy did not reach statistical significance, subgroup analysis demonstrated the efficacy of the drug to slow disease progression, resulting in a partial success (Katsuno et al., 2010).

Furthermore, the recent advance of antisense oligonucleotide technology, which enables to downregulate mRNAs derived from the disease-causative genes or facilitate an exon splicing to enhance translation of the gene products, was applied to the therapy for SMA. Administration of antisense oligonucleotide significantly enhanced the expression level for SMN2 and therefore improved the clinical symptoms and the survival of SMA mice (Sahashi et al., 2013). Based on the findings in the animal model, a clinical trial of antisense oligonucleotide for SMA patients has already showed benefit to the patients, and antisense oligonucleotide as a therapy for SMA has now been approved in many countries (Chiriboga et al., 2016).

Protocols

A brief protocol for extraction of genomic DNA from a mouse tail.

1. The tail is incubated with 500 μL of lysis buffer (0.4 mg/mL Proteinase K, 10 mM Tris, 100 mM NaCl, 10 mM EDTA, 0.5% SDS) at 55°C for 16 hours.
2. Add 500 μL of 1:1 phenol/chloroform solution and mix vigorously.
3. Centrifuge at 15,000 × *g* for 5 minutes. at room temperature.
4. Carefully transfer aqueous supernatant to new Eppendorf tube.
5. Add 40 μL of 3 M sodium acetate and 1 mL 100% ethanol, mix gently, and centrifuge at 16,000 × *g*, 4°C to pellet genomic DNA. Remove supernatant.
6. Wash DNA with 1 mL of 70% ethanol, centrifuge at 16,000 × *g* for 1 minute, remove supernatant, repeat step 6, and air dry for 1 hour.
7. Dissolve DNA with 50–100 μL of TE buffer (pH 8). The DNA is ready to use for genotyping by PCR. Typically, 10%–25% of pups are positive for transgene. These pups are further screened for the transgene copy number by using quantitative PCR methods.

Ethical issues

Nowadays, animal experiments are widely used for understanding disease mechanisms, for developing new medicines, and for testing the safety of drug candidates. As many animal experiments cause pain and compromise the animals' quality of life and life span, researchers should be aware of the ethical problems associated with using laboratory animals.

The "3Rs" are the principles for researchers to follow to reduce the negative impact on animal research and to maintain accountability to society for the use of laboratory animals. The 3Rs are as follows: reduction, refinement, and replacement. Reduction means to reduce the number of animals used in experiments by improving techniques or methods. Refinement means to refine the experimental methods to reduce the suffering of animals by the use of anesthesia or less invasive methods. Replacement means to replace the animal experiments with alternatives such as cell cultures or others. In practice, when mouse tissues are collected for research, we deeply anesthetize the mouse by the inhalational anesthetic drug isoflurane and then euthanize the animals. Similarly, when the animal models reach the end stage of the disease, the animals should be euthanized to reduce pain and suffering from their disabilities due to the inability to move. In accordance to 3R principle, very recently, the researchers are encouraged to follow "ARRIVE guideline," which warrant the reproducibility of animal experiments. Substantial numbers of the journals now ask the authors to follow this guideline (Kilkenny et al., 2010).

All experimental protocols using animals should be approved by the animal care and use committee in each research institute or university. In addition, to maximize safety during distribution of genetically engineered living organisms, more than 90 countries have ratified "the Cartagena Protocol on Biosafety," which has been in effect since 2003. This is an international agreement that aims to ensure the safe handling, transport, and use of living modified organism resulting from modern biotechnology that may have adverse effects on biological diversity, considering the potential risks to human health. When researchers create and use transgenic or knockout rodent models as discussed in this chapter, they should follow this protocol.

Translational significance

The animal models for motor neuron diseases discussed here have been used to test candidate drugs for the therapy. SOD1-ALS models were used for many preclinical studies to test candidate drugs including antibiotics, minocycline, and the cyclooxygenase 2 inhibitor, celecoxib, on their disease courses. To date, more than 20 clinical trials on sporadic ALS patients have been conducted based on the studies using SOD1-ALS rodent models. However, almost all clinical trials did not show efficacies (Benatar, 2007). Failure to translate results of rodent models to sporadic human patients was attributed to several reasons. First, in many preclinical studies, the drugs were administered to animals before onset. However, this is not the case for sporadic ALS patients since human patients are treated after diagnosis. Second, in many cases, the drug effects aiming to extend the survival time of mice were modest, with cohort sizes that were not sufficient. Adequate cohort size (more than 15 animals per genotype) as well as the timing of initiating drug treatment should be carefully considered in the rodent studies. Third, the disease mechanism of mutant SOD1-mediated familial ALS could be different from sporadic ALS. Clinical correlation and translation of other animal models for non-SOD1-ALS, SBMA, and SMA are discussed in "Clinical correlation" section.

In summary, the mouse models for neurodegenerative diseases (especially for motor neuron diseases) are very useful tools to evaluate the efficacy of drug candidates when the protocol is appropriately designed. We hope this chapter provides a better understanding of the applications of animal models for neurodegenerative diseases to research for elucidating pathomechanisms, as well as developing therapies.

World Wide Web resources

Gene Reviews: http://www.ncbi.nlm.nih.gov/sites/GeneTests/review

This open website provides detailed information of human inherited diseases and the research for genetic diseases, including motor neuron diseases.

OMIM (Online Mendelian Inheritance in Man): http://omim.org/

This website provides a comprehensive, authoritative compendium of human genes and genetic phenotypes that is freely available and updated daily. It is maintained by Johns Hopkins University, in collaboration with the National Institute of Health, United Status of America.

International Mouse Strain Resource (IMSR): http://www.findmice.org

The IMSR is a searchable online database of mouse strains, stocks, and mutant ES cell lines available worldwide, including inbred, mutant, and genetically engineered strains. The goal of the IMSR is to assist

the international scientific community in locating and obtaining mouse resources for research.

ALS association: http://www.alsa.org/

The website introduces a nonprofit organization in the United States, which promotes research on ALS and provides support to ALS patients and the community. The detailed information of ALS diseases and research are found.

Families of SMA: http://www.fsma.org/

Like the aforementioned organization, this nonprofit organization provides information about SMA.

Convention on biological diversity: http://bch.cbd.int/protocol/

The website provides information on "The Cartagena Protocol on Biosafety." Researchers who use genetically modified animals should follow these principles.

ARRIVE guideline: https://www.nc3rs.org.uk/arrive-guidelines/

The website provides information on ARRIVE guideline, which in accordance of "3R" policy in animal research.

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Glossary

SOD1 A 153 amino acid, enzyme converts superoxide to oxygen and hydrogen peroxide. SOD1 forms homodimer and each subunit contains copper and zinc. Dominant mutation in *SOD1* gene is the second most frequent cause of familial ALS. Most of them are missense mutations resulting in misfolding of protein. Mutations of the *SOD1* gene (both enzymatically active and inactive mutants) uniformly cause neurodegeneration, not by a loss of enzymatic activity, but rather by a gain of toxicity due to protein misfolding.

TDP-43 TDP-43 is a 414 amino acid, DNA- and RNA-binding protein, which has various roles in RNA metabolism including RNA splicing, stability, transcriptional regulation, miRNA biogenesis, and stress granule formation. TDP-43 has a prion-like domain in its carboxy-terminal; therefore, it is prone to form protein aggregates under disease condition. Abnormal deposition of TDP-43 in cytoplasm is seen in almost all ALS and a part of the patients with FTLD, which is a prominent cause of presenile dementia.

C9orf72 (C9 open reading frame 72) A large hexa-nucleotide GGGGCC repeat extension within intron or 5′UTR of *C9orf72* gene causes dominantly inherited ALS. C9orf72-ALS is the most frequent cause of familial ALS in North America and Europe; however, it is very rare in east Asian ALS patients. Disease hypotheses include (1) abnormal RNA accumulation due to expanded hexa-nucleotide repeats and (2) abnormal dipeptide repeats accumulation due to repeat associated non-AUG (RAN) translation.

SMN SMN is encoded by *SMN1* and *SMN2* genes in human. SMN is localized in the cytoplasm and nuclear gems, forms protein complex with snRNPs, and plays many roles in RNA metabolism including mRNA processing and transcriptional regulation. In most of patients with SMA, homozygous deletion of SMN1 results in unstable SMN protein derived from SMN2. Lower level of SMN protein is causative for SMA. The current therapy for SMA is aimed to increase the amount of SMN protein by correcting the splicing of SMN2 through administration of antisense oligonucleotides.

Abbreviations

ALS	Amyotrophic lateral sclerosis
AR	Androgen receptor
FUS	Fusion in sarcoma
SBMA	Spinal and bulbar muscular atrophy
SMA	Spinal muscular atrophy
SMN	Survival of motor neuron
SOD1	Copper/zinc superoxide dismutase
TDP-43	TAR-DNA-binding protein 43

Long answer questions

1. Neurodegenerative diseases are defined by many elements and contain many diseases. (A) Provide a definition of neurodegenerative disease. (B) Provide several examples.
2. There are many approaches for modeling mouse models for inherited neurodegenerative disease. If the mutation of the gene provokes toxicity in neurons, and mutations are transmitted as autosomal dominant inheritance, which type of genetic engineering of the mouse is appropriate for modeling? Explain your answer.
3. Some inherited disease is mediated by a loss of gene function. (A) Describe the most likely type of inheritance pattern. (B) Which type of genetic engineering of the mouse is appropriate?
4. You would like to disrupt the gene of interest from the mouse. However, you cannot obtain the gene-deficient mouse, because the systemic disruption of the gene of interest causes early embryonic lethality. In such a case, what kind of alternative approaches can you take?

5. When the model mouse for motor neuron disease is used for translational research to develop a medicine, the drugs effective for the mouse models are not always successful for the clinical trial for human patients. Explain the possible causes of failure in translation.

Short answer questions

1. Name a neurodegenerative disease affecting midbrain dopaminergic neurons and describe its inheritance pattern.
2. Describe three representative motor neuron diseases and their genetic inheritance.
3. Why are women carrying mutations of androgen receptor not susceptible for spinal and bulbar muscular atrophy (SBMA)?
4. Which technique is used for conditional gene knockout (cell-type specific gene disruption)?
5. In spinal muscular atrophy (SMA), what type of therapeutic approach is likely to be promising, based on research of animal models?

Answers to short answer questions

1. Parkinson's disease (most cases are not genetically determined, while about 5%–10% of cases are inherited).
2. ALS (10% of cases are inherited and about 18 *ALS* genes are identified), SMA (autosomal recessive inheritance), SBMA (X-linked recessive).
3. Male sex hormone, androgen is required for mutant androgen receptor to provoke toxicity.
4. Cre-loxP system.
5. Increasing expression of functional SMN protein (derived from *SMN2* gene).

Yes/no type questions

1. Do the patients with neurodegenerative diseases have familial cases?
2. Does transgenic mouse usually express the transgene at the lower level than the endogenous gene?
3. If you would like to test the role of essential gene in mice, should the Cre-loxP methods be used?
4. In general, is the mechanism of recessive disease mediated by a loss of gene function?
5. Parkinson's disease selective affects motor neurons in adults. Is it correct?
6. Alzheimer's disease is one of the neurodegenerative diseases. Correct?
7. Spinal bulbar muscular atrophy (SBMA) affects specifically female. Correct?
8. Spinal muscular atrophy (SMA) is caused by a homozygous loss of *SMN2* gene expression. Correct?
9. The strategy of gene therapy for spinal muscular atrophy is to administer antisense oligonucleotide to significantly enhanced the expression level for SMN2. Correct?
10. Researcher who conducts animal experiments should follow the 3Rs, reduction, revision, and replacement. Correct?

Answers for yes/no type questions

1. Yes. In most of neurodegenerative diseases, 1%–10% of patients are familial cases.
2. No. Transgenes are usually expressed at higher level than the endogenous gene.
3. Yes. It is called as a conditional knockout strategy.
4. Yes.
5. No. Parkinson's disease affects dopaminergic neurons in midbrain.
6. Yes.
7. No. SBMA affects male, since it is X-linked recessive inheritance.
8. No. SMA is caused by a homozygous deletion of *SMN1* gene.
9. Yes.
10. No. 3Rs are reduction, refinement, and replacement.

CHAPTER

4

Epigenetics and animal models: applications in cancer control and treatment

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Summary

Epigenetic mechanism is involved in most of the diseases including cancer, cardiovascular diseases, diabetes, neurological disorders, and autoimmune diseases. In contrast to genetics which is based on changes in nucleotide sequences, epigenetics involves gene expression alterations in normal and disease states without changes in DNA sequence. Research conducted in last decade has indicated that cancer is a genetic and epigenetic disease. Genetically engineered tumor-prone mouse models have proven to be powerful tools in understanding many aspects of carcinogenesis, including epigenetics. DNA methylation, histone modifications, noncoding RNA profiling, and chromatin condensation and relaxation (accessibility to chromatin) are major components of epigenetic regulatory machinery. DNA methylation in particular has been the subject of intense interest because of its recently recognized role in disease initiation, development, and progression, as well as in the development and normal function of organisms. Starting from cell line and simple model systems such as *Arabidopsis thaliana*, more complex model systems have led to further insights into role of epigenetics in human diseases. This article focuses on cancer because, compared with other diseases, cancer epigenetics has been studied extensively—from diagnostics to prognosis to therapy and survival, and epigenetic inhibitors have been used successfully in treating specific cancers. Selected epigenetics animal models in brain, breast, liver, lung, blood, colon, and prostate cancers are described. Challenges in the field and potential solutions also are discussed.

What you expect to know

Tumorigenesis is a multistep process and epigenetics plays an important role in tumor initiation and progression, especially by inactivating tumor suppressor genes and activating oncogenes. Epigenetic changes can be reversed by natural nutrients and biological food components (Verma and Srivastava, 2002; Dunn et al., 2003; Verma et al., 2003, 2004; Kumar and Verma, 2009; Khare and Verma, 2012; Mishra and Verma, 2012). Its implication in preventing and treating diseases such as cancer is tremendous (Su, 2012). This article provides advantages of using animal models to understand and implement epigenetic approaches in cancer control and treatment. The implication of animal models in determining the susceptibility to cancer development has also been explained.

Introduction

Cancer is a genetic and epigenetic disease. To understand the underlying basic mechanism, a number of animal models have been created, which are based on animal biotechnology knowledge. The most useful models are the mouse models where epigenetic approaches have been applied. These models are useful because genome-wide methylation patterns and chromatin modifications that are dynamic can be monitored in animal models. They do not persist throughout life but undergo precise, coordinated changes at defined stages of development. Epigenetically altered patterns are involved in the environmentally triggered

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phenotypes. Some of the phenotypes correlate with disease progression. Few sections detail the general epigenetic regulation and animal models in different cancers. Ethical issues, protocols involved, and general questions are also discussed.

History

Both genetic and epigenetic changes occur simultaneously in an organism. Genetic alterations include mutations and single nucleotide changes, deletions, insertions, changes in copy number, and translocations. Epigenetic alterations (methylation, histone and selected nonhistone protein alterations, noncoding RNA alterations, imprinting, and chromatin remodeling) broadly include nongenetic alterations that are capable of being transmitted from one cell generation to another. The epigenome is significantly perturbed in most cancers, raising questions about which epigenetic alterations are functionally important in cancer. The current focus of clinical and basic research is on identifying differentially expressed marks associated with cancer so that they can be used as screening tools (biomarkers) to identify high-risk populations. After successful implication of genome-wide association studies (GWAS) in identifying disease-associated genetic marks, epigenome-wide association studies (EWAS) have also been proposed (Verma, 2016). Such studies are complementary to the Precision Medicine Initiative (PMI) (https://www.nih.gov/precision-medicine-initiative-cohort-program) proposed by the US government.

DNA methylation can occur as hypermethylation or hypomethylation of CpG islands, resulting in gene inactivation or activation. Generally gene-specific methylation is observed in the promoter region, and global methylation is observed in repeat sequences and transposable elements (i.e., short interspersed nuclear elements or SINE, and long interspersed nuclear elements or LINE) that contribute to genomic instability and altered gene expression, leading to disease development. Methylation involves covalent addition of a methyl group at position 5 of cytosine. DNA methylation mainly occurs in CpG islands. These regions are rich in phosphate-linked pairs of cytosine and guanine residues. Gene transcription depends strongly on chromatin structure. Euchromatin, with an open chromatin structure, indicates active transcription; heterochromatin, with a tight chromatin structure, represents transcription repression. Epigenetic mechanisms have evolved to regulate the structure of chromatin and, as a consequence, access to DNA for transcription. These modifications include the post-translational modifications of histones, remodeling of chromatin, polycomb suppressor complexes, and DNA modifications. Active chromatin complex formation involves energy-dependent reactions, specific histone variations, and chromatin remodeling. Few nucleosome remodeling factors, such as SWI/SNF, ISWI, CHD, and IN080, have been characterized. Eukaryotic DNA is packaged in nucleosomes, each consisting of a histone octamer arranged as two H3−H4 tetramers and two H2A−H2B dimers (Fig. 4.1).

Another aspect of epigenetic regulation is noncoding RNAs, especially microRNAs (miRNAs). Genetic and epigenetic alterations in miRNA

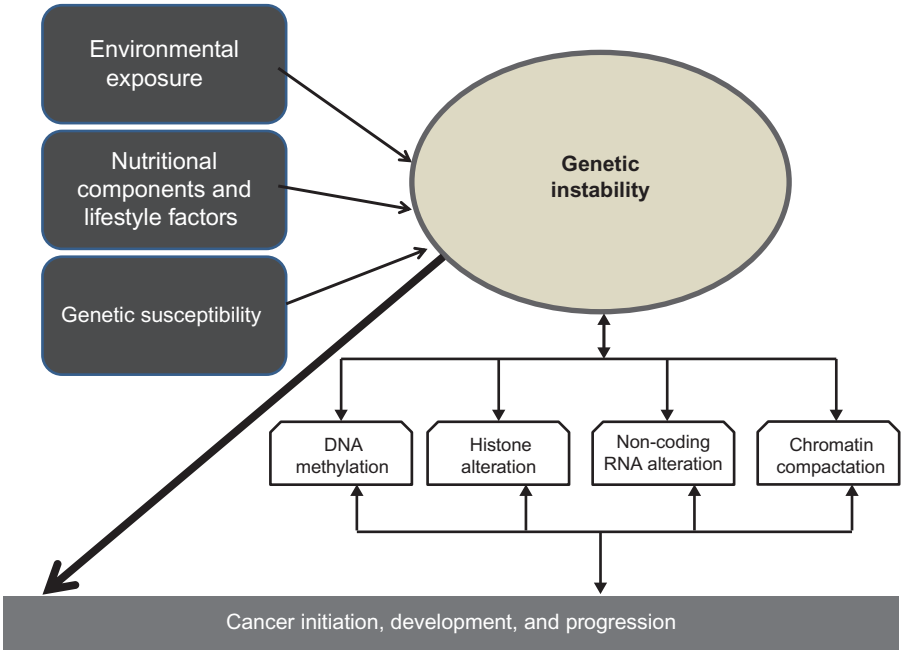


FIGURE 4.1 Factors contributing to the epigenetic regulation of gene expression.

processing contribute to cancer development. MicroRNAs are highly conserved 19–25 nucleotide long nonprotein coding RNAs involved in development and differentiation. These miRNAs bind to mRNAs and degrade them. RNA polymerase II codes miRNAs as a long precursor that is processed from nucleus to cytoplasm with the help of protein complexes. miRNAs are involved in cancer initiation and development. miRNAs are more stable than mRNAs, probably because of their small size. Molecular profiling of miRNAs helps in disease stratification, especially in identifying the stages of disease. The therapeutic potential of miRNAs also is being explored by several investigators.

Epigenetics is different from genetics because epigenetic inhibitors, mostly small molecules, can reverse epigenetic changes. Recently, chromatin-associated proteins have been characterized, which interact with epigenetic inhibitors. A list of selected inhibitors is presented in Table 4.1. The inhibitors of epigenetic target include chromatin-associated epigenomic writers (DOT1L and EZH2), erasers (LSD1), and readers (BRD4; Tanaka et al., 2015). Writers are defined as enzymes that catalyze the addition of a modification to chromatin-associated proteins and erasers are enzymes that catalyze the removal of covalent modification from these proteins.

TABLE 4.1 Epigenetic inhibitors.

Name of the inhibitor/drug	Disease	Comments
Azacytidine	Cancer	Demethylating agent
		Brand name: Vidaza
		FDA approved
Decitabine	Cancer	Demethylating agent
		Brand name: Dacogen
		FDA approved
Vorinostat	Cancer	Histone deacetylase inhibitor agent
		Brand name: Zolinza
		FDA approved
Romidepsin	Cancer	Histone deacetylase inhibitor agent
		Brand name: Istodax
		FDA approved
Belinostat	Cancer	Histone deacetylase inhibitor agent
		Brand name: Beleodaq
		FDA approved
Panobinostat	Cancer	Histone deacetylase inhibitor agent
		Brand name: Farydak
		FDA approved
GSK126	Cancer (diffuse large B cell lymphoma)	From GSK
		Phase I clinical trial
		EZH2 inhibitor
EPZ6438	Cancer	From GSK
		Phase I clinical trial
		EZH2 inhibitor
ORY-1001	Cancer (leukemia)	From Oryzon
		Phase IIa trial
GSK2879552	Cancer (lung)	From GSK
		Phase I trial
OTX015	Cancer (relapsed hematologic malignancies)	From Oncoethix
		Phase I trial
Trichostatin A	Alzheimer disease	Histone deacetylase inhibitor
		Experimental stage

(*Continued*)

Principle

Epigenetics reveals many molecular events of cancer biology. During epigenetic regulation, due to external environmental, dietary, life style, and behavior changes, key components of chromatin, histones, nonhistone proteins and selected factors, DNA in the promoter region, and miRNA expression change and initiate the carcinogenesis process. Technologies exist to measure these changes. By following epigenetic changes, cancer can be detected and intervention and therapeutic approaches can be applied.

Use of mouse models in the epigenetics of cancer

Animal models help us in understanding the underlying epigenetic mechanism during disease development as well as physiological conditions such as stress (Hodes, 2013) and nutritional disorders (Verma, 2013). Rosenfeld (2010) proposed animal models to study environmental epigenetics. Two main approaches are being used in mouse models of epigenetic changes in cancer: (1) models that are genetically manipulated to overexpress or to lack specific genes that are direct

TABLE 4.1 (Continued)

Name of the inhibitor/drug	Disease	Comments
Different HDAC inhibitors	Central nervous system disorders	Animal models show promising results
		Human trials not started yet
Histone methyl transferase inhibitors	Neuroprotection in cerebral ischemia	Animal models show promising results
		Human trials not started yet
Histone deacetylases	Cardioprotective effects	Successful preclinical studies
		Human trials not started yet

regulators of DNA methylation and methylation-related gene expression; and (2) nutrition-, genetic-, or carcinogen-induced mouse models of cancer with assessment of somatic epigenetic alterations that arise in tumors (Mathers et al., 2010). In the first approach, haploinsufficient mice such as $Dnmt1^{wt/-}$ or that have tissue-specific gene alterations are used, which makes interpretation of results easy. For example, the $Apc^{Min/wt}$ mouse model system for intestinal cancer used mice with hypomorphic expression of *Dnmt3b* (DNA methyltransferase 3b) or *Mbd2* (methyl CpG domain-binding protein 2). In this model, *APC* allele modifications contribute to intestinal cancer. To elaborate, in the $Apc^{Min/wt}$ system, the double-mutant mice lack either the ability to establish new methylation patterns (*Dnmt3b* hypomorph) or the ability to repress genes that are associated with methylated DNA (*Mbd2* hypomorph). This provides an excellent system to directly demonstrate the role of methylation in cancer progression. This model also established the fact that de novo methylation and maintenance of methylation are required for disease development. Depletion of DNA methylation caused by a partial loss of the maintenance methyltransferase Dnmt1 leads to aggressive T-cell lymphomas. In another kind of mouse model, a candidate gene approach was adopted. Tumor suppressor genes were identified in specific cancers. These genes are knocked down, and their effects on the development of animals and cancer are investigated. In the following section, selected cancer types and their models are described. The criteria used in selecting these cancers were their high incidence, prevalence, and mortality rates, as well as their complex biology.

Examples with applications

Brain cancer

Medulloblastoma is a pediatric brain tumor. Frequently in this tumor type, Kuppel-like factor-4 (*KLF4*) acquires homozygous deletions and sometimes single nucleotide polymorphisms (SNPs). Functional analysis indicated inactivation of *KLF4* expression at the transcriptional and translational levels. When cells were treated with the demethylating agent 5-aza-cytidine, the *KLF4* gene was reactivated, which suggests the presence of CpG islands in the promoter region of the gene and epigenetic regulation of the gene. This study integrated genetically and epigenetically mediated gene regulation in cancer. Because *KLF4* is also targeted in other neoplasms, this study may provide the fundamental mechanism and model system to understand the underlying mechanism. In another study based on genome-wide methylation and copy number analyses, Hong et al. demonstrated suppression of growth suppressor *SLC5A8* in glioma and its reactivation by demethylating agents. Prenatal malnutrition may lead to reprogramming by epigenetics resulting in memory loss and susceptibility of schizophrenia. Genetically engineered mouse models of medulloblastoma were used for studying genome-wide methylation to identify cancer-associated biomarkers.

Breast cancer

Based on the genome-wide methylation analysis, Demircan et al. (2009) demonstrated similarity in the mouse and human methylomes in breast cancer models. In these experiments, human orthologs of *ATP1B2*, *FOXJ1*, and *SMPD3* were aberrantly hypermethylated in the human disease, whereas *DUSP2* was not hypermethylated in primary breast tumors. In human breast cancer, *BRCA1*, *CDKN2A*, *SFN*, *CDH1*, and *CST6* are regulated epigenetically, but not many mouse genes are regulated epigenetically. Studies by Demircan et al. (2009) identified that the *Timp3*, *Rprm*, *Smpd3*, *Dusp2*, *Atp1B2*, and *FoxJ1* genes are regulated epigenetically. Of all genes studied, only *Wif1 and Dapk1* were found to not be silenced in either species. The protein encoded by *ATP1B2* is a member of the Na^+/K^+ ATPase beta chain protein family, which is an integral membrane protein responsible for establishing and maintaining the electrochemical gradients of Na^+ and K^+ ions across the plasma membrane. *FOXJ1* is a member of the forkhead gene family of transcription factors. Other genes discussed above are tumor suppressor genes.

While conducting studies in breast cancer to evaluate role of epigenetics, the selection of strains of rat is very important because different strains show different

susceptibility to spontaneous breast cancer development but highly prone to extremely severe metastatic tumors (e.g., Fischer rats), estrogen-induced cancer (e.g., ACI rats), and prone to metastasis (e.g., Long-Evans rats). The basal epigenetic profiling in four rat strains has been completed by these investigators, which might help in understanding why few rats are more susceptible to develop cancer compare with other rats. The role of histone methyl transferase, EZH2, in predicting highly aggressive breast cancer was also demonstrated by these investigators.

Colorectal cancer

The epigenetic regulation of colorectal cancer by environmental and host-associated factors has been studied extensively. Epigenetic agents such as folic acid (FA) and sodium butyrate (NaBU) showed potentials to prevent colorectal cancer. In one experimental study, colorectal cancer was induced by 1,2-dimethylhydrazine (DMH) in ICR mice, and FA and NaBU were administered after colorectal cancer developed. A lack of FA in the diet led to the hypomethylation of the total genomic DNA (global DNA) or proto-oncogenes. This also resulted in the development of tumors. Methylation profiling and H3 histone immunoprecipitation were measured in control and treated animals, and the protective effect of these agents was observed. In this study, investigators demonstrated that drug combinations enhanced tumor prevention and, when NaBu and FA were administered concomitantly, the beneficial effect was greater than that observed for either agent administered alone. In mouse models, inhibition of Dnmt3a resulted in inhibition of colorectal cancer initiation.

Esophageal cancer

The methylene tetrahydrofolate reductase (*MTHFR*) gene is polymorphic, and the *p16*, *MGMT*, and *MLH1* genes are methylated during esophageal squamous cell carcinoma (ESCC). A diet rich in FA supplies the methyl group and influences the methylation process. Clinically, dietary FA has been reported to be beneficial for patients with ESCC. Barrett's esophagus is the precancerous form of esophageal adenoma carcinoma, which is a metaplastic condition in which the normal squamous epithelial cells of the lower esophagus are replaced by small intestine-like columnar linings. In a large clinical study, surveillance and early detection of methylation biomarkers (a set of eight genes such as *p16*, *HPP1*, *RUNX3*, *CDH13*, *TAC1*, *NELL1*, *AKAP12*, and *SST*) were found to be very useful in reducing the incidence rate of this cancer. These studies established the stratification strategy for esophageal cancer progression, which might lead to better prognosis of the disease in the future. In another study *naked cuticle homolog 2 (NKD2)* suppression by hypermethylation was demonstrated in esophageal cancer xenografts that involved Wnt signaling pathway.

Gastric cancer

Kikuchi et al. demonstrated that *cycloxygenase-2* (*COX-2*) plays an important role in gastric cancer development. Gastric cancer is very common in Asian countries. It is also well established that *COX-2* plays a major role in inflammation and prostaglandin synthesis via the arachidonic acid pathway. In one model system, hypermethylation of *COX-2* resulted in its inactivation; epigenetic inhibitors reactivated *COX-2*, which suggests the involvement of methylation and histone alterations as key components of gastric carcinogenesis. Gene-encoding protease activated receptor 4 (PAR4) is involved in colon and prostate cancer, but its role in gastric cancer was not known until Zhang et al.(2011) demonstrated its regulation by epigenetics in a model system as well as in human tissues. PAR4s are G protein-coupled receptors that are involved in proteolysis. The involvement of PAR4 in gastric aggression has also been proposed. It has also been suggested that the epigenetic regulation of host genes is affected by *Helicobacter pylori* infection, thereby suggesting its involvement in the initiation and progression of gastric cancer. The involvement of cytidine deaminase has also been proposed by these investigators.

Head and neck cancer

Sun et al. demonstrated the role of transkelolase-like 1 (*TKTL1*) gene in head and neck squamous cell carcinoma (HNSCC). In cell line and xenograft models, functional characterization of *TKTL1* was achieved by following mRNA and protein expression of fructose-6-phosphate, glyceraldehyde-3-phosphate, pyruvate, lactate, and the levels of HIF1α protein and its downstream glycolytic targets. Hypermethylation of *TKTL1* in HNSCC confirmed that epigenetic regulation is an integral part of HNSCC. A combination of protease inhibitor and histone deacetylase therapeutic agents showed promising results (reactivation of transcriptionally inactive genes), which further confirmed epigenetically mediated regulation of HNCCC. Note that proteasome inhibitor PS-341 has emerged as a novel therapeutic agent that works well in combination with other agents as a cancer inhibitor.

Lung cancer

For lung cancer, more information has come from mutations and SNPs than from alterations in epigenetics and proteomics. Its mortality rates are high because the disease spreads to other organs, and little is known about the underlying mechanisms.

The mouse is a good model for use in evaluating the efficacy of chemopreventive agents for lung cancer. Gene silencing by promoter hypermethylation is a critical component in the development and progression of lung cancer and an emerging target for preventive intervention by demethylating agents. Yue et al. (2009) demonstrated that fibulin-5, a vascular ligand for integrin receptors, worked as a suppressor of lung cancer invasion and metastasis by promoter hypermethylation. It inhibited matrix metalloproteinase-7 (MMP-7), a marker of cell invasion. Results were confirmed by classical knock-down approaches. Further research demonstrated that epigenetic silencing of fibulin-5 promoted lung cancer invasion and that metastasis occurred by activating MMP-7 expression through the ERK pathway, which is an integral part of lung cancer development. Belinsky's group demonstrated that methylation events that were observed frequently in human lung cancer could be followed in mouse models. The researchers identified four potential biomarkers for assessing intervention approaches for reversing epigenetically mediated gene silencing.

Lymphoma and leukemia

In a mouse model of leukemia, transgenes were implicated in driving the proliferation of T cells. Some of these cells eventually acquired enough secondary mutations to give rise to T-cell lymphomas or acute lymphoblastic leukemias (ALL). The methylation profile at the genomic level was determined to identify both hyper- and hypomethylation events in the induced benign proliferative state that preceded the formation of the tumors, as well as events in the established tumors.

In hematologic malignancies, epigenetic inhibitors can be used for the treatment of cancer. In one study, zebularine, a demethylating agent, was administered in mice where radiation was used to induce lymphogenesis. Unradiated mice of the matching weight and age were used as controls. Results indicated hypomethylation in lymphoma-associated genes and suppression of lymphoma in zebularine-treated animals.

To determine the early stages in the development of chronic lymphocytic leukemia (CLL), Chen et al. used a well-established mouse model for CLL and followed expression of human *TCL1*, a known CLL oncogene in murine B-cells, which led to the development of mature CD19 + /CD5 + /IgM + clonal leukemia with a disease phenotype similar to that seen in human CLL. Results indicated that the mouse model recapitulated the epigenetic events that were reported for human CLL. These events were detected as early as 3 months after birth, which was close to a year before disease manifestation. Accumulated epigenetic alterations during CLL pathogenesis occurred as a consequence of *TCL1* gene silencing and synthesis of the NFkβ repressor complex. This suggests that NFkβ may be used as a therapeutic target in CLL. The role of miRNA in leukemia has also been explored.

Another well-studied model is an epithelial multistep tumorigenesis model of squamous cell cancer formation. In this model tumors are induced and promoted by chemical exposure, resulting in different progression stages of cancer development. This helps in the molecular classification of tumors. Fraga et al. (2004, 2005) completed the methylation profiling in normal tissues and tumors and identified benign papilloma and invasive carcinoma-related markers, mostly tumor suppressor genes, and established an association between disease and the activation or inactivation of these tumor suppressor genes. Surprisingly, several tumor suppressor genes, including *Cdkn2a*, were found to be consistently methylated very early in disease progression, suggesting that methylation of these genes may be crucial to the formation of tumors in this system. Among early expression genes *Cdkn2a* is a tumor suppressor gene and a few more new genes also were identified.

Prostate cancer

The protective role of FA is well established in colorectal cancer, and the underlying epigenetic mechanism has also been extensively characterized. Such studies have not been conducted in prostate cancer. Recent research demonstrated that similar protection could be obtained in prostate cancer. The transgenic adenoma of the prostate (TRAMP) mouse model was used for this study because the model is very appropriate to follow the aggressiveness of the disease. Generally prostate cancer takes 15–20 years to develop, but in a few cases this cancer is very aggressive. It is clinically significant to distinguish aggressive cancer from normally developing cancer, and the TRAMP model might be very useful in such situations.

Liver cancer

Methylation sources in the body and one-carbon metabolism play important roles in epigenetically mediated gene regulation, and any abnormalities in the process may contribute to cancer initiation and progression. In a recent study of hepatocellular carcinoma (HCC), Teng et al. (2011) reported HCC development and fatty liver due to disturbed choline and one-carbon metabolism by betaine homocysteine-*S*-methyltransferase (BHMT) in a mouse model. This study also demonstrated the integration of genetic and epigenetic alterations that are known to influence each other and contribute to cancer development. The function of BHMT is to transfer the methyl group from

betaine to homocysteine, thereby forming dimethylcysteine and methionine. Mutations in the *BHMT* gene have been reported in breast cancer. To study the effect of mutations in *BHMT*, knockout mice of this gene were generated and functionally characterized. Higher BHMT activity was found in the liver and kidney compared to other organs. Because methionine is the precursor of *S*-adenosylmethionine, which is the major source of methyl donor, it plays an important role in epigenetic regulation. The role of long noncoding RNA (lncRNA) and microRNA (miR-29) in HCC was demonstrated recently by Braconi et al. (2011). These studies were based on the microarray analysis of more than 23,000 lnc RNAs (long noncoding RNAs) and miRNAs. About 3% of RNAs were found to be downregulated in HCC. Furthermore Song et al. (2011) reported heterochromatin histone modifications in liver cancer.

Other approaches

Along with the characterization of methylation patterns, proteins binding to methylated regions have also been characterized. These proteins are identified by methylated DNA immunoprecipitation (methyl DIP), which involves the hybridization of immunoprecipitated methylated DNA to microarrays or deep sequencing of the DNA in the immunoprecipitated DNA complex to assess the pattern at the genome level. However improvements are required to adapt this process on a large scale to address problems such as low resolution when using microarrays, difficulty in obtaining sufficient coverage when deep sequencing is used, and high false-discovery rates. Thus this technique used to assess the cancer methylome in these model systems is one crucial factor to include when interpreting the results of studies of epigenetic alterations in mouse models.

Methodology

Methodologies described below are suitable to assay epigenetic components in animal tissues. Specific methodologies are described below.

Methylation profiling

MethylLight technology, pyrosequencing, and Chip-on-Chip are key technologies to measure epigenetic alterations in cancer. For methylation profiling, quantitative methylation-specific polymerase chain reaction (QMSP) assays followed by pyrosequencing (for confirmation) are performed. Sodium bisulfite treatment followed by alkali treatment is the key for all assays. Bisulfite reacts with unmethylated cytosines and converts them to thymidine. In the PCR reaction all converted Cs behave like Ts. Methylated cytosines and other bases are not affected by bisulfate treatments. MethylLight is the most common method to determine methylation profile in real time. This is a high-throughput quantitative methylation assay that utilizes fluorescence-based real-time PCR technology and does not require any manipulation after PCR reaction. This technology can detect methylation allele in the presence of 1000 unmethylated alleles. The fluorescence signal reflects the amount of PCR amplified DNA.

Histone profiling

Chip-on-Chip is the standard method for determining alterations in histones. Monoclonal antibodies against histone modifications are used in binding to fragmented DNA. The most common histone modifications in cancer are H3K4me1 (monomethylated), H3K4me3 (trimethylated), H3K9/16Ac, and H3K27me3 and monoclonal antibodies are available commercially against these histone-modified proteins. For high throughput, Chip-Seq technique is applied.

High-throughput profiling of miRNA can be achieved by Illumina sequencing.

Nucleosome mapping

The positioning of nucleosomes in the promoter dictates disease initiation and progression of disease and also responsiveness to treatment. Nuclease I digestion of chromatin and analysis by gel analysis is the most common method for nucleosome mapping.

Protocols

Three main protocols are used in epigenetic regulation in animal models.

- *Extraction and sodium bisulfate treatment of genomic DNA*. DNA from tissues is isolated using Qiagen Blood and Cell Culture DNA Isolation Kit (Qiagen, VA) and stored at −20°C before use.
- DNA is modified with EZ DNA Methylation Kit (Zymo Research, CA) using protocols supplied with the kit.
- For sodium bisulfate treatment, 1 μg DNA was incubated with sodium bisulfate at 50°C for 16 hours in the dark (solution of sodium sulfite is supplied with the kit).
- Using columns supplied with the kit, the excess bisulfate is removed and treated DNA is eluted with the elution buffer in a small volume (20 μL).

One microliter is sufficient for PCR amplification for the next step.

- Methylation-specific PCR. Primers are selected based on the gene of interest and PCR amplification is done using Qiagen HotStart Taq DNA Polymerase kit in the amplification buffer.
- Amplified fragments are analyzed by gel electrophoresis.
- Pyrosequencing. Small amount of amplified DNA (2–10 μL) is mixed with streptavidin-coated Sepharose beads based on instructions provided with the lit (GE Healthcare Biosciences AB, Uppsala, Sweden).
- Beads are shaken for 10 minutes at room temperature.
- After centrifugation the supernatant is used to do sequencing using a PyroMark MD System (Biotage, AP, Uppsala, Sweden).
- Analysis of sequencing data is done using the software supplied by PyroMark.
- Analysis of Histone (Acetylated) Content. The procedure is called "Chromatin Immunoprecipitation (ChiP)" protocol. ChiP assay helps in identifying the location of protein-DNA interaction on the chromosome. The most critical step in this assay is the selection of high-quality antibodies directed against specific modifications of histones. The basic steps in the protocol are described below.
- Cells are isolated from animal tissue treated with micrococcal nuclease and fixed for 15 minutes in formaldehyde in PBS on ice (Note that the duration of the incubation and the final concentration of the formaldehyde can affect the efficacy of the procedure in terms of shearing and precipitation of the DNA).
- Cells are fixed one more time in 70% ethanol. Due to large amount of HDAC proteins present in the cell, deacetylation activity is very high.
- All washings are conducted in a refrigerated centrifuge using cold PBS, and finally cells are fixed by keeping them on ice.
- Fixed cells are washed with PBS buffer with 15 BSA and permealized using 0.1% Triton-X100 in PBS for 10 minutes at room temperature.
- After washing with PBS, samples are incubated with 500 μL of 20% normal goat serum in PBS for 20 minutes at room temperature.
- Histone acetylation is detected using monoclonal antibodies antitetra acetylated histone H4 (Upstate Biochemicals, VA) or monoclonals against any specific histones.
- Analysis of results is done by gel electrophoresis and Western Analysis.
- Detection of antibodies is done with FITC-conjugated, affinity purified Fab2 fragment of goat anti-mouse IgG at room temperature in dark.

Ethical issues

For successful development of intervention and treatment drugs, cell lines and animals are used at the initial stages. Animal models are also useful to understand disease biology. Proper handling and treatment of animals is of prime importance in these kinds of research. Few questions relevant to this topic include whether humans are morally more important than all animals and whether humans are placed at higher order than animals. One natural question also emerges whether both humans and animals are morally equal and should be treated equally. Animal-right extremists emphasize that use of animals in research is cruel. However they do not want to address any alternative about how new drugs can be identified and validated if animals are not used for medical research. It would make no sense to experiments with new therapeutic agents directly in humans. Time to time this issue has been raised by different institutes and organization resulting in making policies by major agencies such as National Institutes of Health (http://grants.nih.gov/grants/olaw/olaw.htm) and US Department of Agriculture (https://www.aphis.usda.gov/aphis/ourfocus/animalwelfare). The number of animals used for research is estimated to be 35 million per year (12 million in United States alone). The most frequent species among animals used for research is mice because considerable knowledge about its biology and genetics is known and high genetic homology of mice and human. Sometimes rabbits and rats are also used to test the toxicity and efficacy of drugs. General guidelines for ethical issues are similar in different countries; however each country has its own requirements and policies. The countries where specific guidelines and ethical issues are well documented include United States, Canada, all countries in European Union, Australia, and New Zealand.

To follow proper ethical issues and policies, legislation requires adequate housing conditions, controls on animal pain, and critical review and approval by the institute's review board IRB. The selection of the members participating in the IRB is extremely important because these members should be experienced researchers and policy makers in animal model sciences, and possibly familiar with the recent protocols followed in research institutes. The institute may ask ethical justification for the use of animals in harmful experiments, especially when determining the optimal doses for treatment, as we know that higher doses of chemicals are toxic and fatal. The Public Health Services (PHS) of United States which regulate NIH also developed guidelines to have an oversight system of Institutional Animal Care and Use Committees (IACUCs) to review and evaluate animal

research protocols, care programs, assess and evaluate laboratory technicians and other staff. Random checking of practice in laboratory set up is done by this board and any complaints about misuse or improper use of animals are properly investigated. The challenge is how to regulate private industries and pharmaceutical companies. The government does not provide funds to pharmaceutical companies and drug manufacturing industries, hence cannot implement same rules and regulations that are applied to investigators and institutes supported by federal money. Three "Rs" have also been proposed (replacement, refinement, and reduction) to improve ethical issues.

Translation significance

Huge amounts of data are generated in the analysis of epigenetic results (Liu, 2012). During the last decade, major advances in biology, coupled with innovations in information technology, have led to an explosive growth of biological information. From the genomic revolution and many of its manifestations to recent developments in high-throughput, high-content screening, biomedical scientists have never been exposed before to research data that are so systematically collected, rich, complex, and massive. This situation provides exciting unprecedented opportunities for rapid discovery. At the same time, it presents investigators with the challenge of shifting or separating through mountains of seemingly orthogonal data and transforming them into new knowledge and clinical practice. This challenge is particularly evident in the field of cancer research, where the complexity and heterogeneity of the disease translate to more complex data generation conditions and higher data management and analysis overhead, creating a significant barrier to knowledge discovery and dissemination.

The fast growing field of biomedical informatics offers potential solutions to the "big data" problem. At the intersection of biology, medicine, computer science, and information technology, biomedical informatics concerns the development and application of computational tools to the organization and understanding of biomedical information so that new insights and knowledge can be discerned. The development of computational methods and tools in the form of computer software is a major component of biomedical informatics that is needed now. Application of existing software tools in biomedical research, such as computational data analysis and mathematical modeling, should also be improved. A single, preferably nationwide, coordination center should be formed so that data can be stored and disseminated to interested investigators.

Genetic models of cancer are useful, but they can provide only limited information such as gains, losses, mutations, and rearrangements of chromosomal regions. An integrated genetic and epigenetic approach is necessary to determine the full complement of genes involved in tumorigenesis and to expose hidden therapeutic targets (Roukos, 2011). Gene silencing by aberrant epigenetic chromatin alteration is well recognized as an event that contributes to tumorigenesis. Although a number of examples of animal biotechnology in cancer epigenetics were presented in this article, more work remains to be done. The use of mouse models to assess DNA methylation in epigenetics is still in its early stages, but the approach is showing considerable promise for providing insights into this complex disease. Improvements are needed in areas such as the identification of appropriate mouse models of disease and identification of the most robust techniques for assessing DNA methylation, particularly in relation to technical reproducibility and coverage of the methylome. The use of mouse models is highly effective in clinical applications, especially in determining the toxicity and efficacy of methylation and histone deacetylase inhibitors. Based on animal model results, large human trials can be planned. So far Food and Drug Administration (FDA) has approved seven epigenetic drugs for cancer treatment and in the near future, we expect to see more anticancer drugs that may improve survival of cancer patients and their quality of life. A better understanding of the epigenetic mechanisms may open new avenues for disease prevention and treatment.

Clinical significance

The fact that most of the epigenetic alterations can be reversed by chemicals (drugs) has tremendous significance. The FDA (www.fds.gov) has approved seven specific drugs that are called epigenetic inhibitors (Yang and Yang, 2016).

Web resources

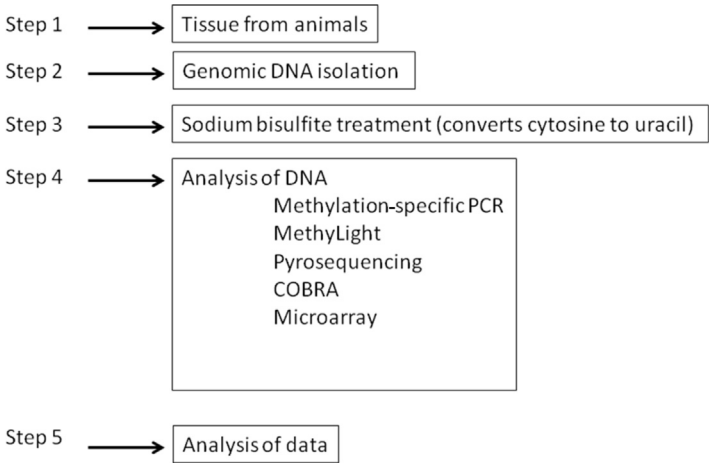
A very common and useful resource of information is World Wide Web (WWW). However no regulatory agency exists that checks the authenticity of the information provided. Our suggestion is to use www for a general background but go for the agency or peer reviewed information (journals and books) for the correctness of the information.

Turning point

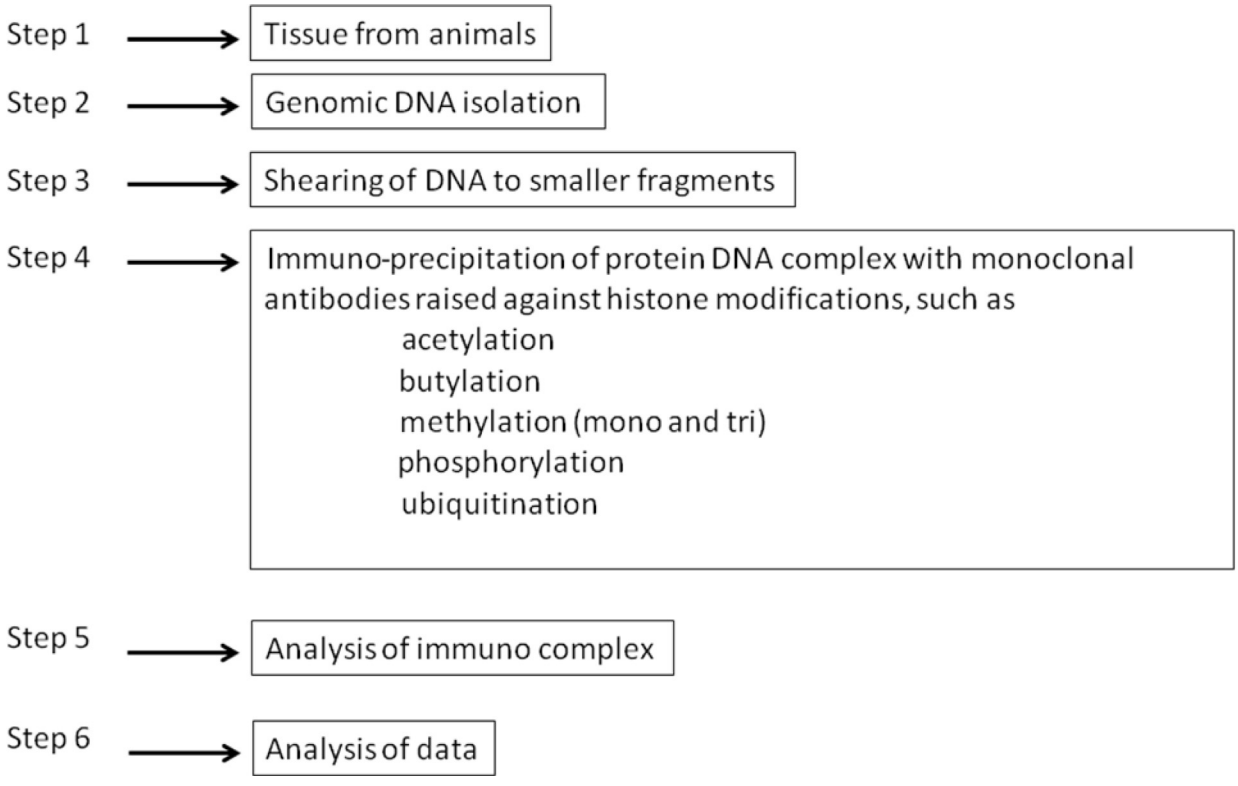
With the advancement of epigenetic information and its clinical implications, epigenetic drugs are used in combination with regular anticancer drugs and few promising results have been observed (Tsai et al., 2012).

Flow chart

Based on the technologies used in animals to understand epigenetic-regulated gene expression in normal and disease states, following flow charts can be used (Flow Charts 4.1 and 4.2).



FLOW CHART 4.1 Epigenetic analysis (methylation profiling).



FLOW CHART 4.2 Histone analysis.

World Wide Web resources

1. https://commonfund.nih.gov/epigenomics/index
2. http://ihec-epigenomes.org/
3. http://www.biolreprod.org/content/82/3/473.full
4. http://www.hendrix-genetics.com/en/better_breeding/breeding/
5. http://www.whatisepigenetics.com/fundamentals/

Something interesting about this chapter

Based on the research on animal models, cell lines, and human tissues and human populations, tremendous progress has been made in cancer epigenetics in the last few years and knowledge has been translated into clinic. Possible ways have been identified in which epigenetic changes may contribute to cancer initiation and progression and ways in which epigenotyping might be cross-correlated with clinical phenotyping in the context of precision medicine. Progress has also been exponential in understanding the chemistry and mechanistic aspects of epigenetic inhibitors, as evident from the interest shown by big pharma companies, academic institutions and government agencies. Epigenetics drugs show potential of cancer treatment, especially when combined with other drugs. However how do these drugs work and what is the underlying mechanism and the area of further investigation? Our understanding of the magnitude and specificity of epigenetic changes in a complex disease like cancer is still very limited. Although not fully explored, the use of epigenetic therapy to sensitize for immunotherapy might have advantages in treating cancer. Target-specific epigenetic therapy also needs improvement. Based on the ongoing efforts in different universities, institutes, industry and government agencies, we expect a huge progress in epigenetic therapy in the coming years. Lessons learned and information gained from epigenetic drugs in cancer treatment facilitate their role in other diseases.

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Glossary (terms used in text with examples)

Epigenetics, cancer, chromatin, histones, methylation, treatment.

Abbreviations

BHMT	Betaine homocysteine-*S*-methyl transferase
COX-2	Cycloxygenase-2
DNMT	DNA methyl transferase
ESCC	Esophageal squamous cell carcinoma
HAT	Histone acetyl transferase
HDAC	Histone deacetylase
HNSCC	Head and neck squamous cell carcinoma
LINE	Long interspersed nuclear elements
lncRNA	Long noncoding RNAs
Mbd2	Methyl CpG domain binding protein 2
Methyl DIP	Methylated DNA immunoprecipitation
MTHFR	Methylene tetrahydrofolate reductase
miRNA	Micro RNA
PAR4	Protease activated receptor 4
SAHA	Suberoylanilide hydroxamic acid
SINE	Small interspersed nuclear elements
TKTL1	Transkelolase-like 1

Long answer questions

1. What is epigenetics and how is it different from genetics?
2. Why epigenetic therapy has worked better in hematologic malignancies and not in solid tumors?
3. Do all modifications of DNA and histone lead to suppression of genes?
4. Which came first, genetics or epigenetics?
5. Where do you see the epigenetics field in the next 10 years and will animal models be useful in future?

Answers to long answer questions

1. Epigenetics regulate genomic changes. While differentiation of stem cells occurs, there are no somatic mutations in stem cells but different epigenetic changes guide stem cells to differentiate into different types of cells and organs. Furthermore unlike genetic changes, epigenetics does not alter genome sequence. Genetic changes cannot be reverted whereas epigenetic changes can be reversed with chemicals and drugs and can be used for therapeutic purposes.
2. Epigenetics inhibitors were first discovered for the treatment of hematologic malignancies and based on the success of these novel therapeutic agents, FDA approved few inhibitors as a first-line therapy for hematologic malignancies. When same agents were used for the treatment of solid tumors (prostate, lung, and colorectal cancer), these agents have minimal beneficial effect or no effect. If a dose (amount) of the agent is increased than toxicity was higher compared with their efficiency. Another important point was that epigenetic therapy was applied in patients when tumors were already metastasized and tumor burden was high. If epigenetic therapy is planned for the treatment of solid tumors, then these agents should be applied at initial stages of tumor development when the tumor burden is low. Sometimes specific organs show distinct properties, for example, ascending colon shown CIPM + phenotype, BRFF mutations and responsiveness EGFR therapy, whereas the descending colon shows CIMP- phenotype, KRAS mutation and no response to EGFR therapy. The bottom line is that therapy should be personalized and designed based on a person's omics profile, genetic background, and response to therapies.
3. Depending on the location on the chromosome and type of modifications, epigenetic alterations can result in activation of a gene or inactivation of a gene. In case of histones, when K4 (Lysine 4) is trimethylated, it results in gene activation, whereas when K27 of histone 3 (H3) is trimethylated, then gene inactivation occurs. In methylation, hypermethylation of tumor suppressor genes result in inactivation whereas hypomethylation of viral oncogenes results in activation of oncogenic viral genes. Another example is of LINE-1 sequences where hypermethylation corresponds to genomic instability whereas hypomethylated provides stability to the genome.
4. Epigenetics and genetics are correlated and both mechanisms can interact and alter disease phenotypes. When systematic studies of genetics (somatic alterations) and epigenetics were conducted with stem cells, starting from the 8-cell stage to organ development, epigenetic marks appeared first and it was after full development of an organ that somatic mutations were observed. This examples supports the view that epigenetics came first.
5. The pace with which epigenetics is growing and public health improvement is the mission of several agencies and countries, epigenetics is likely to develop tremendously. Initial results of cancer treatments where epigenetic inhibitors are used as sensitizers followed by regular drug treatment, promising results have been observed in blood cancers and selected solid tumors. Epigenetic drugs not only affect methylation and histone profiling, but they affect key

pathways in cancer development, such as apoptosis, immune modulation, and cell cycle regulation. Regarding the use of animal models in epigenetics, these models will remain crucial for understanding the underlying mechanisms whenever a disease development is studied. There are not many options to test the toxicity and efficacy of new drugs for the treatment of different diseases.

Short answer questions

1. What is epigenetics and how is it different from genetics?
2. What are the main components of epigenetics?
3. Is it necessary to study epigenetic regulation of cancer in cell line models or animal models?
4. Is cancer the only disease that is regulated epigenetically?
5. Can epigenetic drugs be used for cancer treatment?

Answers to short answer questions

1. Epigenetics is the study of alterations in gene expression without changes in the primary nucleotide sequences. The main feature of genetics is a change in the primary sequence of a gene. Both processes are needed for proper gene expression.
2. The major components are DNA methylation, histone modifications, noncoding RNA expression, and chromatin compactation and relaxation.
3. Epigenetic regulation can be studied directly in humans, but cell line and animal models are useful in identifying the functions of affected genes and pathways involved in the process.
4. No, cancer is one of many diseases that are regulated epigenetically. Other diseases in which epigenetic regulation has been reported are diabetes, cardiovascular diseases, neurological disorders, vision-related diseases, and infectious diseases.
5. Yes, seven epigenetic drugs have been approved by the US FDA and have been used successful in treating blood, lung, colon, breast, prostate, and ovarian cancers in clinical trials (Yang and Yang, 2016).

Yes/no type questions

1. Can you reverse epigenetic changes?
2. Does epigenetic regulation occur in adults only?
3. Are histone methylation and promoter methylation same?
4. Do twins have the same epigenetic profiling throughout their life?
5. Does environment affect epigenetic biomarkers?
6. Are microRNAs (miRNAs) parts of epigenetic machinery?
7. Are histones the only proteins associated with epigenetic regulation of gene expression?
8. Do “shores” and “shells” belong to epigenetics science?
9. Can the same epigenetic drug treat all cancers?
10. Is epigenetic therapy good for cancer only and not for other diseases?

Answers to yes/no type questions

1. Yes
2. No
3. No
4. No
5. Yes
6. Yes
7. No
8. Yes
9. No
10. No

CHAPTER

5

Development of mouse models for cancer research

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Summary

Mouse models are essential in cancer research. They are used to understand the genetic basic of tumor development and cancer progression. They can also be used to test the efficacies of different anticancer agents. This chapter will help students understand the applications of different mouse models in cancer research and provide them the knowledge necessary to carry out the in vivo studies.

What you can expect to know

Numerous mouse models of human cancers have been developed to facilitate our understanding of the key processes in tumor development and as preclinical models to test the efficacies of anticancer agents. These models recapitulate the crucial stages of tumor initiation from normal cells and their progression to aggressive tumors. These models have also aided in the delineation of factors involved in tumor metastasis, tumor recurrence, and therapeutic resistance. Preclinical studies in mouse models have also assisted in in vivo pharmacokinetics (PK), toxicity, and antitumor efficacy monitoring of numerous chemotherapeutic agents. In the genetically engineered mouse (GEM) models, alternations in genes believed to be responsible for human malignancies, are mutated, deleted or overexpressed, and tumor development is monitored. In this model, tumorigenesis and chemotherapeutic responses can be studied in the presence of a normal immune system. However, since mouse tumor cells may not represent the tumorigenic process in humans, another widely used model is human tumor xenografts in immunocompromised mice. In these models, human tumors are generated in different tissues using tumor specimens, cell lines, or cancer stem cells (CSC; $CD44^{+}/CD24^{-}$). However, investigations with tumor xenografts have been unable to address the role of tumor microenvironments, tumor-stroma, and the normal immune surveillance of tumors. Therefore several new strains of humanized mouse models have been developed recently, for example, NOD-SCID-IL2rg$^{-/-}$ mice (NSG) and SCID-huPBL mice, to provide a human immune system. In addition, patient-derived tumor xenografts (PDX) are being used to more accurately address therapeutic efficacy and resistance/recurrence. This chapter provides several protocols and guidelines for the development of mouse models of cancer which will help the student in the initiation and successful completion of these in vivo experiments.

Introduction

To appreciate the successes and failures of clinical investigations using anticancer drugs, one needs to properly understand the implications of modern animal biotechnology as preclinical models for cancer (Cheon and Orsulic, 2011). It is well accepted that tumorigenesis is a complex process involving the accumulation of multiple genetic aberrations that transform normal cells, allowing for their abnormal growth, proliferation, and metastasis (Hanahan and Weinberg, 2000; Müller et al., 2001; Shacter and Weitzman, 2002). In order to fully understand the development and spread of cancer in the body and to elucidate the crucial role of tumor-associated stroma and tumor angiogenesis, it is imperative to carry out research on tumor

Animal Biotechnology.
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TABLE 5.1 Advantages and limitations of mouse models in cancer research.

Advantages	Limitations
Small size, easy to handle and take care of, and short-tumor generation time.	Mice are very different, in terms of size, life span, organ morphology, and physiology and thus differ in drug PK and PD from human beings.
Cheaper than other animal models of cancer, allowing the use of large numbers for statistical measurements.	One critical difference in the mouse is the activity of the telomerase enzyme, which is largely inactive in adult human cells. (Mouse cells transform more readily and thus require fewer genetic alterations for malignant transformation.)
High tumor incidence and relatively rapid tumor growth.	Mouse models tend to develop relatively few metastases or display metastases with different tissue specificity as compared with human tumors.
Many mice can be treated at the same time to observe dose responses.	Differences in metabolic rate and pathways might result in a different drug response in mouse models (e.g., the cytochrome P450 pathway for drug metabolism).
Genetically, the best characterized of all mammals used in cancer research.	Due to a limited number of initiating genetic alterations, mouse tumors are typically more homogeneous, and this can be an obstacle to modeling the heterogeneity of human cancers.

growth in live animals. To discover novel anticancer agents, it is important to first delineate the biology and genetics of a specific tumor, then identify their relationship to tumor initiation, tumor promotion, progression, and metastasis, and lastly tumor recurrence and drug resistance development. This raises the need for in vivo models that resemble the human organ systems, portray the systemic physiology, and closely recapitulate the human disease. These animal models have enabled the testing of new approaches to cancer prevention and treatment, identification of early diagnostic markers, and novel therapeutic targets to prevent, treat, and eliminate the cancerous growth. Unlike the in vitro cell culture systems, the use of in vivo models has been instrumental in obtaining both PK and pharmacodynamics (PD) data relevant to the successful clinical development of numerous chemotherapeutic drugs. The crucial role of both drug-transporters and drug-metabolizing enzymes in dictating the PK and PD of chemotherapeutic agents is also becoming very apparent, and how polymorphisms in these genes can regulate interindividual variability in anticancer drug efficacies form the basis of a new field called pharmacogenomics (PG) (Mondal et al., 2012). Therefore an appropriate animal model system can show the potential relationships among dose, concentration, efficacy, drug–drug interactions, and/or toxicity in humans and is a mainstay of modern cancer research investigations. More than any other animal model systems, mice have revolutionized our ability to study cancer. Recent advances in developing inbred mouse strains, transgenics, and immunocompromised mice have enabled the generation of clinically relevant mouse models that often recapitulate the human disease progression and response to therapy. Therefore it is very important that one understands the rules and regulations, proper protocols and guidelines, as wells as the time and costs that are associated with in vivo studies (Table 5.1). Below is a comprehensive list of the advantages and limitations of the mouse models of cancer.

Although there is no single model that meets the criteria for all different cancers, a combination of the existing models may mimic the clinical features of particular human cancers. It is therefore imperative that one first understands how to decide on the optimum mouse model to be used, either to address specific questions regarding tumor development and/or to determine the therapeutic effects of antitumor agents in vivo.

History

A brief history of the use of mice in cancer research is provided below. As early as 1664, the eminent Microbiologist, Dr. Robert Hooke, had used mice in his studies on infectious diseases. Around 1902, the era of modern mouse genetics began when a Harvard researcher, William Castle, began studying genetic inheritance in different strains of mice. Around the same time, Abbie Lathrop, a mouse breeder and entrepreneur, was generating colonies for mouse hobbyists that were later used in experiments by different researchers. These early studies included inbred mouse strains that were observed to develop tumors frequently. Ms. Lathrop eventually teamed with Dr. Leo Loeb at the University of Pennsylvania, and they authored numerous papers on their investigations using these mouse strains. The laboratory mice used

by Castle and Lathrop are the ancestors of most of the strains that are routinely used nowadays.

Starting in the late 1980s, the advent of transgenic technologies enabled manipulation of mouse germ-line DNA in embryonic stem (ES) cells which brought about the development of numerous strains of GEM models. The early 1990s brought about tremendous advances in gene-targeting approaches which allowed the development of mouse strains with "knockouts" of tumor-suppressor genes or "knockins" of oncogenes that spontaneously developed tumors (Hanahan et al., 2007; Cardiff et al., 2006). However, early studies showed that many of these mutations manifested embryonic lethality in homozygous mice. Furthermore, variability in the time required for tumor formation in different mouse strains posed significant problems. Mutations carried in all somatic cells also confounded the organ-specific tumor development, which is seen in humans. In the late 1990s, the ability to use the *CRE-Lox recombinase* system allowed the development of conditional and inducible systems that addressed the above drawback of GEM models. However, due to species-specific differences in tumor cells, the limitations of GEM in modeling human cancers were clearly evident. In this respect, the human tumor xenografts, either with actual human tumor tissue or with human cell lines, showed key advantages over the GEM models. In these xenografts, a tumor develops in a matter of a few weeks to months, and orthotopic tumor xenografts can be appropriately placed to reproduce the tissue environment in which the tumor grows in humans. One big challenge in using tumor xenografts was the lack of a functional immune system against tumor cells. In recent years, this problem has been partially solved using immunodeficient mice that have been "humanized" by injection of human peripheral blood leukocytes (PBLs) or bone marrow hematopoietic stem cells (HSC, $CD34^+$) which allowed for an almost complete reconstitution of the immune response to the grafted tumors. In addition, limitations of cancer cell lines have led to the development of direct PDX models that more accurately recapitulate the patient's tumor drug responsiveness in vivo.

Principle

Before embarking on studies using mice, it is imperative to develop procedures that reduce, replace, or refine the animal studies. It is important to tailor the experimental designs and procedures and follow the most ethical ways to treat the animals. Housing and feeding are the most important issues to address during the initial stages of in vivo experiment development. Appropriately sized cages, proper nutrition, and environments (temperature, opportunities for socialization, lighting, or water quality) that closely mimic their natural habitat can go a long way in reducing stress and protocol approval by the Institutional Animal Care and Use Committee (IACUC).

Institutional Animal Care and Use Committee approval

All organisms go through stress in many forms but are normally able to adapt and recover. Thus laboratory mice are allowed to acclimatize for at least a week before the start of experiments. However, unlike stress, distress is a negative state wherein the animal fails to adapt and return to its physiological or psychological homeostasis. Age, gender, genetic traits (including transgenic modifications) rearing and postnatal separation, psychological state, and housing conditions can all affect the animal in different ways. Therefore care should be taken to try to relieve stress and eliminate distress as much as possible. In case where alternatives to animal use are not available, it is important that researchers recognize and alleviate distress in laboratory animals. In this respect, it is important to differentiate between stress and distress. Thus the following three Rs (Refine, Reduce, Replace) are important considerations and criteria when writing an IACUC protocol:

1. *Refinement:* This involves careful consideration of the aspects of research methodology and animal maintenance. The in vivo techniques should be thoroughly reviewed so that they cause a minimal amount of pain, stress, or suffering. If it is not possible to alleviate pain by giving medications then a humane endpoint should be defined so that the animal may go through as little suffering as possible. It is also important to have a clearly defined protocol of when to treat or euthanize the animals to reduce their suffering, and a better understanding of distress mechanisms may help to define earlier end points in the experiments.
2. *Reduction:* This aims at limiting the number of animals needed for a study. Pilot studies to optimize techniques, treatments, and conditions are ways of doing so, and the use of good statistical methods may help assess the accurate number of specimens required to get relevant results and limit unnecessary use of large numbers of animals. The development of new techniques can further reduce, or eliminate, the number of animals necessary.
3. *Replace:* This term takes into account that new technologies are developing which provide an alternative to animal testing or at least minimize their use. The use of appropriate cell cultures can eliminate or minimize the use of whole organisms

and still give the required data. As an example, to test the antitumor activity of a drug, tumor cells can be grown in vitro for the majority of tests toward optimizing dosing and treatment regimen, instead of trying to optimize these in vivo. Also, initial toxicology studies should be carried out using cultured liver cells or other normal cell lines such that supportive data are gained to test the efficacy and toxicity prior to animal testing.

Institutional Animal Care and Use Committee guidelines

Before any animal testing can be initiated, the protocol has to be first approved by the IACUC which consists of at least five members appointed by the institution. The IACUC reviews the research protocols and conducts evaluations of the institution's animal care. The IACUC is required to ensure that the proposed work falls within the Animal Welfare Assurance. Furthermore, the approved animal use protocol must be reviewed by the IACUC every 3 years. To obtain an IACUC approval, the protocol needs to address the following points:

1. Identification of mouse strain and an approximate number of mice to be used.
2. Rationale for using mice and the appropriateness of the numbers used.
3. A complete description of the proposed use of mice.
4. A description of anesthetic procedures to minimize discomfort and injury to animals.
5. Animals that experience severe or chronic pain that cannot be relieved will be painlessly killed at the end of the procedure or, if appropriate, during the procedure.
6. A description of appropriate living conditions that contribute to health and comfort.
7. A description of housing, feeding, and nonmedical care of the animals which will be directed by a veterinarian or other trained scientists.
8. Personnel conducting procedures will be appropriately qualified and trained.
9. Euthanasia used will be consistent with the American Veterinary Medical Association (AVMA) guidelines.

Methodology

Inbred mice

In scientific experiments, homogeneity allows for controlled and reproducible experiments which necessitated the development of genetically identical mice by inbreeding. The inbred mice provide the advantage of data reproducibility. The first use of inbreeding in science can be traced back to laboratories of researcher Clarence C. Little, an American geneticist who was exploring coat color inheritance in his studies with the first inbred stock of laboratory mice, the DBA strain (Crow, 2002). Off-springs of these ancestral DBA (Dilute, brown, and non-Agouti) mice are still available to researchers today. Today mice are considered to be stably inbred after 20 generations of the appropriate pairings. The utility of inbred mice as a model system lies in the fact that all the mice have an identical genetic background and are expected to generate similar responses to treatments that allow researchers to accumulate data in standardized collections. Many scientists use a single inbred strain or F1 hybrid in their research because it has a repeatable, standardized, uniform genotype supported by a substantial body of previous research. In fact, the more that is known about a strain the more valuable it becomes and being of a single genotype, it is relatively easier to "know" about inbred strains. Another advantage of inbred strains is that sample size can be reduced in comparison with the use of out-bred stocks. An inbred strain may also be cross-bred with another strain to develop new models. For example, recombinant inbred strains, that have been out-crossed from two separate inbred strains, and then maintained for generations, are utilized for mapping traits and are in wide use (Casellas, 2011). Over 20,000 scientific papers used BALB/c mice between 2001 and 2005, and a further 10,000 used C57BL/6, both are well-defined inbred strains. Additionally, the work on the BALB/c inbred mouse strain led to the development of monoclonal antibodies and the development of ES cells used in developing transgenic mice (Fig. 5.1).

Depending upon the type of cancer being investigated or the application of the tumor model to be tested, either toward basic or translational research, a number of criteria can aid in the decision-making. A brief list of these criteria on different mouse model system to be chosen for cancer investigations is provided in Table 5.2.

Examples where genetic variability within inbred strains have often been used to measure cancer occurrence is *Consomic* strains; wherein an entire chromosome from one inbred strain replaces the corresponding chromosome in another inbred strain. Furthermore, researchers can rapidly breed *Congenic* strains from these consomic strains, such that the mice now carry only a small segment that differs between animals. This allows for the narrowing of the region on a chromosome that can be targeted for gene identification related to oncogenesis. However, the main disadvantage of using a single strain is that it is not representative of the heterogeneity and polymorphisms observed in humans

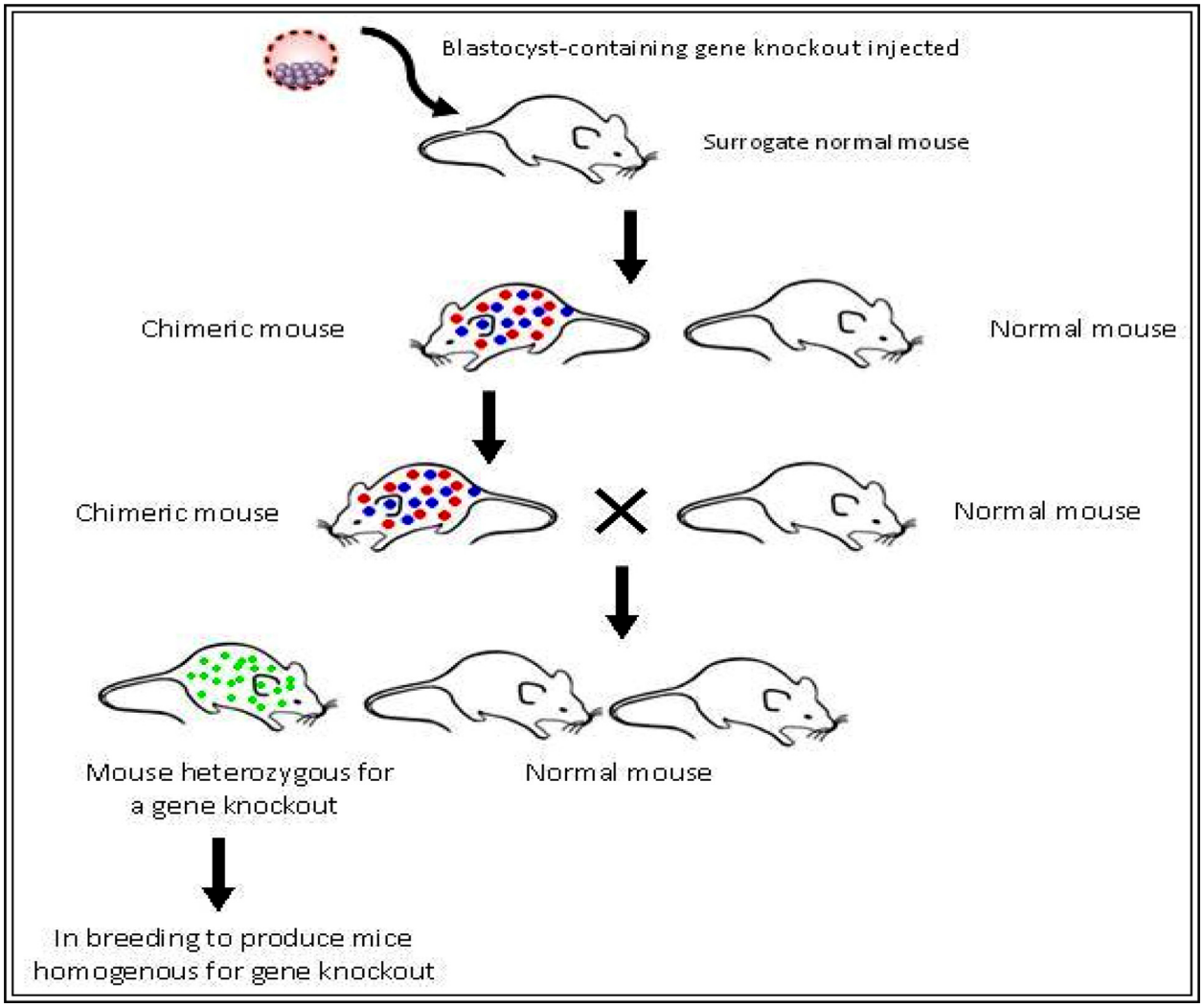


FIGURE 5.1 Generation of inbred strains of transgenic mice.

(Pal and Hurria, 2010; Tian et al., 2011). The following sections provide some examples for each of these mouse cancer models.

Examples with applications

Immunocompetent mice

Spontaneous tumor models

Mice that are genetically engineered to carry predisposed mutations serve as very important tools in the study of cancer initiation and progression following exposure to carcinogens (Fig. 5.2). The role of tumor promoters and or tumor-inducing/silencing agents with specific characteristics can be studied during tumor development. Using these models, the effects of natural and environmental estrogens on normal mammary gland development and on carcinogenesis have been recently reviewed by Pelekanou and Leclercq (2011). This review focused on the role of estrogen receptors (i.e., ERα and ERβ) in regulating growth, apoptosis and differentiation of mammary epithelial cells, and the bidirectional coordination between stroma and cancer cells in the maintenance of tumor growth. Cravero et al. also wrote a recent review on new rodent systems that recapitulate both genetic and cellular lesions that lead to the development of pancreatic cancer (Ding et al., 2010). Interestingly, mice with mutant K-Ras oncogene (G12D) spontaneously develop tumors in the pancreas that are nonmetastatic in nature. However, simultaneous mutation of the p53 tumor-suppressor gene leads to the generation of metastatic pancreatic cancers.

There is also large variability in both the susceptibility and incidence of spontaneous lung tumors between mouse-inbred strains (Piegari et al., 2011). Typically strains with high spontaneous lung tumor incidence

TABLE 5.2 Criteria for choosing the optimum inbred mouse model.

Biologic:

1. Multistage: the model shows clear evidence of progression, enabling analysis of genetic and biological events associated with these transitions.
2. Tumor histology and pathology are similar to equivalent human disease.
3. Tumors metastasize from primary site.
4. Model has specific properties that make it suitable for studies of one of the following:
   - Angiogenesis
   - Apoptosis/survival
   - Tissue invasion
   - Role of the immune system

Genetic:

1. Multiple mutations in specific genes, at least some of which reflect those of equivalent human tumors.
2. Gross chromosomal changes (LOH, deletions, recombinations) in regions orthologous to those altered in human disease.
3. Tumors bear specific alterations in one or more pathways known to be involved in human cancer, for example, p53.
4. Gene expression profiles similar in mouse and human tissues.
5. Tumor shows strong effects of genetic background, enabling studies of genetic susceptibility

Etiologic:

Tumor model reflects exposure to environmental factors known to play a causal role in human tumor induction, for example: Environmental mutagens like chemical, radiation.

Therapeutic:

1. The model is suitable for the testing of new *therapies*:
   - *Chemicals*
   - Viral vectors
   - Immunological targets.
2. The model is predictive of human clinical trial results.

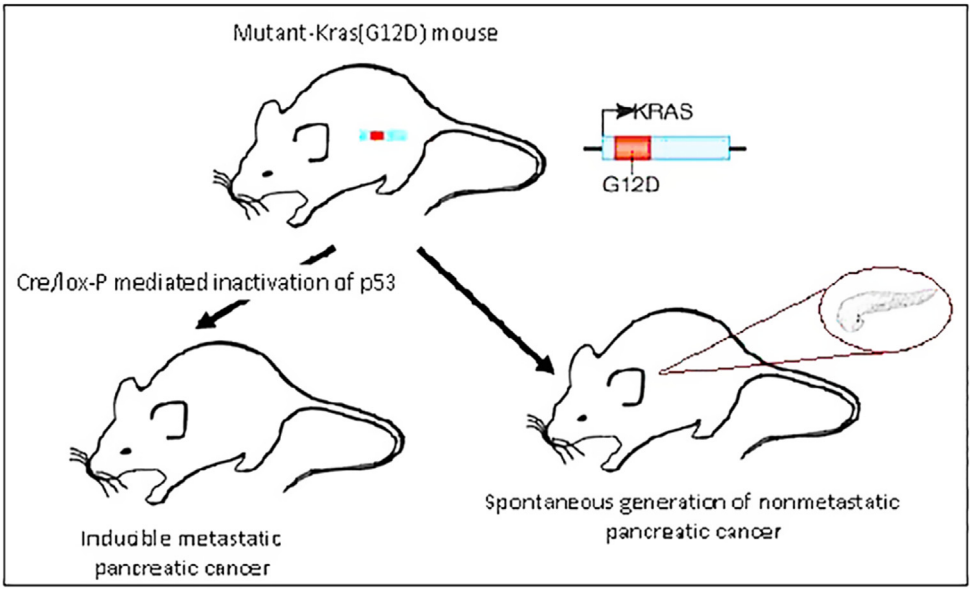


FIGURE 5.2 Generation of spontaneous tumor models for carcinogen studies.

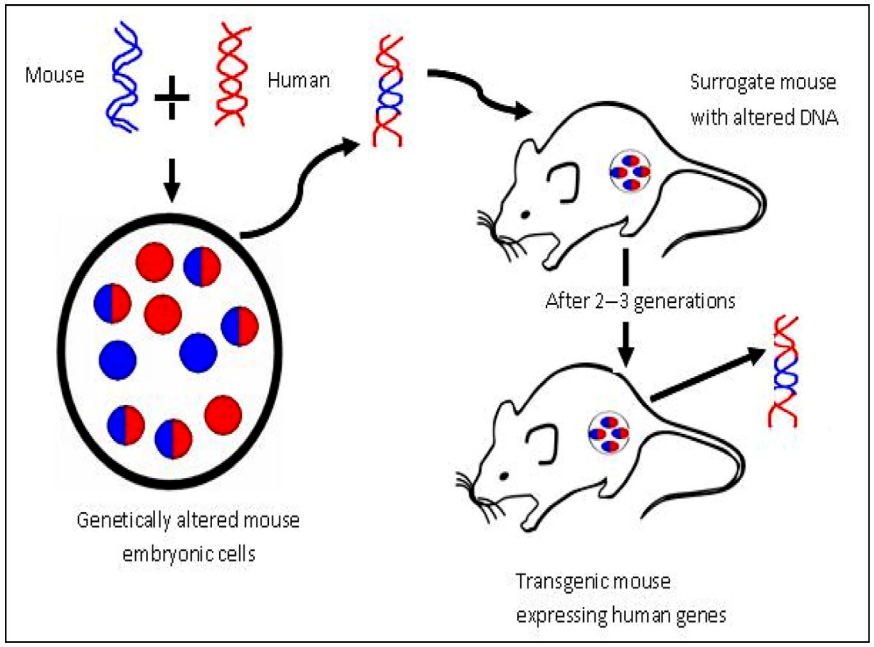


FIGURE 5.3 Generation of GEM models in immunocompetent mice.

are also very responsive to chemical induction of lung tumors, for example, exposure to cigarette smoke, tar, or chemically pure carcinogens. Some strains are more resistant than others, and this difference can often be attributed to an underlying genetic variance. For example, a *Cdkn2a* polymorphism was found between the intermediate-resistant BALB/cJ and susceptible A/J strains. Chemical induction of lung tumors with carcinogens such as polycyclic aromatic hydrocarbons ethyl carbamate (urethane) is very reproducible and invariably results in pulmonary adenoma and adenocarcinomas. In breast cancer research, one of the most commonly used models for the study of cancer preventive agents is the mouse model that develops tumors, either spontaneously or after carcinogen treatment. This model has been successfully used to demonstrate the chemopreventive activity of many agents, including selective estrogen receptor modulators (SERMs) such as Tamoxifen, Raloxifene, and Idoxifene and retinoid compounds. In DMBA-induced mammary tumors, Aryl hydrocarbon receptor (AHR) activation was shown to delay the development of tumors (Wang et al., 2011). In addition to the effects of genetic mutations on cancer susceptibility, the carcinogen-induced tumor models are also useful in understanding the cancers caused by exposure to physical agonists (such as asbestos fibers), chemicals (such as Cadmium, Arsenic), and environmental (UV radiation) carcinogens.

The genetically engineered mouse models

To generate a more reproducible cancer model based on loss or gain of specific genes, scientists in 1987 utilized a procedure called homologous recombination in mouse ES cells allowing them to remove a gene (knockout) or replace it (knockin). These genetically altered mouse models allow cancer researchers to test in the human context, as to how, when, where, and in which combinations particular gene alterations may be involved in the initiation and progression of cancer in immunocompetent animals (Fig. 5.3).

The discovery of oncogenes and their association with the development of human tumors led to the first transgenic cancer models. The "knockin" models of cancer included the overexpression of many oncogenes such as c-myc, v-Ha-ras, or SV40 T-antigen, etc., which clearly indicated their roles in the development of different types of tumors, for example, myelomas, lymphomas, carcinomas, and sarcomas. For example; in a model of brain tumor, derived by delivering the viral oncogene SV40 T-antigen into mouse eggs, the targeting of CD8 + T cells was shown to be sufficient for tumor elimination (Tatum et al., 2008). The SV11 mouse line, which expressed the SV40 T Ag (T Ag) as a transgene from the SV40 enhancer/promoter, leads to T Ag expression and promoted the appearance of small papilloma by 35 days and progressive tumor growth by 104 days. Using this GEM model, investigators showed that immunotherapeutic approaches that target the recruitment of tumor-reactive CD8 + T cells were effective against well-established tumors. Furthermore, donor lymphocytes from transgenic mice expressing a T-cell Ag (epitope IV) specific T cells enabled rapid regression of established tumors.

Another example where GEM mice developing breast cancers were produced by delivering a mutant

human oncogene called c-Myc by MMTV, a mouse virus that infects mouse mammary tissue. These mice developed mammary tumors that resembled human breast tumors. In these models, the role of tumor-suppressor genes such as Rb, p53, and Brca1 was also implicated in the development of cancers (Singh and Johnson, 2006). The GEM models for breast cancers also involved the overexpression of oncogenes such as c-Myc, cyclin D1, Her2, and Wnt1. Of particular importance are tumors overexpressing Wnt1, which showed heterogeneous ER status, making the model very relevant to the study of both ER-positive and ER-negative cancers. Several important mouse models of gastrointestinal cancer have also been developed based on genetic alterations that are known to affect the above pathways. These models clearly showed the process of multistep carcinogenesis where a progressive series of mutations occur during colon cancer development, involving genes such as Wnt1, APC, RAS, p53, and TGF-β (Ramanathan et al., 2012). Indeed, the $Apc^{min/+}$ mouse, developed by Moser and colleagues in 1990, was the first mouse model of intestinal tumorigenesis to be generated by mutational inactivation of the adenoma polyposis coli (APC) gene through random chemical carcinogenesis. The $APC^{min/+}$ mice and other APC mutant mice develop multiple intestinal adenomas, and similar mutations in the APC gene were also found in patients who develop familial adenomatous polyposis. However, this GEM model has some limitations since the location of the polyps is predominantly restricted to the small intestine and some rare but evident occurrence of malignant progression to adenocarcinoma. Despite these limitations, the $APC^{min/+}$ mice model continues to be one of the most used models particularly for chemopreventive studies that involve the gastrointestinal tract.

The Cre/Lox system: a superior genetically engineered mouse model

It has been almost 15 years since the discovery of the Cre/lox system which is now frequently used as a way to artificially control gene expression (Sauer, 1998). This system has allowed researchers to create various genetically modified animals where the gene of choice can be externally regulated. The Cre/*lox* system provides a method to produce a mouse that no longer has a target gene in only one cell type (Fig. 5.4).

In this system, the induction of *Cre-recombinase* enzyme mediates the site-directed DNA recombination between two 34-base pair *loxP* sequences. To achieve this in mice, transgenic mice containing a gene surrounded by loxP sites are mated with transgenic mice that have the *cre* gene expressed in specific cell types. In tissues with no *cre* gene, the target gene functions normally; however, in cells where *cre* is expressed, the target gene is deleted. The correct placement of Lox sequences around a gene of interest may allow genes to be activated, repressed, or exchanged for other genes. Furthermore, the activity of the *Cre* enzyme can be controlled, so that it is expressed in a particular cell type or triggered by external stimulus such as chemical signals or heat shock. This enables the induction of somatic mutations in a time-controlled and tissue-specific manner.

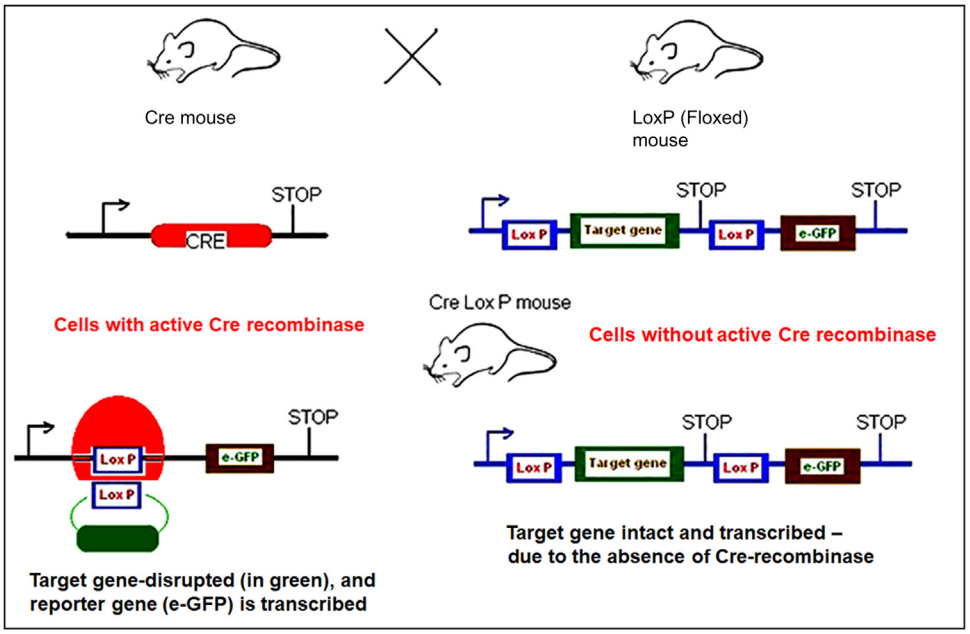


FIGURE 5.4 Generation of the Cre/Lox mouse model. *Source: Modified from Wikipedia images.*

Since a majority of human colorectal cancers exhibit constitutive Wnt activity due to mutations in the APC or β-catenin genes, Wnt-targeted therapeutic strategies in which expression and activity of this gene product are altered specifically in cancer cells, have been successfully demonstrated using the Cre/Lox system (Bordonaro, 2009). By using this system, a mammary-specific deletion on mouse chromosome 11 could be achieved which accelerated Brca1-associated mammary tumorigenesis in comparison to Brca1 conditional knockout mice (Triplett et al., 2008). Similar to human BRCA1-associated breast cancers, these mouse carcinomas were ERα-negative and were of basal epithelial origin. Furthermore, to test the role of p53 in Brca1-associated tumorigenesis, a p53-null allele was introduced into mice with mammary epithelium-specific inactivation of Brca1. The loss of p53 accelerated the formation of mammary tumors in these females. Since the Brca1 knockout mice were found to die during embryonic development, the Brca1 conditional mice-expressing *Cre* in the mammary gland epithelia enabled the development of mammary tumors only during adulthood. Therefore this newly developed homologous recombination system closely models carcinogenesis in humans where tumors evolve from somatic gene mutations in normal cells in a time-controlled and tissue-specific fashion in vivo.

Immunodeficient mice

In order to know whether a patient's tumor will respond to a specific therapeutic regime, it is essential to examine the anticancer response to the human tumor and not a mouse tumor. This is where the human tumor xenograft on athymic nude mice, SCID mice, or nonobese diabetic (NOD)/SCID humanized mice can be helpful. The availability of tumor grafts has made transplanted tumors the test models of choice to investigate anticancer therapeutics (Richmond and Su, 2008). The transplantation models allow us to propagate tumor tissues in vivo. Thus transplanted tumors have contributed in a made major way to our understanding of cancer biology which would be unrealizable with the GEM models. Orthotopic xenografts can reproduce the organ environment in which the tumor grows to mimic the effects of the tumor microenvironment. In addition, stromal cells can be included in the xenograft to more completely mimic the human tumor microenvironment; and xenografts using NOD/SCID mice that have been "humanized" by injection of PBLs or HSCs (bone marrow or cord blood), allow for an almost complete reconstitution of the immune response to the tumor (Fig. 5.5) (Richmond and Su, 2008; Kerbel, 2003).

Both retrospective and prospective studies reveal that human tumor xenografts can be remarkably predictive of cytotoxic chemotherapeutic drugs that have activity in humans, especially when the drugs are tested in mice using clinically equivalent or "rational" drug doses. However, the magnitude of benefit observed in mice, both in terms of the degree of tumor responses and overall survival may be different when compared with the clinical activity of the drug observed in humans. Furthermore, since transplanted tumors allow large quantities of tissue of uniform character, both pathologic and molecular studies can

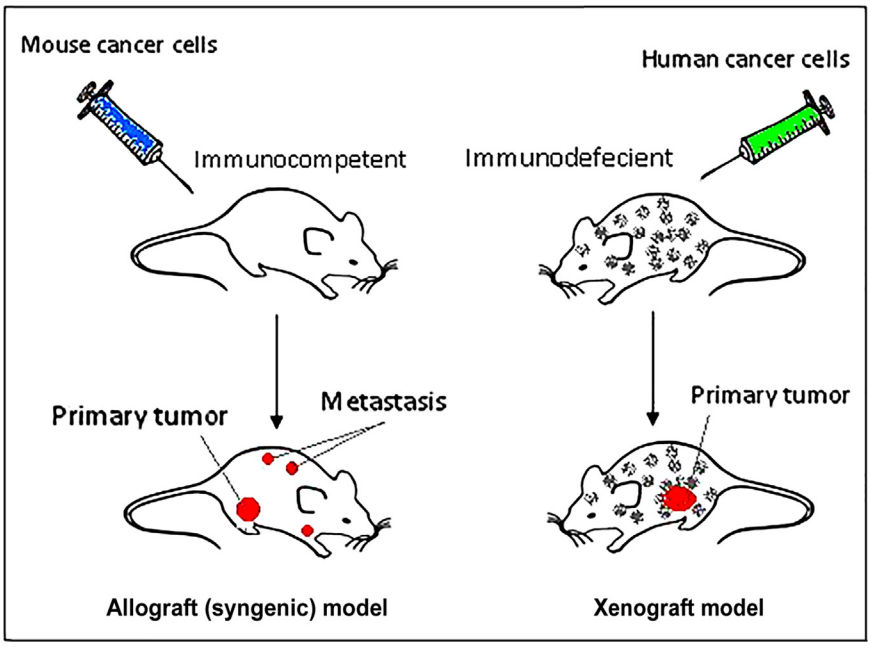


FIGURE 5.5 Generation of allograft and xenograft tumor models.

be easily carried out. The choice of tumor and the site of transplantation must also be validated in advance, so that it is comparable with the normal tissue from which the tumor was derived. A variety of sites has been used for transplantation to mimic that observed in human, but the most commonly used are the subcutaneous (s.c.), intraperitoneal (i.p.), and intramuscular (i.m.) sites. Indeed, in contrast to the "spontaneous" cancers, transplanted tumors are most widely used for assaying the therapeutic effectiveness of a large array of anticancer compounds. Two different tumor transplantation models are currently used in different laboratories.

Allograft transplants

In the Allograft (or Syngeneic) transplantation models, the cancerous cells or solid tumors are itself of mouse origin and are transplanted into another host mouse containing a specific genetic trait, for example, GEM mice. Because the recipient and the cancer cells have the same origin; the transplant is not rejected by mice with an intact immune system. The results of therapeutic interventions can thus be analyzed in an environment that closely recapitulates the real scenario where the tumor grows in an immunocompetent environment. A disadvantage however is that transplanted mouse tissue may not represent in whole the complexity of human tumors in clinical situations (Maulik, 2009). This transplantation strategy circumvents some of the difficulties observed in GEM models complicated breeding schemes, variable tumor latency, and chemotherapeutic drug inefficacy. Tumor fragments from tumor-bearing MMTV-PyMT or cell suspensions from MMTV-PyMT, -Her2/neu, -Wnt1, -Wnt1/p53$^{+/-}$, BRCA1/p53$^{+/-}$, and C3(1)T Ag mice can be transplanted into the mammary fat pad or s.c. into naïve syngeneic or immunosuppressed mice. Tumor development can be monitored and tissues can be processed for histopathology and gene expression profiling, and metastasis can be scored at 40–60 days after removal of original tumors.

In a recent study by Prosperi et al. (2011), APC mutation was shown to enhance PyMT-induced mammary tumorigenesis. In serial passages, regardless of the site of implantation, several other investigators showed that PyMT tumors from anterior glands grow faster than posterior gland-derived tumors. Microarray analysis also revealed genetic differences between these glandular tumors. The differences in transplantation were reproducible using anterior tumors from multiple GEM and tumor growth rates correlated with the number of transplanted cells. Similar morphologic appearances were also observed in both original and transplanted tumors. Metastasis developed in >90% of mice transplanted with PyMT, 40% with BRCA1/p53$^{+/-}$ and wnt1/p53$^{+/-}$, and 15% with Her2/neu tumors. Interestingly however, expansion of PyMT and wnt1 tumors by serial transplantation for two passages did not lead to significant changes in gene expression. Furthermore, PyMT-transplanted tumors and anterior tumors of transgenic mice showed similar sensitivities to cyclophosphamide and paclitaxel. Thus allograft transplantation of GEM tumors can provide a large cohort of mice-bearing mammary tumors at the same stage of tumor development and with a defined frequency of metastasis in a well-characterized molecular and genetic background.

Xenograft transplants

In the xenograft model, human tumor cells are transplanted, either under the skin or into the organ type (orthotopic) in which the tumor originated, into immunocompromised mice such as athymic nude mice, severely compromised immunodeficient (SCID) mice, so that the human cells are not rejected. In this model, human cancer cells or solid tumors are transplanted into a host mouse. These transplants may be orthotopic or they may be s.c. or placed just beneath the host's skin. Because the cancer xenograft is of human origin, it represents the properties and to an extent the complexities of the human cancer. On the other hand, the compromised host immune system is not truly representative of actual patients.

Tumors obtained from patients can be kept viable in the frozen state for prolonged periods. The usual method for preparing solid tumors for freezing is to immerse small pieces in media such as glycerol-glucose, in sealed sterile ampoules. The tumor-medium mixture should be slowly cooled to the final storage temperature and rapidly thawed (at 37°C) on removal and after thawing, tumors should be immediately inoculated in mice. Depending upon the number of cells injected, once the tumor develops to an appropriate size, the response to therapeutic regimes can be studied in vivo.

Using s.c. prostate tumor xenografts of both androgen-dependent (LNCaP) and castration-resistant (HR-LNCaP) prostate cancer cells, Schayowitz et al. (2010) showed that dual inhibition of AR and mTOR can prolong the hormone sensitivity of prostate cancers (Schayowitz et al., 2010). Male SCID mice 4–6 weeks of age (from the National Cancer Institute-Frederick Cancer Research Center) were inoculated with the LNCaP or HR-LNCaP cells (2.0×10^7) along with Matrigel (10 mg/mL) in 100 μL of cell suspension. The mice were then treated with a combination of inhibitors to block the AR and mTOR activation when tumors reached 500 mm^3 for 3–7 weeks. The addition of everolimus (androgen synthesis inhibitor) to bicalutamide (antiandrogen) treatment of resistant tumors

significantly reduced tumor growth rates and tumor volumes and decreased serum prostate-specific antigen (PSA) levels.

In another study by Kataoka et al. (2012), the efficacy of chemoendocrine therapy in both premenopausal and postmenopausal models with ER-positive human breast cancer xenografts was studied (Kataoka et al., 2012). Female 4–5-week-old BALB/c-nu/nu mice were inoculated with a suspension of MCF-7 breast cancer cells (5×10^6 cells/mouse) subcutaneously into the right flank. For the premenopausal breast cancer model, mice were subcutaneously implanted with slow-release estrogen pellets (0.25 mg/pellet 17β-estradiol) the day before tumor cell inoculation. After several weeks, mice bearing a tumor ($\sim$200–400 mm^3) were randomly allocated to control and treatment groups (eight mice each) and received 6-week oral therapy with capecitabine and/or tamoxifen (30 or 100 mg/kg/day). For the postmenopausal breast cancer model, mice were ovariectomized and subcutaneously implanted with slow-release androstenedione pellets (1.5 mg/pellet) the day before tumor cell inoculation. Mice-bearing tumors ($\sim$200–400 mm^3 in volume) were treated for 6-week oral administration of capecitabine (359 mg/kg/day) and/or letrozole (0.1 mg/kg/day). In each model, control mice received vehicle alone. Tumor volumes and body weights were monitored two or three times a week starting from the first day of the treatment. The combination of 5′-deoxy-5-fluorouridine (5′-DFUR; an intermediate of capecitabine) with 4-hydroxytamoxifen (4-OHT; an active form of tamoxifen) or letrozole (aromatase inhibitor) decreased the number of estrogen-responding cells and size of breast tumor xenografts in both premenopausal and postmenopausal models.

Tumor xenografts have been used in the preclinical and clinical development of anticancer therapeutics. In human breast cancer xenografts, several investigators showed that herceptin (Trastuzumab) can enhance the antitumor activity of paclitaxel and doxorubicin against HER2/neu-overexpressing cells, and this led to its subsequent use successful clinical trials. In addition, herceptin is now a standard drug used in the treatment of human epidermal growth factor receptor 2-positive (HER2 +) early-stage breast cancer. Multiple Myeloma has remained an aggressive and incurable cancer, and while melphalan and predinisone provide symptomatic relief, survival rates were only 3 years. However in the past decade, the combination of bortezomib and melphalan was demonstrated as effective for the treatment of multiple myeloma, first in preclinical xenograft trials and then in patients. This has led to the new standard of clinical care for multiple myeloma patients over 65 years of age or those with pre-existing conditions to whom the option of high-dose chemotherapy followed by stem-cell transplantation is an unavailable option. Therefore xenograft models are useful for toxicity studies from targeted therapies and in many cases to predict biomarkers of target modulation. However, several ethical issues need to be first addressed before the testing of toxic chemotherapeutic agents in mouse cancer models.

Humanized mice

Humanized mice are used to model the human immune system in tumor-bearing mice, and enable proper evaluation of therapeutic candidates in an in vivo setting (Shultz et al., 2007). Shultz et al. (2007) pioneered the development of human lymphoid and myeloid cells in NOD/SCID/IL2Rγ null (NSG) mice using CD34 + mobilized HSCs transplanted into the sublethally irradiated NSG mice (Shultz et al., 2007). Humanized mice allowed these researchers to examine xenograft growth in the context of a functional human immune system and resultant tumor microenvironment. Indeed, a number of recent studies have highlighted the increased similarities in tumor growth, metastasis, drug response, and mimic tumor microenvironmental signaling similar to those seen in vivo in cancer patients (Morton et al., 2016). Immunodeficient mice can accept human stem cells due to the lack of host immunity and can thus be used to test promising anticancer therapies (Holzapfel et al., 2015b). In this model, mice are treated with radiation to knockdown the existing blood system, and then human stem cells are introduced to regrow the human immune system. After a few months, the mice become chimeras with human blood cells and the human immune system. Zhou et al. (2014) have published a highly comprehensive review on the use of humanized NOD-SCID-IL2rg$^{-/-}$ mice as a preclinical model for cancer research and its potential use for individualized cancer therapies (Zhou et al., 2014). Humanized CD34 + NSG mice supported multilineage engraftment of human cells including B cells, T cells, dendritic cells, and monocytes. Indeed, the NSG mice injected with human HSCs showed robust engraftment efficiencies as measured by flow cytometry using cell-specific markers of human leukocytes (Morton et al., 2016; Holzapfel et al., 2015b; Zhou et al., 2014).

Traditionally, although the severe combined immunodeficiency (SCID) mouse have been used for humanization purpose, recently the NOD/Shi-SCID IL2rγnull (NOG) mouse and the NOD SCID gamma, that is, NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NSG) mice have been shown to engraft human cells more efficiently (Brehm et al., 2014). In these mice, three options are available for humanization: (1) the HSC engraftment model, which develops a multilineage immune system; (2) the BLT mouse, where the NOD/SCID mice are cotransplanted with human liver and thymus tissues

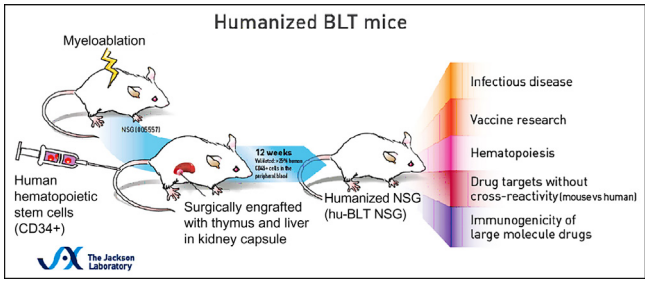


FIGURE 5.6 Generation of humanized mice (https://www.jax.org/strain/013062).

along with autologous CD34 + HSCs; and (3) the PBMC engraftment model, where co-injection of human PBMC with tumor cells can be done at the same site. Ishikawa et al. (2008) had first developed the human hemato-lymphoid system in NSG mice using 10^5 CD34 + Cord Blood cells (depleted of CD3, CD4, CD8, CD11b, CD19, CD20, and CD56, and enriched for CD34 cells) into the sublethally irradiated (100 cGy) NSG newborns (Ishikawa et al., 2008). By using human cord blood HSC (CD34 + cells), McDermott et al. (2010) also showed that the NSG-recipient mouse support greater engraftment of human stem cells (McDermott et al., 2010). Multilineage differentiation of cells (T, B, NK, myeloid, DC, HSC) was clearly observed, and most importantly, no graft versus host disease (GvH) development was seen. In this protocol, 3–4 weeks old mice underwent sublethal irradiation followed by intravenous (i.v.) injection of human CD34 + cells (cord blood, mobilized peripheral blood). These Hu-CD34 + mice clearly showed multilineage reconstitution, consisting of the myeloid lineage, lymphoid lineage, and some mucosal immune function. The human CD34 + cells stably engrafted in the bone marrow, and the engraftment was stable for over 1 year. Both human CD4 + and CD8 + T cells were present in the circulation and in other tissues. Indeed, the engraftment of mature human white blood cells (hCD45 +) was confirmed 12 weeks after injection (Fig. 5.6).

Checklist for a successful in vivo experiment

Prior to initiating the studies, a successful in vivo experiment involves critical planning and addressing of multiple factors. It is very important to make sure that everything is ready and the guidelines and protocols have been well thought out and you have the IUCAC approved animal protocol in place.

1. *Identifying the experimental goals.* It is important to first identify what one plans to answer by using the animal experiments and justify the utility of mouse models. This could be based on either the role of a certain gene or the effect of a certain anticancer therapy. It is also important to pinpoint whether one aims to study tumor initiation, tumor progression, tumor metastasis, or tumor recurrence/resistance. For this purpose, it is imperative that one chooses the best mouse model to be tested and decide on when to initiate the tumor transplant or drug treatment. At this stage, the investigator needs to choose whether an immunocompetent or immunodeficient model would be optimal and whether an easier subcutaneous tumor model or a more difficult orthotopic tumor model would be needed.
2. *Finalizing the experimental setup and budget.* The next most important criteria would be to design an experimental setup based on the budget. Since animal experiments can be very costly, it is important to plan the number of animals available for the studies. Some immunocompetent mouse strains are relatively inexpensive ($10–30 per mouse); however, most of the transgenic strains and especially the immunocompromised (SCID or nu/nu) mice are much more expensive ($60–100 per mouse). Multiple animals (at least 6–10 per group) would be necessary to generate statistically significant effects, and the use of multiple groups to assess drug treatments can become very expensive at the end. Furthermore, right from the beginning, the costs toward animal housing and care in the vivarium should be taken into account. Vivarium charges per day and per animal can add up to be significantly expensive if the experiments are for long-term tumor growth and anticancer efficacy measurements. The investigators should be well aware of the time, efforts, and costs associated with the in vivo experiment themselves and should be able to analyze the validity of the data obtained from these in vivo studies. Therefore time management is very crucial and enough time for performing the in vivo experiment, and dosing of multiple animals will need to be coordinated. Rushing the surgical procedures and drug treatments may ruin the entire experiment.
3. *Training of personnel.* Before starting an experiment, it is important that animal handling workshop/certifications are obtained for all personnel involved. It is crucial that each researcher understands the ethics involved in animal research which will enable them to make important decisions about the protocol, the number of animals required, the end points needed for animal data collection, and the method and timeliness of animal euthanization. Some experiments may take several hours to do,

especially with a large number of replicates and multiple treatment groups. Thus for accurate and efficient progress, it is advisable to have experienced assistance.

4. *Ordering of animals.* Once the experiments are finalized, IACUC approval is obtained, and trained personnel is available, it is advisable to order the animals at least 2 weeks in advance of initiating the experiments. Some experiments may need specific sex, such as prostate cancer model, breast cancer model, etc. The age of the mice is also an important consideration because 6 and 8 weeks old mice may not respond to treatment the same way as mice that are 4 months old. Also, different strains respond to treatment differently, so one treatment that works in one strain may not work in another and *vice versa*. Therefore make the choice of strain, sex, and age according to the type of cancer and the application of experiment.

   There are several different vendors who sell different mouse strains for researchers and the costs may vary significantly, for example, Harlan Laboratories, Charles River Laboratories, etc. It is advisable to shop around for the best prices for the animals, but also be aware of previous publications using the mouse strains from different vendors since minute differences in the genetic makeups of different strains can significantly alter the end results. Before ordering the animals, one needs to contact the vivarium staff in order to secure designated space for the animals, the specifics of animal housing and treatments. In case vivarial staff, for example, veterinarians, surgery specialists are needed, it would be important to schedule and coordinate the experiments in advance.
5. *Preparation of animals for anesthesia.* Mice should be healthy and well-prepared for the experiments. All mice should be housed in a pathogen-free environment under controlled conditions (temperature 20°C–26°C, humidity 40%–70%, light/dark cycle 12 h/12 h). All mice should be allowed to acclimatize and recover from shipping-related stress for at least 1 week prior to the study. The body fat, or lack thereof, age, sex, and strain can all impact a mouse's response to anesthetic agents. Pre-anesthetic fasting is not usually necessary in mice; however, if fasting is employed it should be limited to no more than 2–3 hours prior to the procedure. Because mice have a greater body surface area to body mass ratio than larger animals, thermal support is critical to their survival and successful anesthetic recovery. Parenteral anesthesia may be administered to mice via i.p., s.c., or intravenous injection. Inhalation anesthesia may be delivered by chamber or facemask. Animal anesthesia can be achieved with isoflurane without the use of specialized anesthesia equipment. Alternative methods of anesthesia include injectable anesthesia that is achieved by Avertin (200 mg/kg) or ketamine/xylazine (100/10 mg/kg) i.p. injections. Irrespective of the method used, the mouse should be monitored to avoid excess cardiac and respiratory depression, and insufficient anesthesia.
6. *Preparation of humanized mice.* Roth et al. (2015) used human tumor-infiltrating lymphocytes (TIL) and showed their ability to cooperatively regulate prostate tumor growth in a humanized mouse model (Roth and Harui, 2015). Simultaneous implantation of human PBLs, DC, and tumor results in a huPBL-NSG model that recapitulates the development of human TIL and allows an assessment of tumor and immune system interaction. In this protocol, NSG mice were implanted with 1×10^7 human PBLs (expressing CD14 and/or CD16) and activated T cells (CD25) in combination with 5×10^5 autologous DC by i.p. injection. The PC3 prostate cancer cells (2×10^6) were implanted subcutaneously on the same day. Most interestingly, tumors recovered from these huPBL-NSG mice were significantly smaller than tumors from NSG mice that were not immune reconstituted. Furthermore, the human effector memory T lymphocytes (T-em) were increased in the TIL recovered from tumor-bearing huPBL-NSG animals. These findings clearly emphasized that despite the predominance of CD8 + TIL in the tumors, the CD4 + T cells play an essential role in tumor regression.
7. *Preparation of human tumor cells for xenografts.* For some studies, it is advisable to use labeled tumor cells to be able to measure tumor growth and metastasis without invasive surgery. Therefore fluorescent-labeled cells (GFP or Luciferase) will need to be prepared in advance. Healthy tumor cells in the logarithmic phase of growth are the key to a successful in vivo transplantation experiment. The cell should not be over confluent nor should they be too sparse, during the passage of cells. Three days before the in vivo experiment, seed the cells into a new culture dish or flask, 1 day before the experiment, change to fresh medium, when harvesting the cells, the cells must be 70%–80% confluent (for monolayer culture). These cells should then be used to prepare single-cell suspension. If cells are clumped, disrupt the suspension via aspiration or gentle vortexing. However, only one person should do all the cell injections to mice to minimize experimental errors. The quicker one can inject the cells into animals

after harvesting the better and more uniform will be the tumor growth.

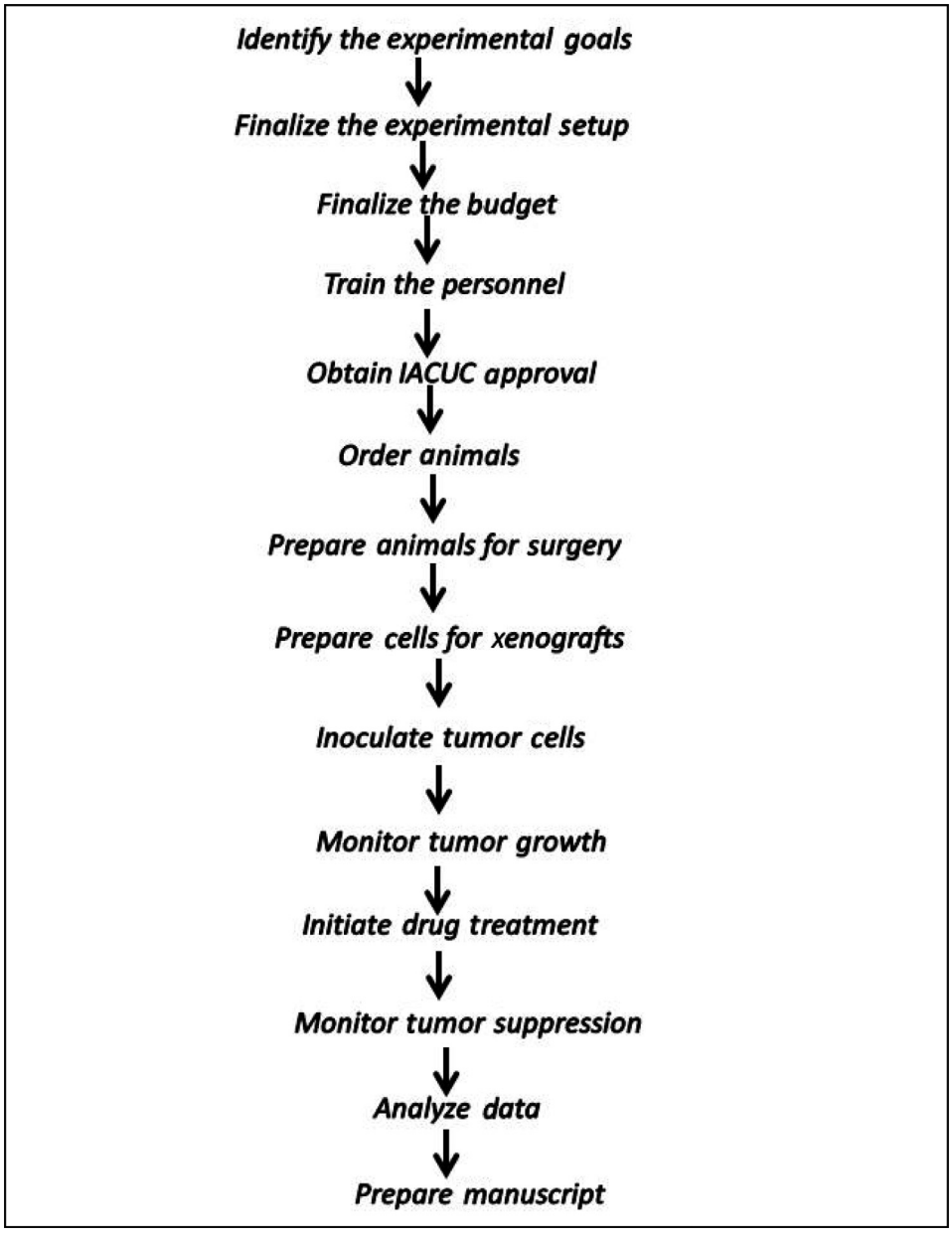
8. *Preparation of surgical instruments and cells.* Depending upon the experimental strategy, the site of injection may be orthotopic, s.c., or directly under the skin. Sterile syringes and needles are required for the procedure along with alcohol swabs. In many cases, matrigel or basement membrane-like matrix is mixed with the human cancer cells in a 1:1 ratio before injecting into mice. Matrigel provides a natural environment for the cancer cells to grow.
9. *Tumor monitoring*. Human tumor usually takes 1–2 weeks to grow in a nude mouse depending upon cancer cell type and the number of cells injected. It is advisable to inject not more than 100 μL of 1–5 million cells per mouse for a xenograft mouse model. Nutritional support is critical during postprocedure recovery periods and moistened rodent chow is recommended to encourage animals to eat following anesthetic events. The health of the mice should be monitored by daily observation. Regular animal monitoring and scheduling of sampling need to be coordinated with all of the personnel involved. When measuring tumor growth using the bioluminescence method, injection of D-luciferin (15 mg/mL) to a final 150 mg D-luciferin/kg body weight is used. At 12 minutes after D-luciferin injection, the animals can be imaged in the dorsal position for 2–5 seconds at field of view. The tumor regions of interest and tissues around the tumor sites are both imaged, and emitted signals are quantified as total photons/second using a Living Image Software. Upon reaching a relevant tumor size, volume mice are randomly separated into treatment groups, and treatment ensues. At the end of the study period, the primary tumors are surgically removed from the animals, isolated from surrounding tissues and weighed. At this stage, other organs such as lung, liver, bone, and lymph nodes can also be dissected to measure metastasis. The mice should be euthanized if there is more than 20% weight loss or the tumors are too big and impede movement or the tumor burden or treatment results in sickness. Scheduling of euthanization needs to be made, and the vivarial staff should be informed in advance.
10. *Analysis of data*. Once promising data with tumor growth or antitumor effects of the drug of interest is obtained in the first set of mice, it is important to repeat a similar experiment in another set of mice. This will facilitate the statistical data analysis and manuscript preparation. Perform statistical analysis utilizing repeated measures analysis of variance (ANOVA) to evaluate the treatment effects on tumor growth. Compare tumor weight measurements between treatment groups utilizing one-way or two-way ANOVA. All data are presented as the mean standard error (SE). Statistical differences are evaluated by Student's *t*-test and ANOVA, followed by Dunnett's post-hoc test. The criterion for statistical significance will need to set at $P < .05$. At this stage, the in vivo data would be ready for publication. A flow chart (Flow Chart 5.1) is provided here to help the researcher to plan ahead. In addition, a detailed checklist that will help the animal researcher initiate their studies is provided below.

Protocols

The above checklist on mouse models of cancer will demonstrate the successes of using the xenografted tumors in many types of human cancers. Similar studies clearly illustrated that information learned from these studies can be translated into successful clinical trials for drugs. Thus the human tumor xenograft models are the most often utilized system to determine anticancer drug efficacies. In the following sections, we are including two examples of protocols using these models where each of the experimental steps is more thoroughly addressed.

An orthotopic mouse model of colorectal cancer

The traditional s.c. tumor model is not ideal for studying colorectal cancer and does not replicate the human disease. Therefore the orthotopic mouse model of colorectal cancer has been very useful for studying both the natural progression of cancer and the testing of new therapeutic agents. In a recent publication by Bhattacharya et al. (2011), the effects of the natural antioxidant supplement selenium on tumor progression and angiogenesis in an orthotopic model of human colon cancer (Bhattacharya et al., 2011). Real-time imaging using both fluorescent protein imaging (FPI) and magnetic resonance imaging (MRI) was utilized to demonstrate tumor progression and angiogenesis. In this model, human colon carcinoma cells (GEO) were fluorescent-labeled with GFP and implanted orthotopically into the colon of athymic nude mice. Beginning at 5 days postimplantation, whole-body FPI was performed to monitor tumor growth in vivo. Tumor-bearing animals were treated with daily oral administration of methylselenocysteine (0.2 mg/day) for 5 weeks. Dynamic



FLOW CHART 5.1 Guidelines for a successful mouse experiment.

contrast-enhanced MRI was performed to examine the change in tumor blood volume following treatment, and CD31 immunostaining of tumor sections was also performed to quantify microvessel density (MVD). Selenium treatment resulted in a significant reduction in blood volume and MVD of GEO-derived tumors and highlighted the usefulness of multimodal imaging approaches to demonstrate antitumor and antiangiogenic therapies against colon cancers. Below are two protocols that have been used to establish the orthotopic model of colorectal cancer. The first involves an injection of a colorectal cancer cell suspension into the cecal wall, and the second technique involves transplantation of a piece of s.c. tumor, obtained from a different mouse, into the cecum.

Design and execution

A. Cell preparation/tumor preparation

1. Colorectal cancer cells (e.g., GEO or HT-29) are grown in culture and harvested when subconfluent, and single-cell suspension is prepared in phosphate-buffered saline (PBS). Alternatively, a mouse with a previously established s.c. colorectal tumor is euthanized, the tumor is removed and divided into 2–3 mm pieces and kept in PBS on ice.

B. Mouse preparation
1. Use inhaled isoflurane (2-chloro-2-(difluoromethoxy)-1,1,1-trifluoro-ethane). Alternatively, use ketamine–xylazine–acepromazine (KXA) combination.
2. The depth of anesthesia is assessed using toe pinch and the absence of withdrawal reflex.
3. The anesthetized mouse is shaved at the site of injection, properly positioned, and the abdomen is prepped with a sterile betadine solution.

C. Laparotomy
1. A small nick is made in the skin, and the abdominal wall musculature is grasped and lifted up to expose the abdominal cavity. The abdominal cavity is entered with a single blade of scissors which is used to push the intra-abdominal contents away from the cecum. At this stage, the abdominal incision can be extended to 2–3 cm to gain better access.

D. Exposure of the cecum
1. The cecum and its pouch are identified and exteriorized. The cecum is isolated from the rest of the mouse and placed on a precut, sterile gauze. Warm saline is continuously used to keep the cecum moist.

E. Injection of cells or tumor pieces
1. *Injection of cells into the cecal wall.*
   a. A 27-G needle is used to inject a 50 μL volume of cells ($2–5 \times 10^6$) into the cecal wall.
   b. The needle is removed, the injection site is inspected to ensure no leakage, and the cecum is returned to the abdominal cavity.
2. *Transplantation of tumors into the cecum*
   a. A "figure of 8" stitch is placed onto the cecum using 6- or 7-sized suture. The cecal wall is lightly damaged and the tumor piece is positioned.
   b. The stitch is tied down and the cecum is returned to the abdominal cavity.

F. Mouse abdominal wall closure.
1. The abdominal wall is closed using three interrupted stitches using 3 or 4 sized suture (alternatively, one can use a simple running stitch). Postoperative analgesics and a fluid bolus may be given at this point.
2. The mouse is allowed to recover from anesthesia.

G. Monitoring tumor development.
1. Tumor formation may vary by cell line, the number of cells inoculated, or size of tumor strips used, but palpable tumors ($\sim 100\ mm^3$) is seen within 4–6 weeks.
2. When animals develop visible tumors at the site of cell implantation, approximately 12 days after cell implantation, obtain images of the animals (ventral position).
3. Remove outlier animals with tumors larger or smaller compared to the majority of mice and randomize the remaining mice into different treatment groups.

H. Monitoring drug effects.
1. Most drug treatments are started at this stage and treatment ended at about 8–10 weeks or when tumors reach a volume of $\sim 500\ mm^3$.
2. For each treatment arm, mice are grouped by 5 or 10 with equal tumor volumes. Tumor size is measured twice weekly with calipers, and tumor volume is calculated by the formula $4/3\pi {r_1}^2 \times r_2$, where r_1 is the smaller radius.
3. At the end of treatments, mice are euthanized and tumors are excised, cleaned, weighed, and stored in $-80°C$ for additional analysis.

Interpretation of results

The time to developing primary tumors and liver metastases may vary depending on the technique, cell line, and mouse species used. Tumor response to anti-cancer therapy can vary dramatically depending on whether cancer cells are implanted in an ectopic (s.c.) versus orthotopic location. However, the orthotopic models of colorectal cancer replicate human disease with high fidelity. The two techniques have unique advantages and disadvantages. The use of colorectal cancer cell lines which have been growing in vitro allows for the inoculation of homogeneous cells. However, these cells may have reduced invasive or metastatic potential after several passages in culture. Transplantation of a piece of s.c. tumor introduces a more heterogeneous population of cancer cells that have been established in vivo. However, since tumors contain stromal cells, this may affect the consistency of the tumors in different mice. Primary, invasive rectal cancers can develop in mice as early as 1 week after injection; however, mice do not usually develop metastatic lesions.

A xenograft model of prostate cancer metastasis

To better understand the process involved in cancer metastasis and the effects of drugs in suppressing breast cancer metastasis, the design, experimental setup, and analysis of tumor-bearing mice are the important aspects to consider. While prostate cancer metastasizes to lymph nodes, lung, and liver, the predominant site of metastatic prostate cancer is bone; approximately 80%–90% of patients with advanced prostate cancer have bone metastases. We are presenting a recent study that

determined the effects of Angiotensin-(1−7) in attenuating metastatic prostate cancer in mice (Krishnan et al., 2012). Angiotensin-(1−7) [Ang-(1−7)] is an endogenous, heptapeptide hormone with antiproliferative and antiangiogenic properties. In vitro studies were performed to establish the role of Ang 1−7 as an antimetastatic agent against advanced prostate cancer. For example, in the in vitro migration studies, Ang-(1−7) mediated a decrease in migration of both PC3 and DU145 cell lines. This suppression of cell migration was blocked by a specific Ang-(1−7) receptor antagonist, D-alanine^7-Ang-(1−7). This suggested that Ang 1−7 may reduce the in vivo metastatis of prostate cancer cells. To demonstrate this in a xenograft mouse model, these investigators used luciferase-expressing PC3 cells injected into the middle of the tibial plateau in 5−6-week old male SCID mice. The SCID mouse does not have an active immune system (as that would lead to the clearance of tumor cells) and thus has a good tumor intake. The ability to make T or B lymphocytes, or activate some components of the complement system, is impaired in SCID mice, and they also cannot efficiently fight infections nor reject tumors and transplants.

Design and execution

A. Cell preparation

1. Grow PC3 cells (CRL-1435; American Tissue Culture Collection) in RPMI1640 medium (HyClone) with 10% fetal bovine serum and antibiotics.
2. Infect PC3 cells with the UBC-GFPLuc lentivirus construct (expressing a GFP-Fireflyluciferase fusion protein behind the human ubiquitin C promoter) and select with 10 mg/mL blasticidin for 2 weeks to establish a stable cell line.

Note: The percentage of GFPLuc-positive cells can be determined by flow cytometry or via the expression of luciferase using a Luciferase Assay kit (Promega).

B. Mouse preparation

Model 1: *in vivo model of prostate cancer metastasis*.

Injection of tumor cells into the left ventricle is a commonly used method to mimic the extravasation of tumor cells from the circulation into metastatic sites.

1. Anesthetize animals with 1% isoflurane. Make a midline incision over the trachea and throat of the mouse. Isolate the carotid artery and insert a catheter (14-mm long) into the artery. Ensure that the tip of the catheter extends down the carotid artery to the aortic arch.
2. Inject one million (1×10^6) PC3^{LUC} cells in 100 mL of Hank's-buffered saline solution (HBBS) into the aortic arch catheter using a 28-G needle.

Model 2: orthotopic intratibial model.

3. Anesthetize animals with 1% isoflurane. Make a 2−3 mm longitudinal incision over the midpatellar region of the right hind limb. Make a percutaneous interosseal bore in the tibia using a 27-G needle.
4. Inject 20,000 (2×10^4) PC3^{LUC} cells into the middle of the tibial plateau.

Note: Hold a cotton swab over the injection site to prevent leakage of cells.

C. Mouse closure

Perform skin closure with a 5-0 coated vicryl suture.

D. Ang(1−7) treatment

Model 1:

1. Two days prior to injection of PC3^{LUC} cells into the aorta, implant a s.c. osmotic minipump to infuse 24 mg/kg/h of Ang-(1−7) in SCID mice or control mice.

Model 2:

1. Two weeks postinjection of PC3^{LUC} cells into the bone, implant a s.c. osmotic minipump to infuse 24 mg/kg/h of Ang-(1−7) in SCID mice or control mice.
2. Treat mice for 5 weeks with Ang-(1−7).

D. Monitor tumor development

1. Monitor tumor metastasis in different organs at 6 weeks after treatment with Ang-(1−7) by bioluminescent imaging of the whole mouse.
2. Measure tumor growth by bioluminescence image analysis and determine tumor volumes in the bone by MRI.
3. At the end of treatments, mice are euthanized and tumors are excised, cleaned, weighed, and stored in −80°C for additional analysis.

Interpretation of results

In model 1 (metastasis from aorta), even at 6 weeks after treatment, no detectable tumors were observed in mice treated with the Ang-(1−7), whereas 83% of the control mice developed metastatic lesions in either the tibia, mandible, or spine. In model 2 (orthotopic tumors), the 5-week regimen of Ang-(1−7) attenuated intratibial tumor growth. Circulating vascular endothelial growth factor (VEGF) levels were significantly higher in control mice when compared with mice administered Ang-(1−7), clearly indicating its role as an antiangiogenic factor. Furthermore, osteoclastogenesis in the bone was reduced by 50% in the presence of Ang-(1−7), suggesting its role in preventing the formation of osteolytic lesions to reduce tumor survival in the bone microenvironment. These findings using two different prostate tumor xenograft models clearly showed that Ang-(1−7) may serve as an antiangiogenic and antimetastatic agent for advanced prostate cancer

and may provide effective therapy for bone metastasis produced from primary tumors sites.

Humanized mouse models for tumor xenografts

Humanized CD34 + mice (hu-CD34) are robust in vivo platform for analyzing the safety and effectiveness of potential new drugs to modulate the immune system (Epperly et al., 2010; Coutelle et al., 2015). The hosts for hu-CD34 and hu-PBMC models are NOD SCID gamma (NSG) mouse (JAX laboratories). These NSG mice combine the features of the NOD background, the SCID, and IL2 receptor gamma chain deficiency ($IL2Rg^{null}$). As a result, these NSG mice lack mature T cells, B cells, or functional NK cells and are deficient in cytokine signaling, leading to better engraftment of human HSC and PBMC cells. Although the hu-HSC mice provide the optimal humanized immune system, the Hu-PBMC models have the fastest engraftment rate and enable short-term studies.

Briefly, the NSG mice (https://www.jax.org/strain/013062) are maintained under specific pathogen-free conditions in sterile microisolator cages. Mice are first irradiated with whole-body gamma irradiation (most commonly performed by exposure to a 137Cs radioactive source). After 24 hours, mice are injected i.v. (tail vein) with CD34 + HSCs ($0.5-2 \times 10^5$ cells/mice) and allowed to reconstitute the human immune system for 12−15 weeks. Successful engraftment of the human immune cells is then checked by staining the PBMCs for the panhuman marker CD45 (huCD45 +). These humanized mice can then be inoculated with the tumor cells (Huang et al., 2012).

Design and execution

The generation of humanized BLT mice first requires the processing of fetal tissue and preparation of human CD34 + HSC, followed by their transplantation in the NSG mice and monitoring of humanization (official strain designation, NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ).

A. Processing of fetal thymus and liver tissue

1. Wash thymus with RPMI + 10% fetal calf serum in a 60-mm dish and shear into 1.5−2 mm pieces with scalpels. Homogenize the tissue with a 16-G blunt needle three or four times. Incubate with the enzymes for tissue digestion: collagenase type IV (1 mg/mL), hyaluronidase (1 mg/mL), and DNase I (2 U/mL).
2. Underlay the homogenized cells with 10 mL of Ficoll. Spin at 2400 rpm for 20 minutes. Transfer the cells to a flask and add 30 mL of RPMI + 10% FCS. Incubate for 1−1.5 hours at 37°C to remove adherent cells.
3. The CD34 + cells can then be sorted using a microBead kit, for example, Miltenyi Biotech's CD34 Direct Kit (http://www.miltenyibiotec.com). The yield of CD34 + cells should be 3%−5% of your total cell counts. *Note*: Younger fetal livers give a higher yield of CD34 + cells.
4. Anesthesia treatment of mice using isoflurane, ketamine/zylazine: weigh all the mice (6−8-week old) and load syringes with a dose of 15 μL/g of ketamine/xylazine and inject (inject 0.3 mL drugs subcutaneously over the shoulder). After mice become sluggish, shave their left side from hip to shoulder.
5. A 16-G needle, with the tip filed round is used to insert the tissue.
6. Locate the spleen under the skin. Pick up the skin over this spot and make a cut parallel to the spleen about 15 mm long. Add a piece of spleen with the needle-nose forceps.
7. Make a little hole in the posterior end of the kidney capsule. Slide the trochar into this hole and along the kidney until the orifice of the trochar is completely covered by the kidney capsule, inject the tissue. Push the kidney back in gently with the closed hemostat.
8. Put one stitch in the peritoneum tied with a double knot. Squeeze the skin up like a purse and put in two wound clips.

B. Transplantation of CD34 + cells

The goal of this second step in the generation of BLT mice is to populate the mouse bone marrow with human CD34 + HSCs. During this secondary transplant, sublethal irradiation of the mice is needed to deplete murine bone marrow cells thus generating "space" for the implantation of the human CD34 + cells.

9. Place NSG mice into an autoclaved, filtered, ventilated housing device for containment during irradiation(^{137}Cs gamma irradiator). *Note*: an autoclaved, filtered, ventilated device for housing mice is needed while in the irradiator chamber.
10. Irradiate mice with 240 cGy whole-body gamma irradiation (most commonly performed by exposure to a ^{137}Cs radioactive source). The mice are then injected (the same day) with CD34 + cells. *Note*: This timeframe is based on the establishment and growth of the thy/liv implant transplanted.
11. Anesthetize a mouse with an isoflurane tube by scruffing it and holding its nose in the tubes for about 20 seconds. Load 28 G insulin syringes with 0.1 mL of cells ($10^5-5 \times 10^5$ cells per 0.1 mL per mouse) for one cage of mice (each cage contains a maximum of five mice).

12. Two to three months later, the mice are bled retro-orbitally (maximum of 200 μL of blood per month per mouse). 50−100 μL of blood should be sufficient for FACS analysis.
13. Blood samples are stained for the expression of the human lymphocyte marker CD45 to assess reconstitution.
14. Blood samples are added into 1 mL red blood cell lysis buffer (160 mM NH_4Cl, 100 mM $KHCO_3$, 0.01 mM EDTA) and incubated for 5 minutes at room temperature. Remove supernatant and resuspend pellets in 100 μL FACS-PBS. Add antibodies to assess human lymphocyte reconstitution, for example, CD45 (human lymphocyte marker), CD8, CD4, CD14, and CD16 for macrophages and monocytes, CD19 for B cells, and CD56 for NK cells. Incubate at 4°C for 30 minutes.
15. If mice are reconstituted (humanized), proceed with tumor injections.

C. Transplantation of tumor cells

16. Tumor cell lines are cultured according to the manufacturer's or laboratory recommendations. Cells are harvested, washed and resuspended in matrigel at a 1:1 ratio (50 μL cells/50 μL matrigel). Samples are kept on ice at all times.
17. Mice are anesthetized and shaved in the area of injection. The area is sterilized using an isopropanol pad. Tumor cells are injected s.c. *Note:* The site of injection should be treated with antibiotic ointment. Tumors should establish and begin growing in 4 weeks.

Ethical issues

Many believe that in vivo experimentations are unacceptable because they cause suffering to animals, and the resulting benefits of such experiments are not clearly proven in human beings. However, another school of thought justifies that animal experiments are necessary for successful drug development and should be acceptable if adverse physical and emotional effects on the animals are minimized. Indeed, animal studies should be avoided wherever alternative testing methods that produce equally valid results and should only be conducted after in vitro studies have been successfully performed on suitable cell lines. Tissue-engineered 3D tumor models have been developed to recapitulate some features of the tumor environment while enabling control of environmental factors and measurement of cell responses (Holzapfel et al., 2015a). Although it is widely agreed upon that since all animal experiments cannot be immediately replaced, it is important to uphold the highest standards of welfare for the care and use of animals (Workman et al., 2010).

Turning point

- 2001: Albert Lasker Award on "Knockout mice as models for human disease."

  Drs. Capecchi, Evans, and Smithies received the Lasker Award for the development of a powerful technology that allowed scientists to create animal models of human disease.

  The Albert Lasker Award recognizes outstanding discovery, contribution, and achievement in the field of medicine and Human Physiology.
- 2007: Nobel Prize for the development of knockout mouse technology.

Drs. Capecchi, Evans, and Smithies received the 2007 Nobel Prize in Physiology or Medicine for their discoveries of specific gene modifications in mice. Their knockout technology has elucidated the role of specific genes in development, physiology, and pathology and has facilitated the discovery of novel therapeutic targets.

Translational significance

Translational research on anticancer drug development is evolving at a very fast pace toward the discovery of novel therapeutics. The in vitro screens are primarily aimed at identifying targets or pathways of interest, then defining the effect a compound on the target(s) or pathway(s). Traditionally, lead compounds are selected for in vivo study based on their in vitro evidence of cytotoxicity and targeted effects on cancer cells. The use of in vivo models to obtain vast quantities of pharmacokinetic (PK) and PD data is a well-established preclinical approach. Before any clinical testing can be initiated in humans, it is important to compare the PK and PD properties of candidate molecules; model potential relationships among dose, concentration, efficacy, and/or toxicity in appropriate animal model systems. Indeed, the in vivo evaluations in numerous mouse cancer models have enabled the success of these screening paradigms which regularly identify diverse types of lead compounds, and their progress toward promising anticancer therapeutics in the clinical setting.

In the past few years, the in vivo models have been able to capitalize on the explosion of new "data mining" technologies to cell-based in vitro assays which have also facilitated the identification of anticancer agents with nonclassical end points such as those which suppress tumor angiogenesis or tumor invasion. The

ultimate goal of in vivo model studies is to form a strictly pragmatic standpoint and the demonstration of unbiased and well understood in vivo activity. Therefore there is a critical need to maximize the utility of the animal model information in selecting agents for translational studies in humans. Many lead compounds that show compromised potency in vitro can turn out to be more effective in vivo because of their favorable PK, for example, greater absorption, better distribution, and stability. Studies are first initiated in small animal models, for example, mouse, rats, and rabbits, to test for acute, subchronic, and then chronic toxicity. In acute toxicity tests, one administration of the drug or chemical is given to each animal in order to generate a safe and effective dose–response curve. Appropriate pharmacological testing in disease models is carried out to determine a 50% effective dose (ED_{50}). Following acute administration, analytical methods are developed for the determination of absorption, distribution, metabolism, and excretion (ADME) of the drug. The subchronic toxicity tests usually involve animals exposed to the drug for 60–90 days duration. Both multiple administrations and/or continuous exposure via food or water to one dose level of a chemical per animal is carried out to measure drug accumulation and possible toxicities. Both genetically altered mice and immunocompromised mice are providing powerful tools for the preclinical evaluation of numerous pharmacological agents. Mutant mice, engineered to mirror the genetic alterations characteristic of human tumors, are also facilitating the identification and validation of biomarkers for early detection of cancer.

However, there are several challenging issues with the interpretation of data obtained in small animal models and to prioritize the lead compounds toward human therapeutics. These are the intrinsic differences in pharmacology and drug metabolism observed between rodents and humans. Although several algorithms exist to predict the drug efficacy in humans, such as the extrapolation of cytochrome p450 (Cyp-450) metabolism or bioavailability features in small animals, even minor differences from the human can translate into decreased relevance for murine dosing and efficacy information as predicting clinical value. This is especially relevant since molecules being tested for cancer treatments are usually exploited at close to their maximum tolerated dose. Indeed, in rodents, the pharmacokinetic parameters such as absorption, plasma protein binding, clearance mechanisms, and intrinsic susceptibility of the host tissues will need to be determined precisely in order to extrapolate a safe and effective dose in humans. It is therefore essential to demonstrate that logarithmic dose increments of the agent can produce a significant parallel reduction in tumor mass. It is becoming clear that in order to manifest the highest antitumor efficacies and ultimately tumor-free survival, agents have to be administered in successive "cycles." Another paradigm that addresses the relationship between tumor cell inoculum to curability is that when antitumor efficacy is not achieved at a constant dose then "adjuvant" treatment programs using multiple drugs that target parallel pathways need to be implemented.

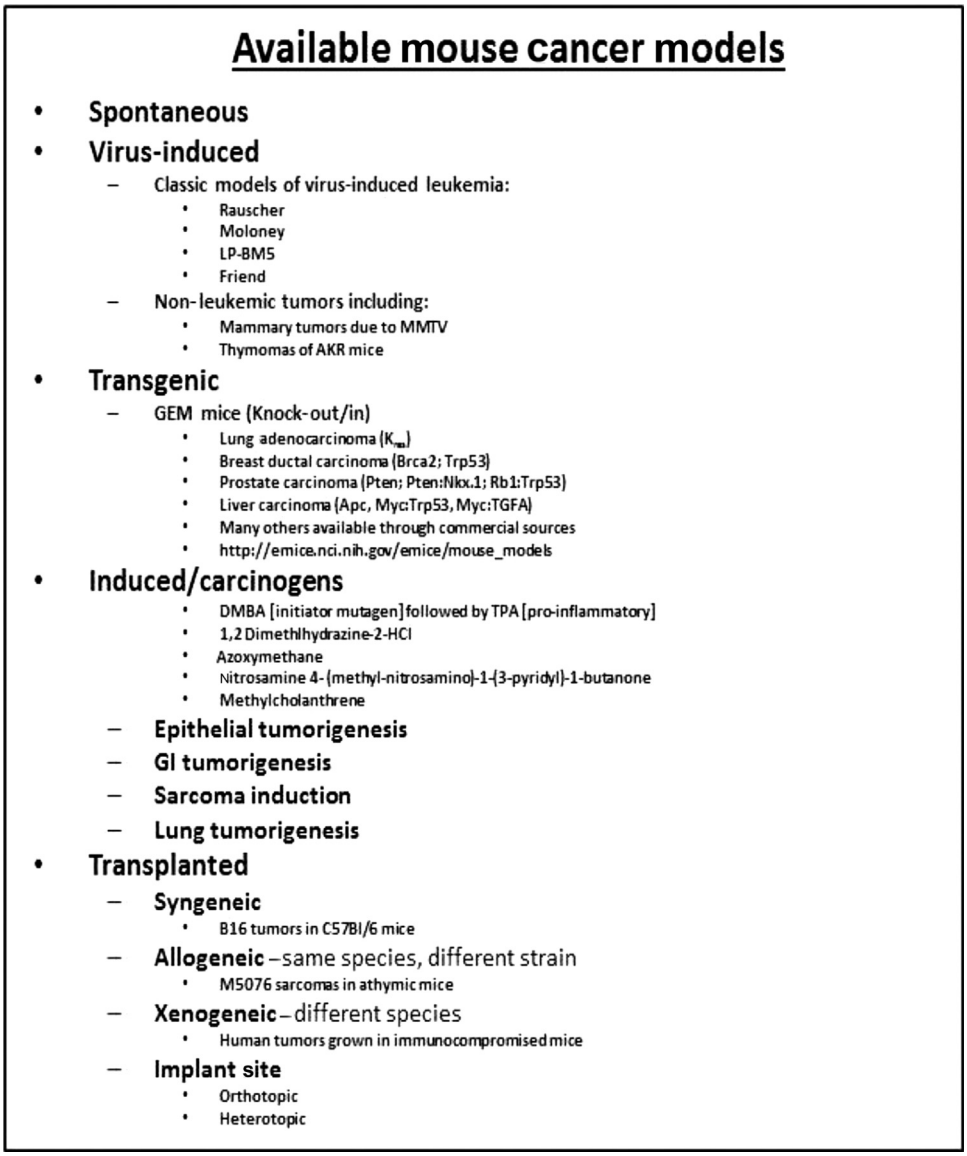
Efficient translational steps taken toward a successful anticancer agent development in mouse models should have a more early integration of pharmacological information, both kinetic and dynamic. In order to be efficacious, the in vivo model should also reflect pharmacological action at a distance and should be able to function across physiological and anatomical barriers. This should be evident at an acceptable therapeutic index and at the clinical proposed dose range and schedule. Thus the ideal in vivo model use should be able to guide therapeutics development, the design and ultimate interpretation of the initial human clinical trial. Therefore from an ethical standpoint, a clear demonstration of in vivo activity in small animal models should form the basis for potentially justifying patient participation in the clinical study. A short list of currently available mouse cancer models used for drug discovery against different types of human cancers is provided (Table 5.3).

World Wide Web resources

Mouse models that mimic human diseases play a vital role in understanding the etiology (cause and origin) of cancer. There are a number of informative web resources that provide guidance and more thorough discussions on the choice of mouse strains to be used in cancer research. Some of these links are provided below:

1. http://www.ncbi.nlm.nih.gov/sites/entrez.
   a. Inactivating mutations in the p53 gene occur frequently in various human cancers. For more information on the study, applications of the human p53 knockin mouse model for human carcinogen testing are provided in the above website.
2. http://emice.nci.nih.gov/aam/mouse/inbred-mice-1
   a. http://emice.nci.nih.gov/
   b. The *electronic Models Information, Communication, and Education* (eMICE) database is maintained by the US National Cancer Institute (NCI) and provides information about a wide variety of animal models of cancer, including mice.
3. http://iospress.metapress.com/content/4806g13r56726741/.
   a. Genetically modified mice are prone to develop specific cancer types. Therefore the

TABLE 5.3 A short list of currently available mouse cancer models.



evaluation of imaging strategies could be undertaken at various stages during the course of cancer progression. Similarly, body fluids could be collected at early to late time points during the course of cancer progression. To learn more about this work, "Cancer Biomarkers, NCI Early Detection Research Network: 5th Scientific Workshop," visit the above website.

4. http://www.informatics.jax.org/mgihome/other/mgi_people.shtml
   a. The *Origins of Inbred Mice* is available online from the Mouse Genome Informatics (MGI) website. This website is maintained by The Jackson Laboratory and integrates access to several databases providing genetic, genomic, and biological data on the laboratory mouse to aid its use as a model of human diseases.
5. http://tumour.informatics.jax.org/mtbwi/index.do
   a. The *Mouse Tumor Biology Database* (MTBD) is part of the MGI database. It integrates data on tumor frequency, incidence, genetics, and pathology in mice to support the use of the mouse as a cancer model.
6. http://phenome.jax.org/
   a. The *Mouse Phenome Database* is also maintained by The Jackson Laboratory and contains strain characterization data (phenotype and genotype) for the laboratory mouse to facilitate translational research.

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Glossary

Allograft A graft between indices, but of different genotypes.
Angiogenesis Blood vessel formation. Angiogenesis that occurs in cancer is the growth of blood vessels from surrounding tissue to a solid tumor.
Benign A swelling or growth that is not cancerous and does not metastasize.
Cancer stem cells A small population of cells inside tumors that have the ability to self-renew while giving rise to different types of cells. Cancer stem cells might be resistant to many cancer drugs and reconstitute a tumor after chemotherapy.
Congenic Relating to a strain of animals developed from an inbred (isogenic) strain by repeated matings with animals from another stock that has a foreign gene, the final congenic strain then presumably differing from the original inbred strain by the presence of this gene.
Consomic An inbred strain with one of its chromosomes replaced by the homologous chromosome of another inbred strain via a series of marker-assisted backcrosses.
DCIS Ductal carcinoma in situ. A precancerous condition characterized by the clonal proliferation of malignant-looking cells in the lining of a breast duct without evidence of spread outside the duct to other tissues in the breast or outside the breast.
Epigenetic Having to do with the chemical attachments to DNA or the histone proteins. Epigenetic marks change the pattern of genes expressed in a given cell or tissue by amplifying or mutating the effect of a gene.
GEM model Genetically engineered mouse model based on the loss or gain of specific genes, to study a disease like cancer in immunocompetent animals.
Germ line Genetic material that is passed down through the gametes (sperm and egg).
Hazard ratio A summary of the difference between two survival curves, representing the reduction in the risk of death on treatment compared to control.
HER2 (human epidermal growth factor receptor-2) The HER2 gene is responsible for making HER2 protein, which plays an important role in normal cell growth and development.
Hyperplasia An overgrowth of cells.
Humanization Immunodeficient mouse models used for effective human cell and tissue engraftment.
Institutional review board (IRB) A board designed to oversee the research process in order to protect participant safety.
Karyotype A photomicrograph of an individual's complete set of chromosomes arranged in homologous pairs and ordered by size. Karyotypes show the number, size, shape, and banding pattern of each chromosome type.
Leukemia Cancer of white blood cells.
Mitosis The process of cell division, resulting in the formation of two daughter cells that are genetically identical to the parent cell.
Neoadjuvant Initial treatment that is not the primary therapy (for instance, chemotherapy or radiation, prior to surgery).
Neoplasm An abnormal new growth of tissues or cells. Neoplasms can be benign or malignant.
NSG mice NOD SCID gamma is a strain of inbred laboratory mice that lack mature T cells, B cells, and natural killer (NK) cells and is the most immunodeficient mice used for humanization.
Oncogene A mutated proto-oncogene that is locked into an active state and continuously stimulates unregulated cell growth and proliferation that leads to tumor development. The normal allele of an oncogene is called a proto-oncogene.
Palliative treatment Treatment aimed at the relief of pain and symptoms of disease but is not intended to cure the disease.
Recurrence The return of cancer, at the same site as the original (primary) tumor or in another location, after the tumor had disappeared.
Remission A decrease in or disappearance of signs and symptoms of cancer. In partial remission, some, but not all, signs and symptoms of cancer have disappeared. In complete remission, all signs and symptoms of cancer have disappeared, although there still may be cancer in the body.
Telomeres Special DNA sequences at the ends of each chromosome that grow shorter each time a cell divides.
Telomerase An enzyme that rebuilds telomeres. Telomerase is overexpressed in many cancer cells, and it contributes to their immortality, or ability to divide endlessly.
VEGF (vascular endothelial growth factor) A protein that is secreted by oxygen-deprived cells, such as cancerous cells. VEGF stimulates new blood vessel formation, or angiogenesis, by binding to specific receptors on nearby blood vessels, encouraging new blood vessels to form.
Xenograft A graft of tissue transplanted between animals of different species

Abbreviations

4-OHT	4-hydroxytamoxifen
ADME	Absorption distribution metabolism excretion
AHR	Aryl hydrocarbon receptor
Ang-(1–7)	Angiotensin-(1–7)
ANOVA	Analysis of variance
APC	Adenomatous polyposis coli
AR	Androgen receptor
ATCC	American Tissue Culture Collection
AVMA	Animal Veterinary Medical Association
Cyp-450	Cytochrome p450
DBA strain	Dilute, brown and non-Agouti strain
DMBA	7, 12-Dimethylbenz(α)anthracene
DNA	Deoxyribonucleic acid
ED_{50}	50% effective dose

eMICE	electronic Models Information, Communication, and Education database
ERα	Estrogen receptor α
ERβ	Estrogen receptor β
ES cells	Embryonic stem cells
FPI	Fluorescent protein imaging
GEM	Genetically engineered mouse
GFP	Green fluorescent protein
HBBS	Hank's-buffered saline solution
HER2	Human epidermal growth factor receptor 2
i.m.	Intramuscular
i.p.	Intraperitoneal
IACUC	International Animal Core and Use Committee
K-Ras	Kirsten rat sarcoma viral oncogene homolog
KXA	Ketamine–xylazine–acepromazine
MGI	Mouse Genome Informatics website
MMTV	Mouse mammary tumor virus
MRI	Magnetic resonance imaging
MTBD	Mouse Tumor Biology Database
mTOR	Mammalian target of rapamycin
MVD	Microvessel density
NCI	National Cancer Institute
NOD	Nonobese diabetic
NSCLC	Non-small cell lung cancer
NSG	NOD SCID gamma
PBS	Phosphate-buffered saline
PD	Pharmacodynamics
PDX	Patient-derived tumor xenograft
PG	Pharmacogenomics
PK	Pharmacokinetics
PSA	Prostate-specific antigen
PyMT	Polyoma virus, medium T antigen
Rb	Retinoblastoma protein
RNA	Ribonucleic acid
s.c.	Subcutaneous
SCID	Severe combined immunodeficiency
SCLC	Non-small cell lung cancer
SE	Standard error
SERM	Selective estrogen receptor modulators
SNP	Single nucleotide polymorphism
SV40	Simian virus 40
UV	Ultraviolet
VEGF	Vascular endothelial growth factor

Long answer questions

1. Discuss the different applications of genetically engineered mouse (GEM) models used in cancer research.
2. Explain how different recombinant DNA vectors can be used to generate the (i) constitutive and (ii) inducible models of transgenic mice used in cancer research.
3. Explain the concept of "humanized mice" and provide an example of how these mice can be used to discover new anticancer agents.
4. Describe in detail how preclinical drug development has led to clinical approval of numerous chemotherapeutic agents, for example, target identification, library screening, in vitro studies, lead candidates, and mouse PK and PD studies in vivo.
5. You have generated a prostate cancer (PC) cell line which grows in the absence of androgen, suggesting that it would be a good model to identify drugs against castration-resistant prostate cancers (CRPC). Using this cell line, how will you study CRPC tumor development and screen for drugs in vivo?

Short answer questions

1. What are the advantages and disadvantages of mouse models in cancer research?
2. What points will need to be addressed to obtain an IACUC approval?
3. Provide an example where GEM models were used to test for tumor-suppressor genes.
4. What are the differences between xenograft and allograft models in cancer research?
5. Discuss the *Cre-lox* model of transgenic mouse development for cancer research.

Answers to short answer questions

1. The small size and short-tumor generation time in mice make them ideal models of cancer studies. They are relatively inexpensive compared to larger animal models which enable the use of large numbers for statistical measurements. Some inbred strains of mice are genetically well-characterized, and several transgenic and knockout mouse models are available for cancer research. However, since the metabolic rates in mice are very different from humans, there are considerable differences in drug efficacies and toxicities, which need to be taken into account. Furthermore, most mouse models develop fewer metastases or display metastases with different tissue specificity as compared to human tumors. Due to a limited number of initiating genetic alterations, mouse tumors are typically more homogeneous, and this can be an obstacle to modeling the heterogeneity of human cancers.
2. The 3Rs (Refine, Reduce, Replace) are important considerations and criteria when writing an IACUC protocol. Refinement involves careful consideration of the aspects of research methodology and animal maintenance. The in vivo techniques should be thoroughly reviewed so that they cause a minimal amount of pain, stress, or suffering. Reduction aims at limiting the amount of animals needed for a study. Replace takes into account that new technologies are developing that provide an alternative to animal testing or at least minimize

their use. The initial toxicology studies should be carried out using cultured liver cells or other normal cell lines such that supportive data are gained to test the efficacy and toxicity prior to animal testing. The protocol needs to first identify the mouse strain, sex, and age, and an approximate number of mice that will be used. A thorough rationale for using mice and the appropriateness and a complete description of the proposed use of mice has to be justified. A description of anesthetic and euthanasia procedures, a description of appropriate living conditions such as housing, feeding, and nonmedical care of the animals will also need to be included.

3. In a recent publication, Huang et al. showed that RUNX3 acts as a tumor suppressor in breast cancer by targeting estrogen receptor α (Huang et al., 2012). *RUNX3*, a runt-related gene family protein, is known to act as a tumor suppressor in breast cancer. To demonstrate the role of *RUNX3* as a tumor suppressor, these investigators used heterozygous female mice with *RUNX3* mutation ($Runx3^{+/-}$) and compared mammary tumor development with control mice. In different age mice, they isolated mammary gland specimens from wild-type and $Runx3^{+/-}$ mice and evaluated RUNX3-immunohistochemical (IHC) staining. Tumor sections were stained with hematoxylin and eosin (HE) and evaluated by a pathologist to designate tumor cells. About one-fifth of the $Runx3^{+/-}$ female mice developed mammary tumors whereas none of the wild-type mice developed mammary tumors. The *RUNX3* genes were overexpressed in human breast cancer cells (MCF-7) and their ability to form tumors in mouse xenografts was also studied. Both vector-MCF-7 (control) or RUNX3-MCF-7 cells were subcutaneously implanted in SCID mice (C.B-17/IcrCrl-scidBR). Compared with vector-MCF-7 cells, RUNX3-MCF-7 cells produced $<80\%$ smaller tumors. These results, using both GEM and xenograft models, indicated that RUNX3 acts as a tumor suppressor.
4. In the Allograft (or Syngeneic) transplantation models, the cancerous cells or solid tumors are itself of mouse origin and are transplanted into another host mouse containing a specific genetic trait, for example, GEM mice. Because the recipient and the cancer cells have the same origin, the transplant is not rejected by mice with an intact immune system. The results of therapeutic interventions can thus be analyzed in an environment that closely recapitulates the real scenario where the tumor grows in an immunocompetent environment. A disadvantage however is that transplanted mouse tissue may not represent in whole the complexity of human tumors in clinical situations. In the xenograft model, human tumor cells are transplanted either under the skin or into the organ type (orthotopic) in which the tumor originated into immunocompromised mice like athymic nude mice (*nu/nu*) or SCID mice, so that the human cells are not rejected. In this model, human cancer cells or solid tumors are transplanted into a host mouse. Because the cancer xenograft is of human origin, it represents the properties and to an extent the complexities of the human cancer. On the other hand, the compromised host immune system is not truly representative of actual patients. Tumor xenografts have been used in the preclinical and clinical development of anticancer therapeutics, and xenograft models are useful for toxicity studies from targeted therapies and in many cases to predict biomarkers of target modulation.
5. The *Cre/lox* system is frequently used as a way to artificially control gene expression in transgenic mice. In this system, the induction of *Cre-recombinase* enzyme mediates the site-directed DNA recombination between two 34-base pair *loxP* sequences. To achieve this in mice, transgenic mice containing a gene surrounded by "lox-P" sites are mated with transgenic mice that have the *cre* gene expressed in specific cell types. In tissues with no *cre* gene, the target gene will function normally; however, in cells where *cre* is expressed, the target gene is deleted. Therefore the correct placement of *Lox* sequences around a gene of interest may allow genes to be activated, repressed, or exchanged for other genes. Furthermore, the activity of the *Cre* enzyme can be controlled, so that it is expressed in a particular cell type or triggered by an external stimulus like chemical signals or heat shock. This enables the induction of somatic mutations in a time-controlled and tissue-specific manner. Therefore this homologous recombination system closely models carcinogenesis in humans where tumors evolve from somatic gene mutations in normal cells in a time-controlled and tissue-specific fashion in vivo.

Yes/no type questions

1. Is the approval of institutional IACUC protocol is necessary before the initiation of any animal experiments?
2. Can immunocompetent mouse models be used to investigate the process of carcinogenesis in human cells?
3. Can human tumor xenografts in immunodeficient mice effectively address the role of tumor

microenvironments and tumor immune surveillance?
4. Is it possible to generate a humanized mouse model to study the role of the immune system in tumor surveillance?
5. Do mice containing the mutant K-Ras oncogene (G12D) can spontaneously develop tumors in the pancreas?
6. Can mouse tumor xenografts be used in the preclinical and clinical development of anticancer therapeutics?
7. Can immunodeficient mouse models be used to measure the pharmacokinetics and toxicity of anticancer agents?
8. Is the age and sex of mice important considerations when designing an experiment?
9. Do all of the mouse tumor models tested show evidence of metastatic lesions?
10. Can an ideal in vivo mouse model guide therapeutics development and aid in the design of human clinical trials?

Answers to yes/no type questions

1. Yes—An approved IACUC protocol is mandatory before starting any animal experiments.
2. No—The immune system of mice will reject the foreign (human) tumor cells, but genetic mutations in mouse cells can be used to study carcinogenesis.
3. No—The tumor microenvironment in immunodeficient mice lacks the necessary antitumor cells, for example, cytotoxic T-cells and natural killer cells.
4. Yes—A humanized mouse model will contain human immune cells that are differentiated from the implanted human hematopoietic progenitor cells (CD34 +).
5. Yes—Mutations in the K-Ras oncogene often lead to the overactivation and/or dysregulation of this critical signal transduction protein and can lead to rapid tumorigenesis.
6. Yes—Although the immune system is important in tumor surveillance, human tumor xenografts can still recapitulate most of the anticancer effects of drugs in clinical development.
7. Yes—The immunodeficient mice, necessary for xenografts, will still contain the barriers needed for drug transport and metabolism, for example, endothelial cells and liver cyp450 enzymes.
8. Yes—The age and sex of mice are very important since significant physiological and hormonal changes may alter the pharmacokinetics and pharmacodynamics of drugs.
9. No—Only a few tested mouse models show evidence of active metastasis to the lungs, bone marrow, and brain. This is most likely due to the non-aggressive nature of cell lines used.
10. Yes—The choice of in vivo tumor models in mice is of utmost importance in designing experiments that will portray the efficacy of drugs in humans.

CHAPTER

6

The clinico-molecular approaches for detection of human papillomavirus

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Summary

Cancer is a multistep process, characterized by uncontrolled growth of cells in the body, invading nearby tissues and distant sites leading to alterations in genetic and molecular pathways of cell. Uterine cervical cancer is the third most common cancer among women worldwide. A large number of risk factors contribute to its high incidence but the most important factor is considered to be the infection of human papillomavirus (HPV). HPVs are small DNA viruses, which are epitheliotropic (infects epithelial cells) in nature and cause a variety of benign epithelial lesions such as warts and condyloma acuminate and neoplasias of the lower genital tracts in humans. More than 100 genotypes of HPV are described and are of high- or low-risk types. The oncogenic potential of HPV is attributed to its E6 and E7 genes. The products of these two genes stimulate cell proliferation by activating the cell-cycle-specific proteins and interfere with the functions of cellular growth regulatory proteins, p53 and pRb.

It is imperative to add that incidence of cervical cancer has been dropped down due to awareness/vaccines and regular Pap screening test. In Western countries, more initiatives have been taken for the development of therapeutic vaccines against HPV but most of them are in infancy and will take time till it becomes a clinical reality.

In this chapter an attempt has been made wherever possible to present the clinical, molecular, and epidemiological aspects of cervical cancer with special emphasis on the diagnostic/prognostic applications for cervical cancer management.

What you can expect to know

- Human papillomavirus (HPV), major risk factor of cervical cancer are common worldwide.
- More than 100 types of HPV include high- and low-risk types and cause cancer.
- HPV is mainly transmitted after the onset of sexual activity.
- Cervical cancer is caused by different types of HPVs.
- Two HPV types (16 and 18) cause 70% of cervical cancers.
- In India cervical cancer is the fourth most common cancer in women living in less developed regions with an estimated 570,000 new cases (1) in 2018 (84% of the new cases worldwide).
- In 2018 approximately 311,000 women died from cervical cancer and more than 85% of these deaths occurring in low- and middle-income countries (Bray et al., 2018).
- Primary prevention (vaccination against HPV), secondary prevention (screening and treatment of precancerous lesions), tertiary prevention (diagnosis and treatment of invasive cervical cancer), and

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palliative care are the measures used for cervical cancer control.
- Three vaccines that protect against HPV are recommended by WHO and have been approved for use.
- To prevent cervical cancer, the most cost-effective method is to do screening and treatment of precancer lesions (with women age of 30 years).
- Early-stage diagnosis of cervical cancer can be cured.

Introduction

Cancer

Cancer refers to a class of disease wherein a cell or a group of cells divide and replicate uncontrollably due to accumulation of both genetic and/or epigenetic changes occurred in a multistep manner. This leads to unregulated cell proliferation, intrude into adjacent cells and tissues (invasion), and ultimately spread to other parts of the body than the location at which they arose (metastasis). These cells continue to grow despite restriction of space, nutrients, and initiating stimulus with a tendency to invade or spread into adjoining and/or distant tissues. Cancer development includes six essential alterations in cell physiology that include malignant growth, self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (Kaivosoja, 2008).

Cervical cancer

Cervical cancer (CA) results from the abnormal growth and division of cells at the opening of the uterus or womb—the area known as the cervix. The progression of cervical cancer is a multistep process. Initially normal cells undergo precancerous changes and ultimately develop into cancer cells. These precancerous conditions include cervical intraepithelial neoplasia (CIN), squamous intraepithelial lesion (SIL), and dysplasia. It takes several years to develop into an invasive cancer and this cancer is preventable if it is detected early. Thus the cervical cancer provides an excellent human model for studying the process of carcinogenesis in vivo.

Noble Prize

Harald zur Hausen was awarded a Nobel Prize in Physiology or Medicine in 2008 for his discovery of "human papillomaviruses causing cervical cancer." He hypothesized against the current dogma that the cervical cancer which is the most common cancer among women is caused by oncogenic human papilloma virus (HPV) and suggested HPV DNA which is present in tumor should be detectable for viral DNA. He concluded that the HPV belongs to the heterogeneous family of viruses and only few of their subtype can cause cancer. This facilitates to understand the molecular mechanisms behind HPV-induced carcinogenesis and further in the development of prophylactic vaccines against HPV.

Historical overview

Cervical cancer is a major health concern for all women. In most developing countries, it is the leading female malignancy and a common cause of death among middle-aged women. In developed populations with good awareness and screening options, invasive cervical cancer is a relatively rare condition, whereas its precursors and the equivocal cytological results represent a major health burden.

The disease has been known since ancient times. However the cause was unknown. In 400 BCE the Greek physician Hippocrates wrote about the disease and even attempted to treat the cancer with a procedure known as the trachelectomy, but could not eradicate it.

Mistaken theories of cervical cancer causation

Epidemiologists working in the early 20th century noted that cervical cancer behaved like a sexually transmitted disease and summarized as follows:

1. Cervical cancer was common in female sex workers.
2. For centuries, doctors were confused as to the cause of cervical cancer. The first theory rose to prominence in 1842 in Florence, when a doctor noticed that married women and prostitutes were susceptible to cervical cancer, but nuns had a very low incidence of the cancer (Rigoni 1841). However because nuns did suffer from breast cancer, it was incorrectly determined that the cause of both diseases was tight corsets.
3. It was more common in the second wives of men whose first wives had died from cervical cancer.
4. It was rare in Jewish women.
5. Syverton and Berry (1935) discovered a relationship between rabbit papillomavirus and skin cancer in rabbits (HPV is species-specific and therefore cannot be transmitted to rabbits).

This led to the suspicion that cervical cancer could be caused by a sexually transmitted agent. Initial

research in the 1940s and 1950s put the blame on smegma (Heins et al., 1958). During the 1960s and 1970s it was suspected that infection with herpes simplex virus was the cause of the disease. In summary HSV was seen as a likely cause because it is known to survive in the female reproductive tract, to be transmitted sexually in a way compatible with known risk factors, such as promiscuity and low socioeconomic status. Herpes viruses were also implicated in other malignant diseases, including Burkitt's lymphoma, Nasopharyngeal carcinoma, Marek's disease, and the Lucké renal adenocarcinoma (ADC). HSV was recovered from cervical tumor cells.

In the 1950s doctors were convinced that cervical cancer was caused by smegma. In the 1970s the prevailing thought in American medicine was that cervical cancer was linked to herpes, which was also incorrect.

The first breakthrough

While the majority of doctors were completely in the dark, in the 1930s, Dr. Richard Shope of the Rockefeller University studied wild rabbits that had developed "horns," which upon further analysis was found to be caused by a virus that could be transmitted. This research eventually led to the discovery that cervical cancer was caused by a papillomavirus.

The development of Dr. George Papanicolaou's, famous smear in the United States, and introduced into practice in the 1940s. He proceeds to tell the story of the development of cervical cytology as an effective screening test, its widespread use in the United Kingdom as early as 1950s, and the development of a national cervical screening program in United Kingdom in 1988, which effectively solved the problem.

In 1951 first successful in vitro cell line HeLa was derived from biopsy of cervical cancer of Henrietta Lacks.

zur Hausen

Dr. Shope paved the way for Dr. Harald zur Hausen's work in the 1980s. The link between genital HPV infections and cervical cancer was first demonstrated in the early 1980s by Harold zur Hausen, a German virologist who cloned two most important high-risk HPV types 16 and 18 and showed the association between HPV infection and cervical cancer (Gasparini and Panatto, 2009). He has done tremendous research on cervical cancer (zur Hausen, 1991, 2002) and has received the Nobel Prize in Physiology or Medicine (2008) for his discovery of HPV. This association is now well established by a large number of clinicoepidemiological, molecular and experimental studies on HPV. With the cause of cervical cancer finally understood, doctors started working on better treatments and vaccines.

In 1988 Bethesda System for reporting Pap results was developed, and in 2006 first HPV vaccine was approved by FDA.

A description of human papillomavirus (HPV) by electron microscopy was given in 1949, and HPV DNA was identified in 1963. It was not until the 1980s that HPV was identified in cervical cancer tissues. It has since been demonstrated that HPV is implicated in virtually all cervical cancers (Eileen., 2003). Specific viral subtypes implicated are HPV 16, 18, 31, 45, and others.

Hence it was not until the 20th century that scientists understood that the disease was caused by exposure to the HPV. Vaccines against some forms of the virus are now widely available, but for most of recorded history, the causes of cervical cancer were completely misunderstood.

Cancer research is becoming multidisciplinary. Complex structural and therapeutic problems require synergistic approaches employing an assortment of molecular cancer biology, which synthesizes the findings of three decades of recent cancer research and proposes a conceptual framework that teaches about these discoveries. This chapter continues to provide a detailed overview of the process that lead to the development and proliferation of cancer cells, including the techniques available for their study. It also describes HPV biology including role of tumor suppressor genes and oncogenes used in the diagnosis and in determining the prognosis of cervical cancer. It will update various methods of cytology screening including colposcopy, detection of HPV 16 and 18, and treatment with ultimate success in reducing cervical cancer mortality. Finally the development of the HPV vaccine is outlined.

Decreased incidence rates for cervical cancer in countries with organized screening, whereas increased rate of cervical cancer has been reported in several populations due to the failures of cytology-based screening programs. Therefore complete awareness and knowledge of current screening approaches of cervical cancer are important to eradicate this cancer. Hence this chapter provides a broad spectrum of current strategies, techniques, and their application to the diagnosis and treatment of cervical cancer. This chapter will help clinicians, virologists, cytologists, epidemiologists, and public health specialists in understanding all clinical, molecular, and epidemiological aspects of cervical cancer. Overall this chapter is comprehensive and offers many pedagogical features such as cervical cancer epidemiology, screening,

and HPV biology, vaccine development, helping readers to hone their analytical abilities and to assimilate and think clearly about complex biological processes.

Prevalence and epidemiology of cervical cancer

Global scenario

Cervical cancer is the major reproductive health problem of women globally being the commonest cause of cancer-related female mortality in developing countries. According to GLOBACAN, 2018, it ranks fourth both by incidence (569,847cases per year) and mortality (number of deaths 311,365) every year with nearly 80% in developing countries. In developed countries, cervical cancer accounts for only 3.6% of new cancers, with a cumulative risk (0–64) of 0.8%. The highest incidence rates are observed in Sub-Saharan Africa, Melanesia, Latin America and the Caribbean, and southcentral and southeast Asia. For example incidence was 38.0 per 100,000 in the Second National Cancer Survey of the United States. Very low rates are also observed in China (6.8 per 100,000) and Western Asia (5.8 per 100,000) and the lowest recorded rate is 0.4 per 100,000 in Ardabil, northwest Iran.

Symptoms of cervical cancer

Precancerous changes and early cancers of the cervix generally do not cause pain or other symptoms. When the disease gets worse, women may notice one or more of the following symptoms:

- abnormal vaginal bleeding,
- bleeding that occurs between regular menstrual periods,
- bleeding after sexual intercourse, douching, or a pelvic examination,
- menstrual periods that last longer and are heavier than before,
- bleeding after menopause,
- increased vaginal discharge, and
- pelvic pain.

Anatomy of female pelvis

The cervix connects the upper body of the uterus to the vagina. The cervix is the lower one-third of the uterus and is composed of dense, fibromuscular tissue lined by two types of epithelium: squamous epithelium and columnar epithelium. It is about 3 cm in length and 2.5 cm in diameter. The endocervix (the upper part which is close to the uterus) is covered by glandular cells, and the ectocervix (the lower part which is close to the vagina) is covered by squamous cells. The stratified squamous epithelium covers most of the ectocervix and vagina. Its lowest (basal) layer, composed of rounded cells, is attached to the basement membrane, which separates the epithelium from the underlying fibromuscular stroma. The columnar epithelium lines the cervical canal and extends outward to a variable portion of the ectocervix. The transformation zone refers to the place where these two regions of the cervix meet. The original squamo-columnar junction (SCJ) appears as a sharp line, with a step produced by the different thicknesses of the columnar and squamous epithelia.

Types of cervical cancer

There are several types of cervical cancer, classified on the basis of where they develop in the cervix. Cancer that develops in the ectocervix is called squamous cell carcinoma (SCC), and around 80%–90% of cervical cancer cases belong to this category arising from the metaplastic squamous epithelium of the transformation zone. The development of cancer in the endocervix is called ADC (10%) arising from the columnar epithelium of the endocervix. In addition, a small percentage of cervical cancer cases are mixed versions of the above two and are called adenosquamous carcinomas or mixed carcinomas.

Risk factors for cervical cancer

A risk factor is anything that increases the chance of getting a disease such as cancer. Various cervical cancer risk factors are illustrated in Fig. 6.1. These are categorized into two factors as follows:

1. Nongenetic factors
   - **a.** Lower socioeconomic status and lack of regular Pap tests
   - **b.** Poor genital/sexual hygiene
   - **c.** Multiple sexual partners or promiscuity
   - **d.** Early age of first sexual intercourse below 18 years
   - **e.** Oral contraceptives use and smoking
   - **f.** Multiple pregnancies and parity
   - **g.** Socioeconomic status
   - **h.** Dietary factors
   - **i.** Religion and ethnicity
2. *Genetic factors*
   - **a.** High-risk type HPVs
   - **b.** Multiple HPV infection
   - **c.** Viral load (severity of a viral infection)
   - **d.** HPV variants
   - **e.** Genetic predisposition
   - **f.** Infections of other STDs like HIV.
   - **g.** Weakened immune system

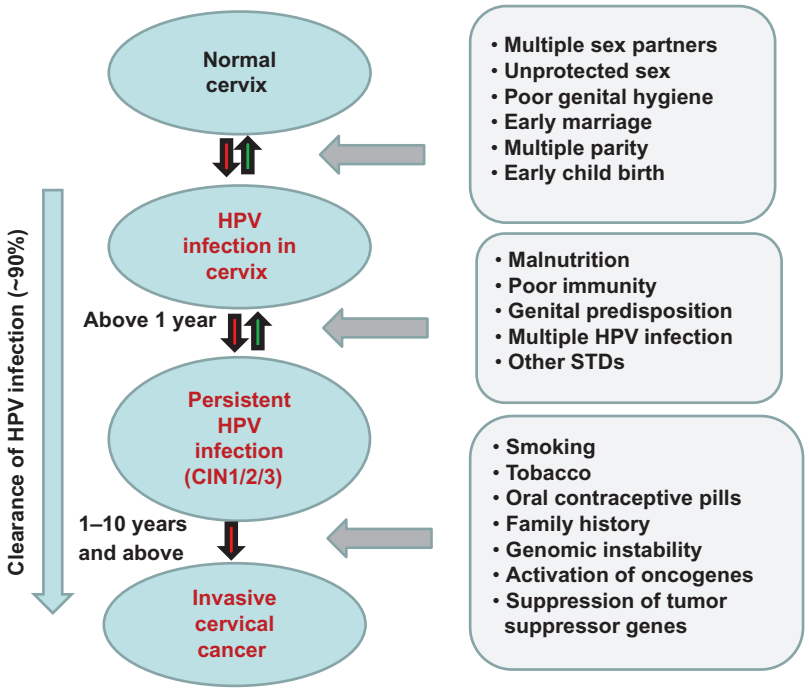


FIGURE 6.1 Integration of HPV and other risk factors leading to cervical cancer.

Human papillomaviruses

HPV infection is a major etiological factor in the development of normal cervical epithelium into cancer (Onon, 2011; Hussain et al., 2012). More than 100 genotypes have been described till date, 15 types are categorized as high-risk (HR-HPVs) types (HPV 16, 18, 31, 33. 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82) that are associated with genital and other epithelial cancers, and 12 low-risk (LR-HPVs) types (HPV 6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81, CP6108), which are responsible for benign tumors and genital warts.

Mainly HPV types 16 and 18 are considered as most prevalent "high-risk" types for cervical cancer, while types 6 and 11 are considered to be the most prevalent low-risk types associated with benign lesions and genital warts. Together HPV 16/18 is estimated to account for more than 80% of invasive cervical cancer. HPV induce hyperplastic, papillomatous, and verruicous squamous cell lesions in the skin and at various mucosal sites in a wide range of hosts including humans. HPV infections have been reported in a number of body sites, including the anogenital tract, urethra, skin, larynx, tracheobronchial mucosa, nasal cavity, conjunctiva, and esophagus. HPVs can be passed from person to person through sexual contact. Most adults have been infected with HPV at some times in their lives.

Genomic organization of human papillomavirus

HPVs are ubiquitous DNA viruses belonging to family papillomaviridae. They are small, nonenveloped DNA viruses with a circular, double-stranded DNA genome of approximately 7200–8000 base pairs (bp). The HPV particles are about 55 nm in diameter and consist of a 72-capsomere capsid containing the viral genome. Capsomeres are composed of two structural proteins: the 57 kD late protein L1, which accounts for 80% of the viral particle, and the 43–53 kD minor capsid protein L2. The genome can be divided into three regions: the long control region (LCR) without coding potential; the region of early proteins (E1–E8), and the region of late proteins (L1 and L2). HPVs have further been classified into subtypes, when they have 90%–98% sequence similarity to the corresponding types and variants when they show no more than 98% sequence homology to the prototype. Some naturally occurring variants have different biological and biochemical properties important in cancer risk. All the putative protein coding sequences called open reading frames (ORFs) are restricted to one strand.

Upstream regulatory region (URR)/LCR: This region constitutes about 10% of the viral genome, varying between 800 and 900 bp. It is the noncoding region, but contains the origin of replication, viral promoters, and enhancer sequences. Viral gene expression is generally regulated by several viral and host–cell transcription factors, which bind to the URR.

The early region: It constitutes nearly 45%–50% of the viral genome lying downstream of URR and consists of 8 ORFs, such as E1, E2, E3, E4, E5, E6, E7, and E8. It encodes regulatory proteins. It is engaged in genomic persistence, DNA replication, and activation

of lytic cycle. E6 functions to activate telomerase and the SRC kinases and inhibit p53 and BAK. E7 inhibits RB, which releases E2F and results in the upregulation of INK4A, but E7 also inactivates INK4A.

The late regions: It spans nearly 40% and is responsible for encoding structural proteins for the production of the viral particles. It contains two ORFs L1 and L2.

Transcriptional regulation of human papillomavirus

Although E6 and E7 themselves possess intrinsic transactivation capacity on their homologous promoter, constitutive expression of E6 and E7 in immortalized or malignantly transformed human keratinocytes is mainly dependent on the availability of a defined set of transcription factors derived from the infected host cell. HPV 16 E6/E7 transcription is regulated by *cis*-acting elements contained within the URR.

HPV has a circular DNA genome in which viral late and early genes are separated by transcriptional control region called URR or LCR. Functionally the 850 bp HPV 16 URR can be divided into three parts:

1. A 5′-terminal portion of unknown function, which only marginally contributes to the activity of E6/E7 promoter.
2. A central 400 bp constitutive enhancer essential for E6/E7 promoter activity.
3. A promoter proximal region containing E6/E7 promoter p97 at its 3′end.

The complete URR as well as the constitutive enhancer regions of the virus have been shown to exhibit a tissue preference for epithelial cells in their transcriptional activity.

Life cycle of human papillomavirus

The HPV life cycle is closely linked to their host cell biology. Normal squamous epithelial cells grow as stratified epithelium, with those in the basal layers dividing as stem cells or transit amplifying cells. After division one of the daughter cells migrates upward and begins to undergo terminal differentiation while the other remains in the basal layer as a slow-cycling, self-renewing population. HPV virions initially infect the basal layers of the epithelium, probably through microwounds and enter cells via interaction with receptors such as α-6 integrin for HPV 16. In infected cells at the basal layer, low levels of viral DNA are synthesized to an episomal copy number of approximately 50−100 genomes per cell. The early HPV genes *E1* and *E2* support viral DNA replication and its segregation so that the infected stem cells can be maintained in the lesion for a long period. As infected daughter cells migrate to the upper layers of the epithelium, viral late gene products are produced to initiate the vegetative phase of the HPV life cycle, resulting in high-level amplification of the viral genome.

As the viral DNA replication almost totally depends on host replication factors except for viral helicase E1, other early genes *E5*, *E6*, and *E7* are considered to coordinate a host cell suitable for viral DNA replication, which sometimes induces host cellular DNA synthesis and prevents apoptosis. In the outer layers of the epithelium, viral DNA is packaged into capsids and progeny virions are released to reinitiate infection. Because the highly immunogenic virions are synthesized at the upper layers of stratified squamous epithelia they undergo only relatively limited surveillance by cells of the immune system. In addition E6 and E7 inactivate interferon (IFN) regulatory factor (IRF) so that HPV viruses can remain as persistent, asymptomatic infections.

High-risk HPV types can be distinguished from other HPV types largely by the structure and function of the E6 and E7 gene products. In benign lesions caused by HPV, viral DNA is located extrachromosomally in the nucleus. In high-grade intraepithelial neoplasias and cancers, HPV DNA is generally integrated into the host genome. In some cases episomal and integrated HPV DNAs are carried simultaneously in the host cell. Integration of HPV DNA specifically disrupts or deletes the E2 ORF, which results in loss of its expression. This interferes with the function of E2, which normally down-regulates the transcription of the E6 and E7 genes and leads to an increased expression of E6 and E7. In high-risk HPV types the E6 and E7 proteins have a high affinity for tumor suppressor genes p53 and pRB, resulting in increased proliferation rate and genomic instability. As a consequence, the host cell accumulates more and more damaged DNA that cannot be repaired. Efficient immortalization of keratinocytes requires the cooperation of the E6 and E7 gene proteins; however the E7 gene product alone at high levels can immortalize host cells. Eventually mutations accumulate that lead to fully transformed cancerous cells. In addition to the effects of activated oncogenes and chromosome instability, potential mechanisms contributing to transformation include methylation of viral and cellular DNA, telomerase activation, and hormonal and immunogenetic factors. Progression to cancer generally takes place over a period of 10 to 20 years.

Functions of human papillomavirus oncoproteins E6 and E7

Both E6 and E7 proteins are essential to induce and maintain cellular transformation, due to their interference

with cell-cycle control and apoptosis. The HPV viral oncogenes, E6 and E7, have been shown to be the main contributors to the development of HPV-induced cervical cancer and increased expression, probably due to integration of the viral DNA in the host cell genome, has been detected in invasive cancers and a subset of high-grade lesions.

Inactivation and degradation of p53 through the E6/E6AP complex

The most important function of the E6 protein is to promote the degradation of p53 through its interaction with a cellular protein, E6-associated protein (E6AP), an E3 ubiquitin ligase (Fig. 6.2). The affinity of E6AP for p53 is likely to be modified in association with E6. The *p53* tumor suppressor gene itself regulates growth arrest and apoptosis after DNA damage. When DNA damage is moderate, a prolonged p53-dependent arrest and DNA repair are induced, but when the damage is severe, apoptosis is provoked. Although aberrant inactivation of pRb family members would also normally induce apoptosis through p53, HPV-infected cells avoid such cell death by E6 inactivation of p53. In addition E6 interferes with other proapoptotic proteins, Bak, FADD and procaspase 8, to comprehensively prevent apoptosis. Alternatively the susceptibility of E6-induced degradation of p53 has been suggested to link the polymorphisms in codon 72 of *p53*.

Inactivation of pRb: E7 is a small nuclear phosphoprotein separated into three conserved regions denoted in an analogous fashion to adenovirus E1A as CR1, CR2, and CR3. E7 is known to bind to the retinoblastoma tumor suppressor gene product, pRb, and its family members, p107 and p130, via a LXCXE (where X represents any amino acid) binding motif conserved in its CR2 region. In the hypophosphorylated state, pRb family proteins can bind to transcription factors such as E2F family members and repress the transcription of particular genes involved in DNA synthesis and cell-cycle progression. Phosphorylation of pRb by G1 cyclin-dependent kinases releases E2F leading to cell cycle progression into the S phase. Because E7 can bind to unphosphorylated pRb, it may prematurely induce cells to enter the S phase by disrupting pRb–E2F complexes. Most recently it was found that E7 promotes *C*-terminal cleavage of pRb by the calcium-activated cysteine protease calpain and that this cleavage is required before E7 can promote the proteasomal degradation of pRb (A. Suhrbier, personal communication, 2007). The E7 protein function enables HPV replication in the upper layers of the epithelium where uninfected daughter cells normally differentiate and completely exit the cell cycle (Fig. 6.2). One cyclin-dependent kinase inhibitor, p16INK4a, which prevents the phosphorylation of pRb family members, is reported to be overexpressed when pRb is inactivated by HPV E7. Normally over-expression of p16INK4a results in cell cycle arrest, but it is overcome with E7 expression. Thus over-expression of p16INK4a is suggested to be a useful biomarker for evaluating HPV pathogenic activity in cervical lesions.

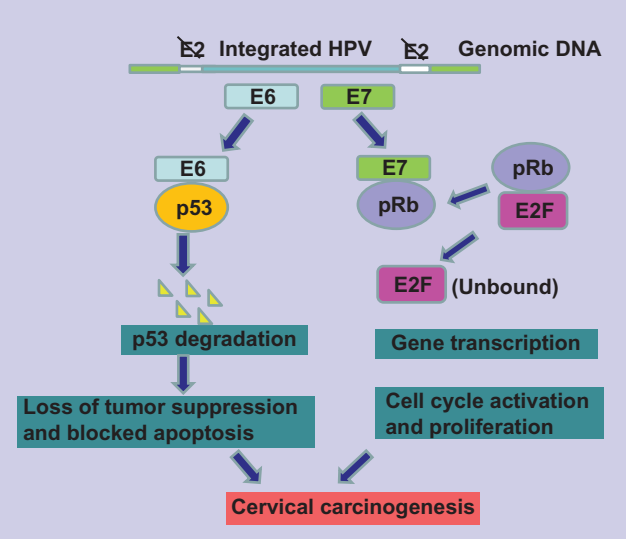


FIGURE 6.2 Role of HPV in cervical cancer E6 and E7 proteins of HPV inactivates p53 and pRb.

Screening and diagnostic methodologies of cervical cancer

Screening

Screening is a public health intervention used on a population at risk or target-population. Screening can detect abnormalities before they become cancer. Also if cancer itself is detected early, it can be cured with proper treatment. Cervical cancer screening aims to test the largest possible proportion of women at risk and to ensure appropriate follow-up for those who have a positive or abnormal test results. Such women will need diagnostic testing and follow-up or treatment. Colposcopy and biopsy are often used to reach a specific diagnosis of the extent of the abnormality in women with a positive screening test. Decisions on the target age group and frequency of screening are usually made at the national level based on the local prevalence and incidence of cervical cancer-related factors such as HIV prevalence and availability of resources and infrastructure.

Methods used for screening/diagnosis of cervical cancer

Visual methods

Two visual methods are available:

- visual inspection with acetic acid (VIA) and
- visual inspection with Lugol's iodine (VILI).

Abnormalities are identified by inspection of the cervix without magnification, after application of dilute acetic acid (vinegar) (in VIA) or Lugol's iodine (in VILI). When vinegar is applied to abnormal cervical tissue, it temporarily turns white (acetowhite) allowing the provider to make an immediate assessment of a positive (abnormal) or negative (normal) result. If iodine is applied to the cervix, precancerous and cancerous lesions appear well-defined, thick, and mustard or saffron-yellow in color, while squamous epithelium stains brown or black, and columnar epithelium retains its normal pink color. VIA and VILI are promising alternatives to cytology where resources are limited. They are currently being tested in large, cross-sectional, randomized controlled trials in developing countries. In research settings VIA has been shown to have an average sensitivity for detection of precancer and cancer of almost 77%, and a range of 56%–94%. The specificity ranges from 74% to 94% with an average of 86%. They are both short procedures, less costly, and cause no pain. Assessment is immediate and no specimen is required.

Indications

VIA and VILI are indicated for all women in the target age group specified in national guidelines, provided that:

- They are premenopausal. Visual methods are not recommended for postmenopausal women, because the transition zone in these women is most often inside the endocervical canal and not visible on speculum inspection.
- Both SCJ s (i.e., the entire transformation zone) are visible. If the patient does not meet the above indications and no alternative screening method is available in the particular clinical setting, then the patient should be referred for a Pap smear.

Other screening tests

The other screening tests that can help to prevent cervical cancer or find it early are as follows:

1. *The Pap test (or Pap smear)* looks for precancers, cell changes on the cervix that might become cervical cancer if they are not treated appropriately. It is recommended for all women, and the doctor will use a plastic or metal instrument, called a speculum, to widen your vagina. It is most important that an adequate sample be taken from the squamo-columnar junction (the transformation zone), transferred to the cytology slide, and immediately fixed with a commercial fixative. The location of the squamo–columnar junction can be identified by a change in color and texture between the squamous and columnar epithelia. The squamous epithelium appears pale pink, shiny, and smooth. The columnar epithelium appears reddish with a granular surface. Pap smear screening is a rapid method for detecting cervical dysplasia and in situ cancer, as well as invasive cancer. A Pap smear evaluates cells harvested from the ectocervix and endocervix for abnormal changes associated with the development of cervical cancer.
2. *Thin Prep Pap test*: The Thin Prep Pap Test, which has been approved by FDA has been described as more effective than a conventional Pap smear, is a liquid-based test that employs a fluid medium to collect and preserve cervical cells.
3. *Speculoscopy*: Recently approved by the US FDA, this procedure involves the use of a magnifier and a special wavelength of light. Speculoscopy allows the physician to see cervical abnormalities that would otherwise be undetectable when performing a Pap test.

Reporting systems terminology

Fig. 6.3A–C represents normal human cervix, histopathological changes in precancerous lesions and in cancerous tissue of human cervix. The reporting of Cervical Pap smears varied tremendously from one laboratory to another, because different classification systems put forth at different times added due to the lack of uniformity. An outline of all classifications is mentioned below.

Precancer classification

WHO classification

According to this classification, cervical lesions can be classified into dysplasia, carcinoma in situ (CIS), and invasive cancer. Cervical dysplasia can be further graded into mild, moderate, and severe dysplasia based on the degree of involvement that is the ratio between the thicknesses of abnormal cervical epithelium and its complete thickness.

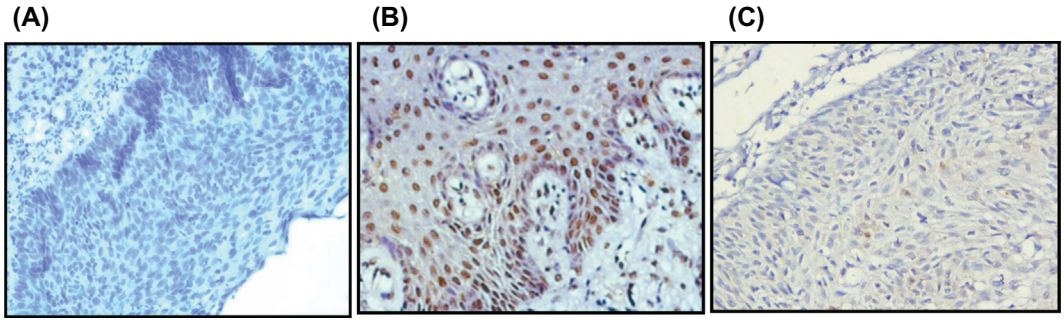


FIGURE 6.3 (A) Normal human cervix; (B) precancerous lesion of human cervix (Dysplasia); (C) invasive squamous cell carcinoma.

CIN classification (Bhambhani., 2007)

1. *Richart* (in 1968) introduced the term CIN which was used to distinguish severe dysplasia from carcinoma in situ. It is further classified into three groups namely CIN1, CIN2, and CIN3, which are compatible with WHO classification in which mild dysplasia corresponds to CIN1, moderate to CIN2, and CIN3 includes both severe dysplasia and carcinoma in situ.
2. *Reagen and associates* promulgated the use of the term "dysplasia" to designate these intraepithelial lesions and classified further severe dysplasia and carcinoma in situ suggested as separate disease and were clubbed to CIN III, CIN II to moderate dysplasia and CIN 1 to mild dysplasia. Cervical cancer follows a progressive course from epithelial dysplasia to carcinoma in situ to invasive cancer. It may take years for dysplasia to turn into carcinoma in situ or microinvasive cancer, but once this process occurs, cancer can quickly become invasive and spread deeper into lymph nodes, nearby tissues, or other organs, such as the bladder, intestines, liver, or lungs.

Bethesda classification

In December 1988, the National Cancer Institute sponsored a workshop to develop a uniform reporting system for cervico-vaginal cytology, which is known as "The Bethesda System." In 2001 it classifies squamous cell abnormalities into following categories (Apgar et al., 2003).

1. Atypical squamous cell (ASC) including lesions that have several abnormalities suggestive of SIL.
2. Low squamous intraepithelial lesions (LSIL) encompassing cellular changes associated with HPVs and mild dysplasia or CIN1.
3. High squamous intraepithelial lesions (HSIL) include moderate dysplasia or CIN2, severe dysplasia and CIS as CIN3.

Subsequently modifications were made after a second meeting convened in 1991. The Bethesda System has the following advantages:

- A uniform diagnostic terminology to improve communication both among cytopathologists and between cytopathologists and health care providers.
- A descriptive diagnosis of ASC s of undetermined significance (ASC-US) and of atypical glandular cells of undetermined significance (AGUS), which refers to glandular cell nuclear enlargement, hyperchromasia and/or architectural abnormalities.
- The inclusion of changes associated with HPV such as koilocyctosis along with CIN within the category of low-grade squamous intraepithelial lesion (LGSIL). In other words the use of terminology that reflects current understanding of the pathogenesis and biology of cervical neoplasia.
- Evaluation of specimen adequacy as an integral part of the report.

Cancer classification

Cancer staging is one of the fundamental activities in oncology and is of pivotal importance to the modern management of cancer patients. Tumor classification is generally conceived so that the clinical and/or pathological spread is stratified into four stages: Stage I refers to a tumor strictly confined to the organ of origin, hence of relatively small size; Stage II describes disease that has extended locally beyond the site of origin to involve adjacent organs or structures; Stage III represents more extensive involvement, that is wide infiltration reaching neighboring organs; and Stage IV represents clearly distant metastatic disease. These four basic stages are then classified into substages, as a reflection of specific clinical, pathological, or biological prognostic factors within a given stage.

The invasive cervical cancer can be classified by the FIGO system (International Federation of Gynaecology and Obstetrics, Montreal, 1994) (Table 6.1).

TABLE 6.1 Figo staging in cervical cancer.

Stage 0	Carcinoma in situ. It is found only in the top layer of cells in the tissue that lines the cervix.
Stage I	The carcinoma is strictly confined to the cervix (extension to the corpus would be disregarded).
	Ia Invasive carcinoma which can be diagnosed only by microscopy. *Ib* All macroscopically visible lesions – even with superficial invasion – are allotted to Stage Ib carcinomas. Invasion is limited to a measured stromal invasion with a maximal depth of 5.0 mm and a horizontal extension not wider than 7.00 mm. Depth of invasion should not be more than 5.0 mm taken from the base of the epithelium of the original tissue should not change the stage allotment.
	Ia1 Measured stromal invasion of not more than 3.0 mm in depth and extension of 7.0 mm. *Ia2* Measured stromal invasion of more than 3.0 mm and not more than 5.0 mm with an extension of 7.0 mm.
	Ib Clinically visible lesions limited to the cervix uteri or preclinical cancers greater than Stage Ia *Ib1* Clinically visible lesions 4.0 cm. *Ib2* Clinically visible lesions 4.0 cm.
Stage II	Cervical carcinoma invades beyond uterus, but not to the pelvic wall or to the lower third of vagina
	IIa No obvious parametrial involvement. *IIb* Obvious parametrial involvement.
Stage III	The carcinoma has extended to the pelvic wall. On rectal examination, there is no cancer-free space between the tumor and the pelvic wall. The tumor involves the lower third of the vagina. All cases with hydronephrosis or nonfunctioning kidney are included, unless they are known to be due to other causes.
	IIIa Tumor involves lower third of the vagina, with no extension to the pelvic wall. *IIIb* Extension to the pelvic wall and/or hydronephrosis or nonfunctioning kidney.
Stage IV	The carcinoma has extended beyond the true pelvis or has involved (biopsy proven) the mucosa of the bladder or rectum. A bullous edema, as such, does not permit a case to be allotted to stage IV.
	IVa Spread of the growth to adjacent organs. *IVb* Spread to distant organs.

Source: Quinn, M.A., Benedet, J.L., Odicino, F., Maisonneuve, P., Beller, U., Creasman, W.T., et al., 2006. Carcinoma of the cervix uteri. Int. J. Gynecol. Obstet. 95 (Suppl. 1), S43.

Colposcopy and biopsy

Colposcopy is the procedure of viewing the cervix, vagina, and vulva with a magnifying lens (colposcope) to identify abnormal epithelial patterns. The colposcopy procedure begins by wiping away cervical mucus with normal saline. Inspection of the cervix is done with a colposcope that magnifies the tissues with filtered and unfiltered light. A 3%–5% acetic acid solution is then applied to the cervix and upper vagina. If an epithelial abnormality is identified, a biopsy is done. If the entire lesion is not visualized, an endocervical curettage is indicated. During pregnancy colposcopy is done in order to exclude the presence of invasive cancer and to reassure the woman that her pregnancy will not be affected by the presence of an abnormal Pap test.

1. *Endocervical curettage*: This procedure which generally is performed at the same time as colposcopic biopsy removes cells from the endocervix (part of the cervix that opens into the uterus). A woman who has endocervical curettage may experience menstrual-type cramping or light bleeding for a short time afterward.
2. *Cone biopsy*: This procedure consists of removing a cone-shaped piece of tissue from the cervix. Tissue is removed from the "transformation zone" area between the ectocervix (the part of the cervix that connects with the vagina) and the endocervix (the part of the cervix that opens into the uterus). It will only be safe to have a cone biopsy if the cancer cells are only in the cervix, the cancer is less than 3–5 mm deep into the tissue of the cervix, the area affected is no bigger than 10 mm across at any point, and there is absolutely no sign of any cancer in a blood vessel, lymphatic vessel, or lymph gland. The two methods commonly used to perform cone biopsy are:
   a. *Loop electrosurgical excision procedure (LEEP)*: A wire heated by electrical current is used to remove cervical tissue for laboratory analysis. This procedure takes about 10 minutes. Mild cramping may occur during and after the procedure. Mild or moderate bleeding may persist for several weeks.
   b. *Cold knife cone biopsy*: The physician uses a surgical scalpel or laser (intense, focused light beam) to remove abnormal cervical tissue.

A woman who undergoes the procedure can go home the same day but may experience cramping and bleeding for a few weeks afterward.

New technologies

New technologies provide adjuncts to cervical cancer screening.

1. *Autopap* provides automated cytology scanning with interpretation. It is primarily used as a secondary screening to enhance laboratory quality control. Autopap rescreens all satisfactory slides read as "within normal limits" and selects 10% of the slides most likely to be false negatives for review by cytotechnologists.
2. *Papnet* provides computerized selective rescreening of all slides read as "within normal limits," creates a digitized image of the entire slide, and selects 128 of the most questionable fields of each slide for review by a cytotechnologist or pathologist.
3. *Cervicography* is a visual adjunct to cervical screening. Because one limitation of the Pap smear is the false-negative rate, cervicography is being considered for screening and/or secondary triage. The advantages include the following: it is simple to perform, less expensive and noninvasive than colposcopy and biopsy. If cervicography is performed concomitantly with a screening Pap smear, it may improve the false-negative rate. If it is used to distinguish women with an abnormal Pap who should be referred for immediate colposcopy from those for whom follow-up with repeat Pap smears is appropriate, it may also effectively decrease the false-negative rate. Cervicography is not currently FDA approved for primary screening.

DNA cytometry

Dysplastic lesions progress to more severe lesions or regress to normalcy through an unknown series of changes in the abnormal epithelium. Microspectrophotometric determination of nuclear DNA content has contributed valuable information in understanding the pathogenesis of cervical dysplasia. An increased aneuploidy rate with increase in grade of CIN has been observed by number of investigators and aneuploidy has been regarded as malignancy-specific marker. Quantitative cytochemical DNA measurements in individual cell showed a correlation between progressive morphological alterations with progressive increase in nuclear DNA content. Hence aneuploid DNA has been considered as risk indicator of malignant potential of dysplastic tissues.

Human papillomavirus DNA-based screening methods (protocol)

Reliable diagnosis of HPV infection, particularly the "high-risk" types (16/18), may facilitate early identification of high-risk populations for developing cervical cancer and may augment the sensitivity and specificity of primary cervical cancer screening programs by complementing the conventional Pap test. HPV is not generally used on its own as the primary screening test.

Advantages: It is mainly used in combination with cytology to improve the sensitivity of the screening or as a triage tool to assess the women with borderline. New screening procedures are based on the detection of high-risk HPV DNA in vaginal or cervical smears or tissue biopsies (Flow Chart 6.1).

Urine-based noninvasive human papillomavirus DNA detection method

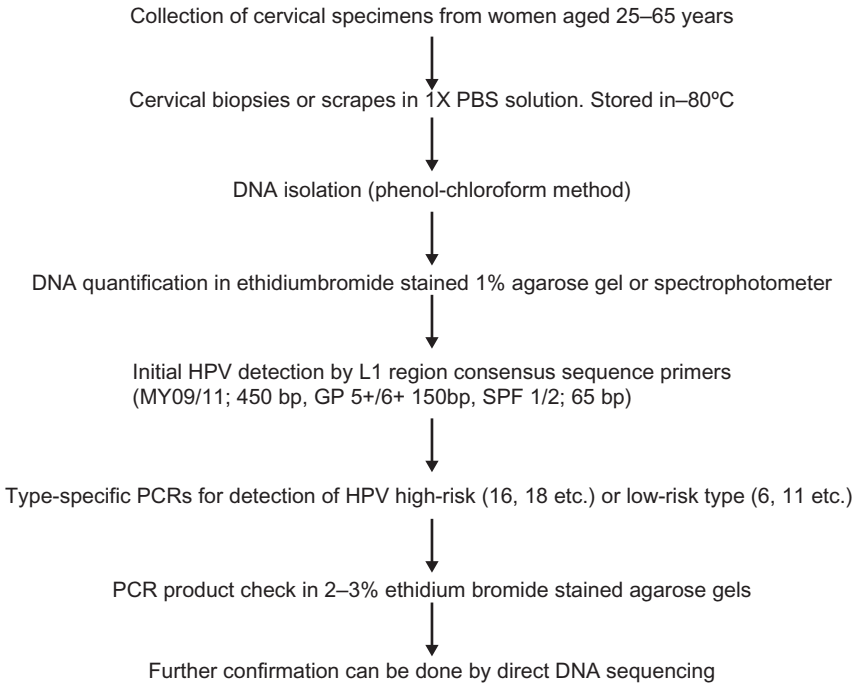
Conventional testing for genital HPV infections requires the collection of smears, scrapes, or tissue biopsies from the cervico-vaginal region, which involves pelvic examination and invasive procedures in a gynecologic/cancer clinic. Such invasive methods are not only unsuitable for large-scale population screening, but are strictly prohibited for adolescent or unmarried girls due to strong sociocultural and religious reasons in developing countries.

Advantages: The use of noninvasive urine sampling for detection of various genital infections including HPV has developed which has been validated over and over again to confirm its utility, sensitivity, and reliability by comparing the results of urine with that of biopsy specimens or cervical scrapes/swabs of the same patients. Exfoliated cells containing HPV DNA or virions shed into the urine from epithelial lesions of the cervix could be detected by a highly sensitive technique such as PCR.

Simple "paper smear" method for rapid detection of human papillomavirus infection

A simple "paper smear" method has been developed for dry collection, transport, and storage of cervical smears/scrapes at room temperature for subsequent detection of HPV DNA by a simple PCR assay. This method requires several types of biological specimens such as imprint biopsies, blood, and fine-needle aspirates.

Advantages: It is simple, rapid, and cost effective, and can be effectively employed for large-scale population screening, especially for regions where the specimens are to be transported from distant places to the laboratory. This method is under US patent application.



FLOW CHART 6.1 HPV DNA testing.

Detection of HPV by multiplex PCR and RFLP

A low-cost method was developed for the detection of HPV types 6, 11, 16, 18, and 33 including coinfections from the cervical swabs of the females attending gynecological outpatient departments and cancer clinics. The method detects the five most prevalent HPV types commonly associated with cervical abnormalities.

Principle: This technique involves restriction fragment length polymorphism (RFLP) of the approximately 450 bp amplicon, obtained after the amplification of L1 region of HPV genome by MY09/11 consensus primers. MY09/11 primers are used routinely for HPV detection covering a broad spectrum of HPV types as compared with general primers GP5 + /GP6 + . About 90% of the cervical carcinoma that contain some high-risk HPV types, HPV 16, 18, and 33 and few others are associated with CIN and cervical cancer, whereas HPV types 6 and 11 are associated with genital warts (condyloma accuminata and flat genital warts). Hence the detection of coinfection is equally important to understand the biological behavior of HPVs. This method detects the above five HPV types by digesting the PCR product of MY09/11 primers with Rsa-1 and resolving on 8% nondenaturing polyacrylamide gel.

Advantages: This method has advantage over other conventional methods of HPV typing, as it saves the cost and time for second PCR by type-specific primers. Most of the PCR-RFLP studies show either use of multiple restriction enzymes with two rounds of PCR. Hence it was found to be less combursive, low cost, and user friendly for the detection of HPV DNA from cervical swabs, both at clinical and research level.

High-quality screening programs are also important to prevent cervical cancer among unvaccinated older women. The WHO recommends the screening of women aged 30–49 years—either through visual inspection with acetic acid in low-resource settings, papanicolaou tests (cervical cytology) every 3–5 years, or HPV testing every 5 years—coupled with timely treatment of precancerous lesions.

Statistical information

Chi-square (χ^2) test for trend is used to evaluate if there is a statistical significant increase in the HPV detection rate due to the technique applied (one-step PCR, RT-PCR, nested PCR, and double-nested PCR). The results were considered to be statistically significant with a P value below .05.

Hybrid Capture II Method

Hybrid Capture II (HCII, Digene Corp., Gaithersburg, Maryland, United States) is a semiquantitative assay for signal amplification, which was first licensed and approved methodology for screening purposes and ASC-US triage by the US FDA. Like many PCR methods, HPV DNA can be detected, with similar analytic sensitivity. All the emerging technologies for HPV DNA detection are clinically validated in comparison to HCII.

Principle

The HCII high-risk HPV DNA test using Hybrid Capture2 technology is a nucleic acid hybridization assay with signal amplification that utilizes microplate chemiluminescent detection. Specimens containing the target DNA hybridize with a specific HPV RNA probe cocktail. The resultant RNA:DNA hybrids are captured onto the surface of a microplate well coated with antibodies specific for RNA:DNA hybrids. Immobilized hybrids are then reacted with alkaline phosphatase conjugated antibodies specific for the RNA:DNA hybrids, and detected with a chemiluminescent substrate. Several alkaline phosphatase molecules are conjugated to each antibody. Multiple conjugated antibodies bind to each captured hybrid resulting in substantial signal amplification. As the substrate is cleaved by the bound alkaline phosphatase, light is emitted which is measured as relative light units on a luminometer. The intensity of the light emitted denotes the presence or absence of target DNA in the specimen.

Applications

Next-generation sequencing

The information on viral genome sequence classifies papillomaviruses. A distinct HPV type is established when the viral DNA sequence of the L1 ORF differs from any other closely related type by at least 10% of this region of the viral genome. Major advances in HPV genome analyses are being made with next-generation sequencing (NGS) that constitutes a multiple highly parallel sequencing technique that is able to sequence individual molecules from small amounts of DNA. It has been progressively applied to HPV typing and has proven to be highly accurate, reproducible, with high sensitivity to detect and identify multiple HPV type infections, and/or detect uncharacterized HPV types. Target enrichment combined with NGS allows the detection of integrated forms of HPV DNA with mapping of viral–cellular junctions to host chromosomes. Hence NGS makes it possible to document and describe HPV integration events.

Clinical correlations

Previously our study revealed that 85% (109/128) of total (precancer and cervical cancer) HPV-L1 positivity, 80% (24/30) of precancerous lesions, and 86.7% (85/98) of tumor biopsies were infected with HPV (Tripathi et al., 2014). Subsequent PCR-based HPV typing using type-specific primers revealed that a 96%(82/85) of HPV-L1 positive cervical tumors harbored high-risk HPV (HR-HPV) type 16; however 7.0%(6/85) of cancer ones were found to be infected with HPV 18 and 3.5% (03/85) of L1 positive cervical tumor cases showed coinfection with HPV type 16 and 18 and all the precancerous HPV-L1 positive lesions were infected with HPV type 16. Further the analysis of HPV infection was correlated with clinicopathological parameters. Tumor vaginal involvement was found to be associated with 89.2% of HPV 16 ($P = .03$), tumor histopathological degree, moderately and poorly differentiated ISCC had higher percentage (94.2%) of HPV 16 positivity as compared to well-differentiated ISCC (71.7%, $P = .003$) and 89.2% of HPV 16 with progressed FIGO stage (III + IV) and 79.2% in FIGO stage-I + II ($P = .03$) (Tripathi et al., 2014). Hence HPV 16 confirms its significant involvement with clinical progression of cervical cancer.

Treatment

If a biopsy shows persistent LSIL or HSIL, or if there is a question of invasive cancer, further evaluation and/or treatment is indicated. Choice of treatment modality depends on several factors such as age of patient, type of lesion, experience of attending surgeon, facilities available with the hospital, and others. Other possible therapeutic modalities are both ablative and excisional. The ablative techniques include laser and cryosurgery. The excisional modalities include cold knife conization, laser conization, loop electrocautery excision, and hysterectomy.

Ablative techniques

- *Laser ablation*: Laser stands for light amplification by stimulated emission of radiation. Laser ablation is a procedure in which a carbon dioxide laser directed through a microscope is used to vaporize the cervical transformation zone. This procedure is done under local anesthetic and takes 15–20 minutes to perform. Post therapy the patient may experience pain, some uterine cramping and bloody discharge/vaginal spotting.
- *Cryotherapy*: Among ablative techniques, cryotherapy is a procedure performed under direct visualization in which a probe is placed against the cervix. The probe freezes the affected tissue, which in turn results in the destruction and sloughing of cervical cells. The entire procedure takes about 15 minutes. During the procedure the patient may experience uterine cramping. Subsequently the patient may expect to have a profuse watery discharge for 7–10 days. Recurrence rate is low and 4 months after cryotherapy, the reevaluation of pap smear is done.

Excisional techniques

- *Electrosurgical excision (LEEP)*: Electrosurgical excision of the transformation zone is a procedure in which an electrical current generating a radiofrequency is passed through a wire loop that excises the tissue and cauterizes the base. This procedure can usually be performed in an outpatient setting with the use of local anesthetic. Depending on the size of the loop and the lesion, either the transformation zone or a "cone-like" specimen can be obtained. The patient must be grounded for safety purposes, because an electrical current is used. The electrical current also generates heat, which can cause distortion of the surgical margins, thus making accurate interpretation of the regions difficult, if not impossible. Risks of all excisional procedures include bleeding, cervical stenosis, cervical incompetence, decrease in cervical mucus, and possible infertility.
- *Laser conization*: Laser conization is an operative procedure requiring an anesthetic in which the carbon dioxide laser is used as a knife to generate the same type of specimen obtained with cold knife conization. Postoperatively the patient will experience cramping and some bleeding.
- *Cold knife conization*: A cold knife conization (CKC) is an operative procedure requiring either a regional or general anesthetic. During the procedure the involved ectocervix and endocervix are excised using a circumferential excision. Postoperatively the patient may have cramping and bleeding. Postoperative infection requiring antibiotic therapy may also occur.

Follow-up for excisional/ablative treatment

Following excisional/ablative treatment, a woman will need a follow-up Pap smear in 3 to 4 months. If the cytology report is normal at subsequent visits, the Pap smear should be repeated every 6 months for the first 2 years and then annually thereafter as long as the cervical smear is normal. If, however, a cervical smear is reported abnormal (based on the Bethesda Classification), the patient should be reintroduced into the observation/treatment protocol.

Hysterectomy

Historically hysterectomy was performed either vaginally or abdominally for CIN III of the cervix. Currently with the advent of colposcopy and good excisional therapy, hysterectomy is indicated in only 5%–10% of patients. There are still situations where hysterectomy is acceptable management of the patient. In the presence of the more aggressive recurrent high-grade squamous intraepithelial lesions (HGSIL) in women who have coexisting gynecologic disease and have completed childbearing, hysterectomy may be an option. If hysterectomy is performed for cancerous lesions, the patient's postoperative follow-up care should include a vaginal smear of the upper 1/3 of the vagina every 6 months for 2 years and then annually. Once the patient has had her first postoperative return visit (which should occur within 6 weeks of treatment) she should follow an observation or follow-up protocol because of her increased risk of vaginal neoplasia. The role of HPV testing in this situation requires further investigation.

Stage-wise management of cervical cancer

Treatment of microinvasive carcinoma

Stage IA1 and IA2

Cone biopsy is done in cancer-suspected lesions with clear margins at stages IA1 and 1A2. IA1 stage with clear margins is followed by simple observation if fertility is desired or simple hysterectomy if fertility is not desired. However IA2 stages with clear margins is followed by radial trachelectomy and pelvic lymph node dissection. Repeat cone biopsy or modified radial hysterectomy plus pelvic lymph node dissection is done in patients with stage 1A1 and 1A2 where margins involved with cancer/CIN3 (Souhami, and Tobias, 2005).

Treatment of early invasive cancer (stage IB1 and IIA < 4 cm)

When the tumor is more extensive but predominantly situated in the cervix, possibly with some vaginal involvement, surgical removal is preferred by radial hysterectomy/pelvic lymphadenectomy followed by simple observation in negative nodes and pelvic teletherapy $\pm$ brachytherapy $\pm$ chemotherapy (cisplatin, 30–40 mg/m^2 per week) in positive nodes. Treatment with radiotherapy as for early bulky disease is done in medically unfit patient.

Treatment of early bulky disease (stage IB2 and IIA > 4 cm)

Treatment is done according to skills and resources, either by pelvic teletherapy $\pm$ brachytherapy $\pm$ chemotherapy or by radial hysterectomy/pelvic lymphadenectomy.

Treatment of extensive disease: stages IIB–IIIB

These patients are managed by radical (curative intent) radiotherapy, comprising teletherapy and brachytherapy. The role of chemotherapy has not yet been proven in developing country settings.

Treatment of stage IVA

Stage IVB (5% of cases) indicates the presence of distant haematogenous metastasis and is incurable by any currently known means such as pelvic teletherapy and/or brachytherapy.

Treatment of stage IVB or recurrent disease

Cases with pelvic metastasis with no prior radiotherapy are treated with radiotherapy/chemotherapy. Such patients who had prior radiotherapy and have tumor in central pelvis are treated with pelvic exenteration, radial hysterectomy if $\leq$ 2 cm, and palliative care, and patients with tumor in pelvic sidewall undergo palliative care. Patients with extrapelvic metastasis undergo palliative radiotherapy/resection of isolated metastasis/palliative care.

Pelvic exenteration is infrequently used as it has major sequel of urinary and colonic diversions, both of which is difficult to care for in developing countries, and are unacceptable to many patients when it is not possible to offer a cure.

Human papillomavirus vaccines

Prophylactic human papillomavirus vaccines

Currently three successful prophylactic HPV vaccines quadrivalent "Gardasil" (HPV 16/18/6/11) developed by Merck while bivalent "Cervarix" (HPV 16/18) by Glaxo SmithKline (GSK) are recommended for vaccinating young adolescent girls at or before onset of puberty. In these vaccines viral capsid proteins are present in the form of spontaneously reassembled virus-like particles (VLPs) expressed either in yeast for Gardasil or in baculovirus for Cervarix. These two vaccines protect from infection with two of the most common cancer-causing HPV types 16 and 18 and more than 70% of cervical cancer cases are associated with these two HPV types. Both the vaccines were found to be highly immunogenic, safe, well-tolerated, and effective in preventing incident and persistent HPV infections including developing precancerous lesions.

Turning point

Three vaccines Gardasil, Cervarix, and Gardasil 9 that prevent infection with different HPV types has been approved by the FDA. All three vaccines prevent infections with high-risk types HPV 16 and 18 that cause about 70% of cervical cancers. Gardasil (which causes 90% of genital warts) also prevents infection with HPV types 6 and 11. Recently a new vaccine Gardasil 9 for HPV has achieved 100% protection in young women against nine HPV types, such as HPV 16, 18, 31, 33, 45, 52, 58, 6, and 11.

As of May 2017, Gardasil 9 is the only HPV vaccine available for use in the United States. Cervarix and Gardasil are still used in other countries.

Although these vaccines are expected to provide protection against other malignancies such as vaginal, anal, vulvar, oral, esophageal, and laryngeal papillomatosis that are associated with these HPV types, against which vaccines are developed, they will certainly not provide protection against about 10%–30% of cervical cancer that arise due to infection of other high-risk HPV types (31, 35, 39, 45, 51, 52, 56, 58, etc.). It is suspected that some of these HPV types may take lead because of change in microenvironment.

Therapeutic human papillomavirus vaccine

Although prophylactic vaccines appear to be successful, it would take decades to perceive the benefits because it takes 10–20 years to develop invasive cervical cancer. Therapeutic vaccines are to bridge the temporal deficit by attacking already persistent HPV infections and to treat cervical cancer in women. Several animal studies showed promising results and indicated that therapeutic HPV vaccine may regress disease progression. As a result several therapeutic HPV vaccines are in phase I and II clinical trials (Das et al., 2008) (Table 6.2). Most efforts have been directed toward the early proteins, HPV E6 and E7, or small peptides derived from them, mainly because these are the major transforming viral proteins that are invariably retained and expressed throughout the full spectrum of HPV-related disease progression and cervical carcinogenesis. Financial concerns are compounded by health care structures that can impede a woman's timely progress to the appropriate provider and may limit her access to the vaccine.

Genetic-based DNA vaccine

- No risk of infection
- Cheap to produce
- Stable product

TABLE 6.2 Summary of the current status of the selective human papillomavirus vaccines (Das et al., 2008).

Antigen used	Type of vaccine	Nature of vaccine	Current status	Mode of administration
Quadrivalent HPV types HPV(16/18/6/11)L1 (Gardasil)	Prophylactic	Virus-like particle(VLP), protein	US FDA approved	Injectable
HPV 16/18 L1 (Cervarix)	Prophylactic	VLP, protein	Applied for US FDA approval	Injectable
HPV 16 E6/E7	Therapeutic	Fusion protein	Phase I clinical trial	Injectable
HPV 16 E7	Therapeutic	Peptide	Phase I clinical trial	Injectable
HPV 16/18 E6/E7	Therapeutic	Recombinant Vaccinia virus	Late-stage cervical cancer	Injectable
Second generation vaccine	Prophylactic	VLP, protein		Injectable
Other high-risk (HPV31,45,33)L1				
HPV 16 L1	Prophylactic	Capsomeres (pentameric) protein	Animal model	Intranasal delivery
HPV 16 L1 in plant	Prophylactic	VLP, protein	Animal model	Oral delivery
HPV 16 L1 as recombinant bacteria	Prophylactic	VLP produced in recombinant Lactobacillus, protein	Animal model	Mucosal
HPV 16 L1	Prophylactic	DNA based	Animal model	Parenteral, oral
HPV 16 E7	Therapeutic	DNA based	Clinical trial	Injectable with Micro particles (ZYC101)
HPV 16 E7 Detox (Sig/E7 detox/HSP70)	Therapeutic	DNA based	Phase I	Injectable
HPV 16 L1/L2-E7	Chimeric (prophylactic/ therapeutic)	Fusion protein	Animal model	Injectable
HPV 16L2E7E6	Chimeric (prophylactic/ therapeutic)	Fusion protein	Phase I, II clinical trial	Injectable

Source: Das, B.C., Hussain,, S., Nasare, V., Bharadwaj, M., 2008. Prospects and prejudices of human papillomavirus vaccines in India. Vaccine, 26, 2669–2679.

- High antigenicity
- All proteins present in best effectiveness

Issues/unanswered questions associated with human papillomavirus vaccine

Vaccine efficacy

- Will HPV vaccine work for lifelong?
- How long it will last?
- Whether booster shots needed?
- How long will it protect from HPV infection?
- End point of vaccine trial.

Vaccine protection

- Does HPV vaccine ultimately prevent development of cervical cancer?
- Needs long-term monitoring of vaccinated woman (to reach 35–45 years) to prove that vaccine prevents HPV-related cancers.

Who should be vaccinated

- What age group of females to be vaccinated?
  - Childhood (1–5 years)
  - Young adolescent (9–19 years)
  - Immediately after marriage (18–25 years)
- Whether men also to be vaccinated?

Ethical issues

With screening implementation

In recent years medical ethics has become an undisputed part of medical studies. Cervical screening

programs do have the potential to save lives at minimum risk but at considerable cost. One of the biggest limitations at present is that few of the cervical screening programs seem to address these ethical imperatives, and many causing more harm than good. Types of cancer screenings have created the bioethical dilemmas. Debates over research and screening ethics have until recently revolved around two related questions: the voluntary, informed consent of subjects and the appropriate relationship between risk and benefit to subjects in the experiment. Every patient has a right to full and accurate information about his or her medical condition. Hence in planning a screening program physicians must also think about the ethical responsibilities entailed. Ethical principles to be followed in cancer screening programs are intended mainly to minimize unnecessary harm to the participating individuals. The dilemma facing physicians today is to decide whether the current method of cervical screening is justified in the light of increasing evidence that these programs can be damaging to patients.

Risk

There is now growing evidence to suggest that cervical screening programs cause psychological harm to patients, particularly increased anxiety, embarrassment, and fear of outcome. False-positive results can lead to considerable distress, and perhaps unnecessary treatment. Negative results can produce false reassurance to women. These negative effects of screening are probably quite common, and in some cases, can continue to cause long-term anxiety and distress. In order to choose between screening and not screening, physician has to establish a balance to ensure that the benefit of screening is maximized and the risks minimized. This can be accomplished in several ways, such as rethinking how the program is organized, providing effective training of operators to ensure high-quality smears, and maintaining an effective quality control system. One of the most crucial ways of reducing anxiety is to ensure that before, during, and after the screening process, the patient is fully informed and thus involved in the decision-making process. Individual women require varying amounts of information with differing levels of detail. It is therefore ethically imperative that all screening programs attend to these details.

Benefit versus cost

Socioeconomic status, access to care, and lack of health insurance coverage correlates with delay in diagnosis, advanced stage, and impaired survival. It is widely believed that screening programs attract worried healthy patients, particularly in the higher socioeconomic groupings, and that the patients most at risk are habitually missed. It has also been calculated that it takes 40,000 smears and 200 excision biopsies to prevent one death from cervical cancer. This has been calculated to be equivalent to $600,000 per life saved. However the return is not worth the effort, especially as that effort is not directed to the population of women at greatest risk, which is primarily those that habitually fail to respond to screening invitations.

Patient autonomy and coercion

"Autonomy" means leaving the decision to have a cervical screening entirely up to the patient. Screening will be effective only if a high proportion of women are screened, and such a proportion can be achieved only by infringing patient autonomy and using coercion. In this case certain dangers have to be considered, as some patients may visit too often, use resources too frequently, and perhaps receive false reassurance. Some will not come at all, who are most at risk. So, a patient should be properly informed prior to screening.

With vaccine implementation

Cervical cancer and HPV vaccine awareness among school, undergraduate students, and also to their parents was found to be very low in both urban and rural school population of Noida and Delhi, India (Hussain et al., 2014) The level of awareness and education appears to be insignificant determinants in rural compared with urban setup. In a survey of parents of school girls in developing countries, it has been discovered that the majority of parents are unaware of HPV and perceived that their children were not at a risk of acquiring sexually transmitted HPV infection as they have good family backgrounds and the children are not allowed to be involved in premarital sexual activity (Das et al., 2008). Some parents have diverse opinions that HPV vaccines would make sex safe, leading to freedom for promiscuity, and risky sexual behavior, which is not very common in this region of the globe due to sociocultural factors. They also suspected that the vaccine itself might cause infection in children. Therefore it is extremely important to raise general awareness about HPV, destigmatization of HPV infection and subsequently to gain acceptance for a mass vaccination program for preadolescent and adolescent girls in India. Better health education is needed to maximize public awareness for cervical cancer prevention (Hussain et al., 2015).

Potential strategies may therefore include vaccination of school girls (which may miss the more vulnerable girls not attending school), through mother–daughter initiatives or other existing

community outreach programs. Although boys do not develop genital cancer, they may develop other HPV-associated diseases such as penile, anal, and oral cancers and genital warts.

Translational significance

It is an established fact that HPV infection is necessary but insufficient to cause malignancy. Furthermore persistence of HPV 16 or 18 in women does not necessarily result in cancer. Persistence indicates the importance of other factors for malignant conversion of high-grade HPV infection. Progression of the HPV-infected cell to a malignant phenotype involves further modification of host gene expression and/or mutations. The appearance of chromosomal aberrations can lead to mutational inactivation or loss of tumor suppressor genes, activation and amplification of oncogenes play central role in cancer progression.

The process of gene expression in response to physiological and environmental entities is mainly regulated at transcriptional level where transcription factors bind to *cis* regulatory DNA sequences. Transcription factors are proteins involved in the regulation of gene expression that bind to the promoter elements upstream of genes and either facilitate or inhibit transcription. They control and regulate gene expression through this process. Transcription factors are composed of two essential functional regions: a DNA-binding domain and an activator domain. The DNA-binding domain consists of amino acids that recognize specific DNA bases near the start of transcription. The activator domains of transcription factors interact with the components of the transcriptional apparatus (RNA polymerase) and with other regulatory proteins, thereby affecting the efficiency of DNA binding.

HPV 16 E6/E7 transcription is regulated by *cis*-acting elements contained within the URR. The URR is a transcriptional control region in HPV. It is an 850 bp region which is functionally divided into three parts, a 5′ terminal portion of unknown function, central 400 bp constitutive enhancer for E6/E7 promoter activity, and a terminal 3′ region containing the E6/E7 promoter. All the transcription factors are known to bind the URR region. These transcription factors do not function in isolation, but form regulatory networks in which several factors interact with them at a DNA-binding domain also called transactivation domain that mediates the interaction between host and environment including viruses. A number of oncogenic signaling pathways all seem to converge on a limited set of nuclear transcription factors. These transcription factors are the final “switches” that activate the gene expression patterns that ultimately lead to malignancy. Targeting a single transcription factor can block the effects of a multitude of upstream genetic aberrations that cause its persistent activation. Certain transcription factors such as signal transducer and activator of transcription, activator protein-1 (AP-1), and nuclear factor-κB (NF-κB) have been identified as important components of signal transduction pathways leading to pathological outcomes such as inflammation and tumourigenesis and are current molecular targets for cancer therapy. These transcription factors are normally modulated at the level of expression and/or their activation. Host transcription factors in association with viral factors are likely to dictate viral latency, vegetative replication, or oncogenic transcription during HPV infection.

The multistep cervical carcinogenesis process is amendable to molecular therapeutics such as therapeutic nucleic acids (TNAs). TNA-based therapies for cervical carcinoma include ribozymes, antisense oligonucleotides (AS-ODNs), micro RNA (mRNA), and small interfering RNAs (siRNAs). In vitro experiments with TNAs have successfully inhibited E6/E7 expression and caused induction of apoptosis and/or senescence in cervical carcinoma cells. Early ribozyme and AS-ODN approaches showed promise as therapeutic moieties for cervical cancer. Viral early genes E6 and E7 from high-risk HPV types are responsible for the transformation of epithelial cells, and their continuous expression is essential for ongoing cervical cancer cell survival as they function as oncogenes. Therefore E6 and E7 are ideal targets for RNAi therapy. In recent years there have been a number of publications showing the potential use of RNAi as a treatment for cervical cancer.

MicroRNAs and siRNAs belong to a family of small noncoding RNAs that bind through partial sequence complementarity to 3′-UTR regions of mRNA from target genes, resulting in the regulation of gene expression. MicroRNAs have become an attractive target for genetic and pharmacological modulation due to the critical function of their target proteins in several signaling pathways, and their expression profiles have been found to be altered in various cancers.

Rapid advances in the study of microRNA expression profiles and siRNAs for silencing gene expression have led to many ongoing efforts to exploit these molecules as biomarkers and therapeutic agents, respectively, in the treatment of several cancers. A key feature of microRNAs and siRNAs is that they are not translated into proteins but rather function in the regulation of gene expression. The new knowledge has contributed to an improved understanding of the mechanism of microRNAs biogenesis and an emerging consensus about the function of microRNAs and their targets in several species including humans. One of

the most successful approaches utilizes microRNAs as biomarkers in several diseases. The siRNAs, on the other hand, represent a fast, cost-effective, and relatively simple tool for inducing downregulation of virtually any gene sequence in many species. Therefore siRNA-based drugs may be the next generation of biochemical compounds because they are highly gene specific due to nucleotide complementarity and have less challenging pharmacodynamics because siRNAs are biologic molecules.

MicroRNA expression profiles in cervical cancer

By comparing microRNA expression profiles between normal and tumor tissues, studies have identified deregulated microRNAs and mRNAs, demonstrating an aberrant microRNA expression pattern in various malignancies. However it is unclear whether microRNA expression is altered at the onset of cell transformation or as a consequence. Since microRNAs regulate the expression of their mRNA targets, it is expected that the over- or underexpression of microRNAs would have an effect on cellular phenotype (Díaz-González et al., 2015). Several microRNAs with altered expression in cervical cancer have been identified and put forth as oncomirs or tumor suppressor genes. For instance, miR-10a, miR-106b, miR-21, miR-135b, miR-141, miR146, miR-148a, miR-214, and miR-886-5p have been proposed to act as oncomirs in cervical cancer, contributing to the development of cancer through dysregulation of gene products involved in cell proliferation, apoptosis, or cell−cell adhesion (Li et al., 2011; Long et al., 2012).

Recently our laboratory has identified 383 miRNAs that were differentially expressed in cervical cancer cases, ($P < .0001$) of which 350 miRNAs were upregulated and 33 miRNAs were downregulated. We also observed that 182 miRNAs were differentially expressed ($P < .0001$) in HPV 16/18-positive (SiHa/HeLa) cell lines compared with HPV-negative (C33A) cell line. In addition we identified the novel microRNAs such as miR-892b, miR-500, miR-888, miR-505, and miR-711 in cervical precancerous lesions and cervical cancer cases in Indian population. Taken together our study demonstrates a crucial role of microRNAs in cervical cancer, which may serve as potential early diagnostic markers for cervical carcinogenesis (Sharma et al., 2015).

siRNAs for human papillomavirus oncogenes as potential gene therapy for cervical cancer

The silencing of genes by siRNAs is a potential mechanism to inactivate foreign DNA sequences and may be employed to silence the expression of HPV oncogenes in cervical cancer. The first studies carried out with synthetic siRNAs to silence HPV 16 E6 and E7 oncogene expression were described by Jiang and Milner (2002). Also, E6 silencing induced expression of the p53 gene and transactivation of the p21 gene and decreased cell proliferation, while silencing of E7 induced cell death by apoptosis. These findings demonstrated for the first time that the expression of HPV E6 and E7 oncogenes may be specifically silenced by siRNAs in human tumor cervical cells.

Recent investigation has focused on silencing the HPV E6-E7 bicistron with siRNAs. These oncogenes are transcribed jointly, as a bicistron, which is the result of alternate splicing. The effect of siRNAs against the HPV 16 E6 oncogene on the E6-E7 bicistron has been studied in vitro and in vivo. Administration of siRNAs for E7 induces silencing of both oncogenes, while siRNAs for E6 inhibit E6 expression but do not affect E7 expression (Lea et al., 2007). However siRNA-based targeting requires further validation of its efficacy in vitro and in vivo, for its potential off-target effects, and of the design of conventional therapies to be used in combination with siRNAs and their drug delivery vehicles.

Chemotherapeutic drugs and siRNAs

Although the effect of chemotherapeutic drugs on p53 expression in cervical cancer cells is known, new research has focused on the association between the activation of p53 gene, the cytotoxic effect of drugs, and the silencing of HPV oncogenes with siRNAs. Different groups have analyzed the expression of p53 in HeLa cells (HPV 18 +) treated with siRNAs for HPV 18 E6, combined with carboplatin, cisplatin, doxorubicin, etoposide, gemcitabine, mitomicine, mitoxantrone, oxaliplatin, paclitaxel, and/or topotecan treatment (Koivusalo et al., 2005). The researchers observed silencing of HPV 18 E6 and E7 oncogenes, as well as an increase in p53 protein expression and changes in cytotoxicity dependent on the nature of each chemotherapeutical compound. This evidence suggests that the silencing of HPV E6 and E7 oncogenes with siRNAs can increase cellular sensitivity to the cytotoxic effects of drugs and that combined treatment may have a synergistic effect and reduce resistance to chemotherapeutical drugs, representing an advantage for treatment.

Cancer stem cells (CSCs) within a tumor possess the capacity to self-renew and to cause the heterogeneous lineages of cancer cells that comprise the tumor. CSC population that fuels the tumor growth have emerged a new therapeutic intervention in cervical cancer

progression. Cervical cancer contains a heterogeneous population of cancer cells. Several investigations have identified putative stem cells from solid tumors and cancer cell lines via the capacity to self-renew and drive tumor formation. Cervical CSCs have a 10-fold higher binding capacity for papillomavirus-like particles than any other cervical epithelial subpopulations, suggesting that HPVs might indeed preferentially bind and infect cervical CSCs cells in vivo. CSCs are responsible for tumor initiation and maintenance and are attractive target for advanced cancer therapy. Sphere-forming cells (SFCs) from cervical cancer cell lines HeLa and SiHa have been isolated. These cells showed an expression pattern of CD44high/CD24low that resembles the CSC surface biomarker of breast cancer. HeLa-SFCs expressed a higher level (6.9-fold) of the HPV oncogene E6, compared with that of parental HeLa cells. Silencing oncogene expression in cervical cancer stem-like cells inhibits their cell growth and self-renewal ability.

Conclusion

Cervical cancer provides a unique window to study the deregulation of important molecular gate keepers that are important for normal cell cycle events. However tumorigenic transformation of cervical epithelial cells takes 10–15 years to develop histopathologically well-characterized precursors and cancerous lesions. So the question arises why it takes so long to develop cervical cancer compared with other cancers that have poor prognosis. Cervical intraepithelial lesions is a common precancerous condition among HPV-infected cases and are known to progress to invasive cancer. To intensify the scenario, until date there is no standard therapeutic modality available that can cure these viral infections. Therefore for effective therapeutic intervention of HPV and to prevent cervical cancer development at an early stage, it is important to develop understanding of molecular mechanisms involved in HPV-mediated cervical carcinogenesis. Therefore it is mandatory to address important questions that would eventually help us to understand the molecular basis of cervical cancer.

World Wide Web resources

The American Cancer Society Southwest Division

Tel: (505) 260–2105
WWW: http://www.cancer.org/

Address: 5800 Lomas Boulevard, NE, Albuquerque, NM 87110

The ACS is a nonprofit, nationwide organization that supports research, conducts educational programs, and offers a variety of services to people with cancer and to their families. ACS helps women with cancer through various patient services and support groups (Albuquerque, 1997).

CancerNet

E-mail: Cancernet@icic.nci.nih.gov

CancerNet is a way to obtain PDQ information summaries and other NCI information via Internet and selected electronic information services. To use CancerNet, send a mail to the address above. Enter the word "HELP" as the text of the message to receive materials in English; enter "SPANISH" to receive the information in Spanish.

People Living Through Cancer

Tel: (505) 242–3263
Fax: (505) 242–6756
Address: 323 Eighth Street SW, Albuquerque, NM 87102

PLTC was founded by and for those coping with a cancer diagnosis or with the cancer of a friend or loved one. PLTC cervical cancer resources.

American Institute for Cancer Research

Provides information on cancer and nutrition. Publishes a newsletter, cookbooks, and diet/nutrition brochures. AICR is also a hotline for nutrition-related cancer inquiries, where callers will be connected with a registered dietitian.

American Society of Plastic and Reconstructive Surgeons

For referrals to a plastic surgeon for corrective or reconstructive procedures, contact the American Society of Plastic and Reconstructive Surgeons for a list of local board-certified plastic surgeons.

Asian and Pacific Islander American Health Forum

The Asian and Pacific Islander American Health Forum is a national advocacy organization dedicated to promoting policy, program, and research efforts for the improvement of health status of all Asian American and Pacific Islander Communities. The

Women's Health Information Network strives to increase the public understanding of Asian and Pacific Islander women's health status including cancer. Translations include Chinese, Tagolog, Vietnamese, Korean, Hindi, Gujurati, Urdu, Farsi, Thai, and Cambodian.

Avon's Breast Cancer Awareness Crusade

Avon's Breast Cancer Awareness Crusade, a national initiative of Avon Products, Inc., provides women, particularly low-income, minority, and older women, with direct access to a full range of breast cancer education and early detection services.

Cancer Information Service

CIS interprets and explains research findings to the public in a clear and understandable manner. The Northwest Regional Cancer Information Service serves Alaska. The CPCD provides bibliographic citations, abstracts of journal articles, book chapters, technical reports, papers, materials, curricula, and descriptions of cancer prevention programs and risk reduction activities at national, state, and local levels in English, Spanish, or on TTY equipment.

Cancer Mail

National Cancer Institute information about cancer treatment, screening, prevention, and supportive care. To obtain a contents list, send e-mail to cancermail@i-cicc.nci.nih.gov with the word "help" in the body of the message.

CancerNet

CancerNet contains material for health professionals, patients, and the public, including information from PDQ about cancer treatment, screening, prevention, supportive care, and clinical trials and CANCERLIT a bibliographic database.

Cancer Patient Education Database

Provides information on cancer patient education resources for cancer patients, their family members, and health professions.

Cancer Research Foundation of America

Women's health ages 18–27, 28–39, 40–49, 50 + years (breast cancer and cervical cancer).

Cancer source.com

Cancer Types: Cervical Cancer

Here you will find information and resources related specifically to cervical cancer.

Further information

Division of Cancer Studies, University of Birmingham

http://www.birmingham.ac.uk/schools/cancer/index.aspx

The School of Cancer Sciences is one of the world's premier translational cancer research institutes. Underpinned by excellent basic science across the University campus, we work with many clinical partners to improve the outlook for cancer patients.

Keywords

1. *Cancer*: It refers to a class of disease wherein a cell or a group of cells divide and replicate uncontrollably due to accumulation of both genetic and/or epigenetic changes occurred in a multistep manner. This leads to unregulated cell proliferation, intrude into adjacent cells and tissues (invasion) and ultimately spread to other parts of the body than the location at which they arose (metastasis). Different types of cancer include cervical lung, breast, oral, colon, prostate, and ovarian cancers.
2. *Cervical cancer*: It results from the abnormal growth and division of cells at the opening of the uterus or womb—the area known as the cervix. The progression of cervical cancer is a multistep process. Initially normal cells undergo precancerous changes and ultimately develop into cancer cells. These precancerous conditions include cervical intraepithelial neoplasia (CIN), squamous intraepithelial lesion (SIL), and dysplasia. It takes several years to develop into an invasive cancer.
3. *Human papilloma virus (HPV)*: These are ubiquitous DNA viruses belonging to family Papillomaviridae. They are small, nonenveloped DNA virus with a circular, double-stranded DNA genome of approximately 7200–8000 base pairs (bp). HPV infection is a major cause of uterine cervical cancer and benign epithelial lesions such as warts and condyloma acuminate in lower genital tracts in humans. More than 100 genotypes have been described till date, 15 types are categorized as high-risk (HR-HPVs) types (HPV 16, 18, 31, 33. 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82) that are associated with genital and other epithelial cancers, and 12 low-risk (LR-HPVs) types (HPV 6, 11, 40, 42, 43, 44,

54, 61, 70, 72, 81, CP6108), which are responsible for benign tumors and genital warts.

4. *Proto-oncogene and oncogene*: A proto-oncogene is a normal gene that can become an oncogene due to mutations or increased expression. The resultant protein may be termed an oncoprotein. Proto-oncogenes code for proteins that help to regulate cell growth and differentiation. Proto-oncogenes are often involved in signal transduction and execution of mitogenic signals, usually through their protein products. Upon *activation*, a proto-oncogene (or its product) becomes a tumor-inducing agent, an oncogene that has the potential to cause cancer. Examples of proto-oncogenes include RAS, WNT, MYC, ERK, and TRK. The MYC gene is implicated in Burkitt's Lymphoma. In tumor cells they are often mutated or expressed at high levels. Since the 1970s dozens of oncogenes have been identified in human cancer. Many cancer drugs target the proteins encoded by oncogenes.
5. *Tumor suppressor gene*: These genes normally function to inhibit cell growth/division and prevent cancer. If a tumor suppressor gene is deleted or becomes mutated, this can contribute to cancer development. Examples include the BRCA1 gene that is mutated in some breast cancers and RB1 which is mutated in retinoblastoma. A tumor suppressor gene, or antioncogene, is a gene that protects a cell from being cancerous and encodes the proteins that either have a dampening or repressive effect on the regulation of the cell cycle or promote apoptosis, and sometimes do both. When this gene is mutated to cause a loss or reduction in its function, the cell can progress to cancer, usually in combination with other genetic changes example: p53 tumor suppressor protein encoded by the TP53 gene. Homozygous loss of p53 is found in 70% of colon cancers, 30%–50% of breast cancers, and 50% of lung cancers. Mutated p53 is also involved in the pathophysiology of leukemias, lymphomas, sarcomas, and neurogenic tumors.

   Care taker genes: Genes involved in repair or prevent DNA damage and arise by deletion, point mutation, or methylation (mutation is generally recessive). Their inactivation results in genetic instabilities causing an increased mutation rate affecting all genes including DNA repair genes (XPD, XRCC).
6. *Screening*: Screening is a public health intervention used on a population at risk, or target-population. Screening is not undertaken to diagnose a disease, but to identify individuals with a high probability of having or developing a disease. Screening can detect abnormalities before they become cancer. Also, if cancer itself is detected early, it can be cured with proper treatment. For example, cervical cancer screening aims to test the largest possible proportion of women at risk and to ensure appropriate follow-up for those who have a positive or abnormal test result. Such women will need diagnostic testing and follow-up or treatment. Methodologies include colposcopy and biopsy.

Key points to remember

- HPV infection is the cause of almost all cases of cervical cancer and also associated with the development of carcinoma of other organ sites.
- HPV is one of the common sexually transmitted infection (STIs) but may not be an indicator of sexual practice or promiscuity.
- HPV infections are often asymptomatic but may transmit infection to sex partners.
- Majority (~90%) of HPV infections resolve spontaneously.
- Persistent infection of HPV is essential before initiation of events in host cells.
- Most predominant is the high-risk HPV type 16, and HPV 16 and 18 account for more than 80% cervical cancer.
- High-risk HPV types 16/18 can immortalize human squamous epithelial cells in vitro.
- Systemic immunization with HPV-VLP can confer protection against HPV infection.

Key points for effective cervical cancer screening program in low-resource settings

- Cervical cancer can be prevented if precancerous lesions are identified early through screening.
- Every woman should undergo cervical Pap smear screening or HPV DNA testing at least once in her life time.
- An optimal age for cervical cancer screening to achieve maximum effect is 30–40 years.
- Visual inspection with acetic acid (VIA) and cytology-based Pap smear test should continue for screening but HPV DNA testing must be incorporated for confirmation particularly in unequivocal cases till an affordable, cost-effective and reliable HPV test is available.

Ablative techniques

- *Laser ablation*: Laser stands for light amplification by stimulated emission of radiation. It is a procedure in

which a carbon dioxide laser directed through a microscope is used to vaporize the cervical transformation zone. This procedure is done under local anesthetic and takes 15–20 minutes to perform. Post therapy, the patient may experience pain, some uterine cramping and bloody discharge/vaginal spotting.

- *Cryotherapy*: Among ablative techniques, cryotherapy is a procedure performed under direct visualization in which a probe is placed against the cervix. The probe freezes the affected tissue, which in turn results in the destruction and sloughing of cervical cells. The entire procedure takes about 15 minutes. During the procedure the patient may experience uterine cramping. Subsequently the patient may expect to have a profuse watery discharge for 7–10 days. Recurrence rate is low and 4 months after cryotherapy, the reevaluation of pap smear is done.

Excisional techniques

- *Electrosurgical excision (LEEP)*: Electrosurgical excision of the transformation zone is a procedure in which an electrical current generating a radiofrequency is passed through a wire loop that excises the tissue and cauterizes the base. The procedure usually can be performed in an outpatient setting with the use of local anesthetic. Depending on the size of the loop and the lesion, either the transformation zone or a "cone-like" specimen can be obtained. The patient must be grounded for safety purposes, because an electrical current is used. The electrical current also generates heat, which can cause distortion of the surgical margins, thus making accurate interpretation of the regions difficult, if not impossible. Risks of all excisional procedures include bleeding, cervical stenosis, cervical incompetence, decrease in cervical mucus, and possible infertility.
- *Laser conization*: It is an operative procedure requiring an anesthetic in which the carbon dioxide laser is used as a knife to generate the same type of specimen obtained with cold knife conization (CKC). Postoperatively the patient will experience cramping and some bleeding.
- *Cold knife conization*: It is an operative procedure requiring either a regional or general anesthetic. During the procedure, the involved ectocervix and endocervix are excised using a circumferential excision. Postoperatively, the patient may have cramping and bleeding. Postoperative infection requiring antibiotic therapy may also occur.
- *Hysterectomy*: Historically hysterectomy was performed either vaginally or abdominally for CIN III of the cervix. Currently with the advent of colposcopy and good excisional therapy, hysterectomy is indicated in only 5%–10% of patients. There are still situations where hysterectomy is acceptable management of the patient. In the presence of the more aggressive recurrent high-grade squamous intraepithelial lesions (HGSIL) in women who have coexisting gynecologic disease and have completed childbearing, hysterectomy may be an option. If hysterectomy is performed for cancerous lesions, the patient's postoperative follow-up care should include a vaginal smear of the upper 1/3 of the vagina every 6 months for 2 years and then annually. Once the patient has had her first postoperative return visit (which should occur within 6 weeks of treatment) then the patient should follow an observation or follow-up protocol because of her increased risk of vaginal neoplasia. The role of HPV testing in this situation requires further investigation.

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Glossary

DNA Deoxyribonucleic acid is a molecule composed of two chains that form a double helix carrying the genetic information used in the growth, development, functioning, and reproduction of all known organisms and many viruses.

HCII Hybrid capture II is a quantitative HPV nucleic acid test that uses an efficient signal amplification strategy with a chemiluminescent readout.

HPV HPV stands for human papillomavirus. It is the most common sexually transmitted infection that causes cervical cancer.

miRNA A microRNA is a small noncoding RNA molecule found in plants, animals, and some viruses, which functions in RNA silencing and post-transcriptional regulation of gene expression.

Oncogene An oncogene is a gene that has the potential to cause cancer. In tumor cells, they are often mutated or expressed at high levels.

p53 Tumor protein p53, also known as p53, cellular tumor antigen p53, phosphoprotein p53, tumor suppressor p53, is any isoform of a protein encoded by homologous genes in various organisms, such as TP53 and Trp53. It is a gene that codes for a protein that regulates the cell cycle and hence functions as a tumor suppression.

pRb The retinoblastoma protein is a tumor suppressor protein that is dysfunctional in several major cancers.

siRNA Small interfering RNA, sometimes known as short interfering RNA or silencing RNA, is a class of double-stranded RNA molecules, 20–25 base pairs in length, similar to miRNA, and operating within the RNA interference pathway.

Tumor suppressor gene A tumor suppressor gene, or antioncogene, is a gene that protects a cell from one step on the path to cancer. When this gene mutates to cause a loss or reduction in its function, the cell can progress to cancer, usually in combination with other genetic changes.

Vaccine A vaccine is a biological preparation that improves immunity to a particular disease.

Abbreviations

ACS	American Cancer Society
ADC	Adenocarcinoma
AP-1	Activator protein-1
AS-ODNs	Antisense oligonucleotides
BAK	BCL1-antagonist/killer-1
BRCA1	Breast cancer type 1
CC	Cervical cancer
CIN	Cervical intraepithelial neoplasia
CIS	Carcinoma in situ
CR	Conserved region
CIS	Cancer information service
CPCD	Cancer prevention and control database
CSC	Cancer stem cell
CKC	Cold knife conization
DNA	Deoxyribonucleic acid
ERK	Extracellular signal regulated kinase
E6AP	E6-associated protein
FADD	Fas-associated protein with death domain
FDA	Food and Drug Administration
FIGO	International Federation of Gynaecology and Obstetrics
G1	GAP1
GP	General primers
GSK	Glaxo SmithKline
HPV	Human papilloma virus
HGSIL	High-grade squamous intraepithelial lesions
HSIL	High squamous intraepithelial lesions
HCII	Hybrid capture II
IFN	Interferon
IRF	Interferon regulatory factor
LCR	Long coding region SIL
LSIL	Low squamous intraepithelial lesions
LEEP	Electrosurgical excision
miRNA	Micro RNA
NCI	National Cancer Institute

NF-κB	Nuclear factor-κb
PLTC	People living through cancer
PDQ	Physician data query
PCR	Polymerase chain reaction
PBS	Phosphate-buffered saline
RB	Retinoblastoma
RFLP	Restriction fragment length polymorphism
RAS	Renin angiotensin system
SIL	Squamous intraepithelial lesion
S	Stationary
SCC	Squamous cell carcinoma
STATs	Signal transducer and activator of transcription
siRNA	Short interfering RNA
SFC	Sphere-forming cell
STIs	Sexually transmitted infection
TRK	Tropomyosin receptor kinase
TP53	Tumor protein 53
TNA	Therapeutic nucleic acids
TTY	Tele-type writer (text telephone)
URR	Upstream Regulatory Region
US	United States
VIA	Visual inspection with acetic acid
VILI	Visual inspection with Lugol's iodine
VLP	Virus-like particle
WHO	World Health Organization
WNT	Wingless integrated
XPD	Xeroderma pigmentosum group D
XRCC	X-ray repair cross-complementing group

Long answer questions

1. What are the visual methods used for diagnosis of cervical cancer?
2. What are the HPV vaccines available commercially? Are they therapeutic or prophylactic in nature?
3. How HPV can be detected by multiplex PCR and RFLP?
4. What are siRNAs and how they are used as potential gene therapy?
5. What ablative techniques are used to treat cervical cancer?

Answers to long answer questions

1. Visual methods used for screening/diagnoses of cervical cancer are as follows:

- Visual inspection with acetic acid (VIA);
- Visual inspection with Lugol's iodine (VILI).

Abnormalities are identified by inspection of the cervix without magnification, after application of dilute acetic acid (vinegar) (in VIA) or Lugol's iodine (in VILI). When vinegar is applied to abnormal cervical tissue, it temporarily turns white (acetowhite) allowing the provider to make an immediate assessment of a positive (abnormal) or negative (normal) result. If iodine is applied to the cervix, precancerous and cancerous lesions appear well-defined, thick, and mustard or saffron-yellow in color, while squamous epithelium stains brown or black, and columnar epithelium retains its normal pink color. VIA and VILI are promising alternatives to cytology where resources are limited. They are currently being tested in large, cross-sectional, randomized controlled trials in developing countries. In research settings VIA has been shown to have an average sensitivity for detection of precancer and cancer of almost 77%, and a range of 56%–94%. The specificity ranges from 74% to 94% with an average of 86%. They are both short procedures, less costly, and cause no pain. Assessment is immediate, and no specimen is required.

•VIA and VILI are indicated for all women in the target age group specified in national guidelines, provided that:

•They are premenopausal. Visual methods are not recommended for postmenopausal women, because the transition zone in these women is most often inside the endocervical canal and not visible on speculum inspection.

•Both SCJs (i.e., the entire transformation zone) are visible. If the patient does not meet the above indications and no alternative screening method is available in the particular clinical setting, then the patient should be referred for a Pap smear.

2. HPV vaccines are of two types: prophylactic and therapeutic. Two successful prophylactic HPV vaccines -quadrivalent "Gardasil" (HPV 16/18/6/11) developed by Merck while bivalent "Cervarix" (HPV 16/18) by GSK are recommended for vaccinating young adolescent girls at or before onset of puberty. In these vaccines viral capsid proteins are present in the form of spontaneously reassembled virus-like particles (VLPs) expressed either in yeast for Gardasil or in baculovirus for Cervarix. These two vaccines protect from infection with two of the most common cancer-causing HPV types 16 and 18 and more than 70% of cervical cancer cases are associated with these two HPV types.

   Therapeutic vaccines are to bridge the temporal deficit by attacking already persistent HPV infections and to treat cervical cancer in women. Several animal studies showed promising results and indicated that therapeutic HPV vaccine may regresses disease progression. As a result several therapeutic HPV vaccines are in phase I and II clinical trials (Das et al., 2008). Most efforts have been directed toward the early proteins, HPV E6 and E7 or small peptides derived from them, mainly because these are the major transforming viral proteins that are invariably retained and

expressed throughout the full spectrum of HPV-related disease progression and cervical carcinogenesis.

3. This method detects the five most prevalent HPV types commonly associated with cervical abnormalities. The technique involves RFLP of the approximately 450 bp amplicon, obtained after the amplification of L1 region of HPV genome by MY09/11 consensus primers. MY09/11 primers are used routinely for HPV detection covering a broad spectrum of HPV types as compared to general primers GP5 + /GP6 + . About 90% of the cervical carcinoma that contain some high-risk HPV types, HPV 16, 18, and 33 and few others are associated with CIN and cervical cancer, whereas HPV type 6 and 11 are associated with genital warts (condyloma accuminata and flat genital warts). Hence the detection of coinfection is equally important to understand the biological behavior of HPVs. The method detects the above five HPV types by digesting the PCR product of MY09/11 primers with Rsa-1 and resolving on 8% nondenaturing polyacrylamide gel. This method has advantage over other conventional methods of HPV typing, as it saves the cost and time for second PCR by type-specific primers. Most of the PCR-RFLP studies show either use of multiple restriction enzymes with two round of PCR. Hence it was found to be less combursive, low cost, and user friendly for the detection of HPV DNA from cervical swabs, both at clinical and research level. High-quality screening programs are also important to prevent cervical cancer among unvaccinated older women. The WHO recommends the screening of women aged 30−49 years—either through visual inspection with acetic acid in low-resource settings, Papanicolaou tests (cervical cytology) every 3−5 years, or HPV testing every 5 years—coupled with timely treatment of precancerous lesions.
4. The silencing of genes by siRNAs is a potential mechanism to inactivate foreign DNA sequences and may be employed to silence the expression of HPV oncogenes in cervical cancer. The first studies carried out with synthetic siRNAs to silence HPV 16 E6 and E7 oncogene expression were described by Jiang and Milner (2002). Also, E6 silencing induced expression of the p53 gene and transactivation of the p21 gene and decreased cell proliferation, while silencing of E7 induced cell death by apoptosis. These findings demonstrated for the first time that the expression of HPV E6 and E7 oncogenes may be specifically silenced by siRNAs in human tumor cervical cells.

   Recent investigation has focused on silencing the HPV E6-E7 bicistron with siRNAs. These oncogenes are transcribed jointly, as a bicistron, which is the result of alternative splicing. The effect of siRNAs against the HPV 16 E6 oncogene on the E6-E7 bicistron has been studied in vitro as well as in vivo. Administration of siRNAs for E7 induces silencing of both oncogenes, while siRNAs for E6 inhibit E6 expression but do not affect E7 expression. However siRNA-based targeting requires further validation of its efficacy in vitro and in vivo, for its potential off-target effects, and of the design of conventional therapies to be used in combination with siRNAs and their drug delivery vehicles.
5. The ablative techniques include laser and cryosurgery. The excisional modalities include cold knife conization, laser conization, loop electrocautery excision, and hysterectomy.

Short answer questions

1. What is the status of cervical cancer incidence worldwide?
2. What are the symptoms and risk factors of cervical cancer?
3. What are the risk factors of cervical cancer?
4. What oncogenes in the HPV genome which are involved in cervical cancer and what are their functions?
5. What is Pap test?
6. What are miRNA and siRNA?

Answers to short answer questions

1. According to GLOBOCAN 2018, it is the fourth most frequent women malignancy for both incidence and mortality with an estimated 5,30,232 new cases and 275,008 deaths every year with nearly 80% in developing countries. Incidence rates are now generally low in developed countries.
2. Women may notice one or more of the following symptoms: abnormal vaginal bleeding, bleeding that occurs between regular menstrual periods, bleeding after sexual intercourse, douching, menstrual periods that last longer and are heavier than before, bleeding after menopause, increased vaginal discharge, and pelvic pain.
3. The risk factors are categorized into two categories as follows:
   a. *Nongenetic factors*

      Lower socioeconomic status and lack of regular Pap tests, poor genital/sexual hygiene, multiple sexual partners or promiscuity, early age of first sexual intercourse below 18 years, oral

contraceptives use and smoking, multiple pregnancies and parity, socioeconomic status, dietary factors, religion, and ethnicity.

b. *Genetic factors*

High-risk type human papillomaviruses (HPVs), viral load (severity of a viral infection), HPV variants, genetic predisposition, infections of other STDs like HIV, weakened Immune system.

4. Both E6 and E7 proteins are essential to induce and maintain cellular transformation, due to their interference with cell-cycle control and apoptosis. The HPV viral oncogenes, E6 and E7, have been shown to be the main contributors to the development of HPV-induced cervical cancer and increased expression, probably due to integration of the viral DNA in the host cell genome, has been detected in invasive cancers and a subset of high-grade lesions. Both E6 and E7 HPV oncogenes interact with and inhibit the activities of tumor suppressors p53 and/or retinoblastoma protein (pRb), which is a common event for the carcinogenesis of human cells. This appears to result from deregulation of Plk1 by the loss of p53 through E6, and pRb family members by E7, overcoming the safeguard arrest response. Acute loss of pRb family members by E7 has also shown to induce centrosome amplification and aneuploidy, In addition E6 and E7 cause deregulation of cellular genes controlling the G2/M phase transition and progression through mitosis, such as the genes controlling centrosome homeostasis.
5. The Pap test looks for precancers, cell changes on the cervix that might become cervical cancer if they are not treated appropriately. An adequate sample should be taken from the squamo-columnar junction (the transformation zone), transferred to the cytology slide, and immediately fixed with a commercial fixative. Pap smear screening is a rapid method for detecting cervical dysplasia and in situ cancer, as well as invasive cancer. A Pap smear evaluates cells harvested from the ectocervix and endocervix for abnormal changes associated with the development of cervical cancer.
6. MicroRNAs and siRNAs belong to a family of small noncoding RNAs that bind through partial sequence complementarity to 3′-UTR regions of mRNA from target genes, resulting in the regulation of gene expression. By comparing microRNA expression profiles between normal tissue and tumor tissue, studies have identified deregulated microRNAs and mRNAs, demonstrating an aberrant microRNA expression pattern in various malignancies. However it is unclear whether microRNA expression is altered at the onset of cell transformation or as a consequence. Since microRNAs regulate the expression of their mRNA targets, it is expected that the over- or underexpression of microRNAs would have an effect on cellular phenotype. Also the silencing of genes by siRNAs is a potential mechanism to inactivate foreign DNA sequences and may be employed to silence the expression of HPV oncogenes in cervical cancer. However, siRNA-based targeting requires further validation of its efficacy in vitro and in vivo, for its potential off-target effects, and of the design of conventional therapies to be used in combination with siRNAs and their drug delivery vehicles.

"Yes/no" type questions

1. Cervical cancer is the major reproductive health problem of women globally.
2. HPV is discovered by Françoise Barré-Sinoussi and Luc Montagnier.
3. HPV oncogenes are E6 and E7.
4. Low-risk HPV does not involve in causing cervical cancer.
5. A proto-oncogene is a normal gene that helps to regulate cell growth and differentiation.
6. High-risk (HR-HPVs) types are associated with genital and other epithelial cancers.
7. Benign tumors and genital warts are also caused by HPV infection.
8. HPV infection can be diagnosed.
9. Till date, no micro RNA has been identified in cervical cancer.
10. There is no need of cervical Pap smear screening until any problem persists.

Answers to yes/no type questions

1. Yes—It is the commonest cause of cancer-related female mortality in developing countries. According to GLOBACAN, 2012, it is the third most frequent women malignancy worldwide with an estimated 5,27,624 new cases and 2,65,653 deaths every year with nearly 80% in developing countries.
2. No—HPV is discovered by Harald zur Hausen who got noble prize in Physiology or Medicine 2008.
3. Yes—E6 and E7 are the main contributors to the development of HPV-induced cervical cancer due to integration of the viral DNA in the host cell genome and apoptosis.
4. No—Both low-risk and high-risk HPV are involved in causing cervical cancer.

5. Yes—A proto-oncogene is a normal gene that can become an oncogene due to mutations or increased expression and then regulate cell growth and differentiation. Its resultant protein may be termed an oncoprotein.
6. Yes—15 types are categorized as high-risk (HR-HPVs) types (HPV 16, 18, 31, 33. 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82) and are associated with genital and other epithelial cancers.
7. Yes—12 low-risk (LR-HPVs) types (HPV 6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81, CP6108), are responsible for benign tumors and genital warts.
8. Yes—Reliable diagnosis of HPV infection, particularly the high-risk HPV DNA in vaginal or cervical smears or tissue biopsies is done. It can also be diagnosed by urine-based noninvasive HPV DNA detection method, simple paper smear method, by multiplex PCR and RFLP.
9. No—Several microRNAs with altered expression in cervical cancer have been identified and put forth as oncomirs or tumor suppressor genes like miR-10a, miR-106b, miR-21, miR-135b, miR-141, miR146, miR-148a, miR-214, and miR-886-5p have been proposed to act as oncomirs in cervical cancer, contributing to the development of cancer through dysregulation of gene products involved in cell proliferation, apoptosis, or cell−cell adhesion.
10. No—Every women should undergo cervical Pap smear screening or HPV DNA testing at least once in her life time.

CHAPTER

7

Human DNA tumor viruses and oncogenesis

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Summary

Viruses with transforming abilities can change a normal cell into a cancer cell when persist in the infected cells. Tumor viruses are subclassified as either DNA viruses, which include Epstein–Barr virus (EBV), Kaposi's sarcoma–associated herpesvirus (KSHV), human papillomavirus (HPV), hepatitis B virus (HBV), and Merkel cell polyomavirus (MCPyV), or RNA viruses, such as hepatitis C virus (HCV) and human T-cell lymphotropic virus (HTLV-1).

What you can expect to know

Cancer involves the deregulation of multiple cell-signaling pathways that govern fundamental cellular processes such as cell death, proliferation, differentiation, and migration (Abhik Saha et al., 2010). The biological pathways that lead to cancer are more complex and intertwined (Hanahan and Weinberg, 2000). Globally, it is estimated that 15%–20% of all cancers are linked to oncogenic viruses (Parkin, 2006). However, most viral infections do not lead to tumor formation as several other factors influence the progression from viral infection to cancer development. Some of these factors include the host's genetic makeup, mutation occurrence, exposure to cancer-causing agents, and immune impairment. Initially, viruses were believed to be the causative agents of cancers only in animals. It was almost half a century before the first human tumor virus, Epstein–Barr virus (EBV), was identified in 1964 (Moore and Chang, 2010). Subsequently, several human tumor viruses were identified. Tumor viruses are subcategorized as either DNA viruses, which include EBV, Kaposi's sarcoma–associated herpesvirus (KSHV), human papillomavirus (HPV), hepatitis B virus (HBV), and Merkel cell polyomavirus (MCPyV), or RNA viruses, such as hepatitis C virus (HCV) and human T-cell lymphotropic virus (HTLV-1) (Abhik Saha et al., 2010). The normal cell is transformed into a cancer cell on persistent viral infection, either by integrating or retaining its genome as an extrachromosomal entity. The infected cells are regulated by the viral genes, which have the ability to drive the abnormal growth. The virally infected cells are either eliminated via cell-mediated apoptosis or they persist in a state of chronic infection. Importantly, the chronic persistence of infection by tumor viruses can lead to oncogenesis (Damania and Pipas, 2009). This chapter specifically focuses on the major tumor DNA viruses associated with human cancer and their mechanism of oncogenesis.

History and methods

Oncogenesis or tumorigenesis, that is, the development of cancer, begins with the accumulation of disruptions in several normal cellular activities that can eventually transform normal cells into cancer cells. These disruptions upset the normal balance between cell proliferation and death, allowing cells to acquire certain capabilities essential to malignant growth and spread. These general hallmarks of cancer include self-sufficient growth, insensitivity to antigrowth signaling, evasion of apoptosis, limitless replication, tissue invasion/metastasis, and angiogenesis (Hanahan and Weinberg, 2000). The progression stage of oncogenesis occurs when cells acquire a combination of these

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abilities, which allows conversion of a normal cell into a cancer cell (Hahn et al., 1999). As the repertoire of capabilities continues to build, the next stage of oncogenesis is observed when cells acquire the ability to degrade the local basement membrane, allowing them to spread and invade the surrounding tissues. In the case of solid tumors, these now invasive cancer cells can acquire the ability to induce blood vessel growth (i.e., a blood supply) from preexisting vessels *via* angiogenesis (Folkman, 2002). This provides the cancer with nutrients and oxygen needed to further grow and spread. At this point, the cancer cells may concurrently acquire the ability to metastasize to distant sites (such as other organs) as tumor-mediated angiogenesis can provide primary tumor cells with a mode of transport to metastasize. This metastatic process includes the successful intravasation of cancer cells into blood/lymphatic vessels, transit, extravasation out of blood vessels, and finally, establishment of a secondary site of the tumor growth. These cancer cells can then either lie dormant or can aggressively propagate into a secondary tumor. Each of these successive phases of carcinogenesis increases the likelihood of cancer-related morbidity and mortality, the metastasis stage representing the largest contributor. In addition, promoting a permissive microenvironment is crucial to the progression of carcinogenesis, one major example being the modulation of immune responses (Hanahan and Weinberg, 2000). Similar to environmental and host-related oncogenic events, human tumor–associated viruses can lead to malignancies by providing viral mechanisms that promote one or more general hallmarks of cancer (Damania and Pipas, 2009). Furthermore, it is recognized that chronic inflammation and immunosuppression provide a microenvironment more conducive to the progression of oncogenesis (Goedert, 2001). This chapter specifically focuses on human DNA tumor viruses that are known to associate with various cancers and also highlights mechanisms of virus-induced oncogenesis.

Transformation and oncogenesis

Studies on DNA tumor viruses have been instrumental to our understanding of basic cell biology and how the perturbations of cellular pathways contribute to the initiation and maintenance of cancer. DNA tumor virus infection leads to immortalization of the infected cell through deregulation of multiple cellular pathways via expression of many potent oncoproteins (Abhik Saha et al., 2010) as shown in Fig. 7.1. Research on various viral oncoproteins has revealed many of their novel cellular targets that are directly associated with cellular signaling, cell-cycle control, and the host's defense system (Abhik Saha et al., 2010; Stevenson, 2004) (Table 7.1). Tumor viruses reprogram the host quiescent, G0 cell into the S phase of the cell cycle, allowing viral access to the nucleotide pools and cellular machinery that are required for viral replication and transmission. The host cellular innate immune responses respond to viral infection by activating tumor-suppressor proteins, pRB1 and p53, to induce cell death. However, the tumor viruses have evolved the means to inactivate these signaling pathways for their own benefits (Goedert, 2001; Bouvard et al., 2009). Importantly, p53, the "guardian of the genome," and its downstream effectors are inactivated in 50% of human cancers. Herpesvirus family members, EBV- and KSHV-encoded oncoproteins, have been shown to manipulate p53 and pRb functional activity to block apoptosis during tumor progression. The EBV-encoded proteins, EBNA3C and LMP1, modulate p53 function either by repressing its transcriptional activity or by blocking p53-mediated apoptosis (Abhik Saha et al., 2010). Several studies have demonstrated that EBNA3C recruits MDM2 E3-ubiquitin ligase activity for augmenting proteasome-dependent degradation of p53. Recently, EBNA3C has been shown to bind and stabilize Gemin3 expression, which is crucial for inhibiting p53-dependent transcriptional activity and apoptosis. EBNA3C has also been shown to induce pRb degradation, thus leading to an establishment of latent infection (Moore and Chang, 2010). Similarly, KSHV-encoded latency-associated nuclear antigen (LANA) and K8 proteins, block p53-mediated host cell death through their interaction with p53. The KSHV-encoded LANA can also directly interact with pRb and enhance E2F-dependent transactivation activity and contribute to KSHV-induced oncogenesis by targeting the pRb-E2F regulatory pathway (Abhik Saha et al., 2010; Verma et al., 2007). Likewise, other DNA tumor virus-encoded oncoproteins also target tumor-suppressor proteins; HPV-encoded E6 protein has been shown to bind and degrade p53 through the ubiquitin-proteasome pathway (Cai et al., 2010; Enrique et al., 2010). In addition, HPV E7 oncoprotein bypasses cell cycle arrest through binding to the hypophosphorylated form of pRb, thereby inducing the degradation of pRb through a proteasome-mediated pathway (Moore and Chang, 2010). Also, HBV-encoded HBx interacts with p53 to inhibit its functional activity, which leads to the development of human hepatocellular carcinoma (HCC). In addition, the HBV-encoded HBx oncoprotein destabilizes pRb by upregulating the E2F1 promoter activity (Abhik Saha et al., 2010; Damania, 2007). Recent studies show that KSHV-encoded LANA and HBV-encoded HBx can downregulate *von Hippel-Lindau*, a tumor-suppressor gene, along with p53 (Damania, 2007; Colin et al., 2006). The association

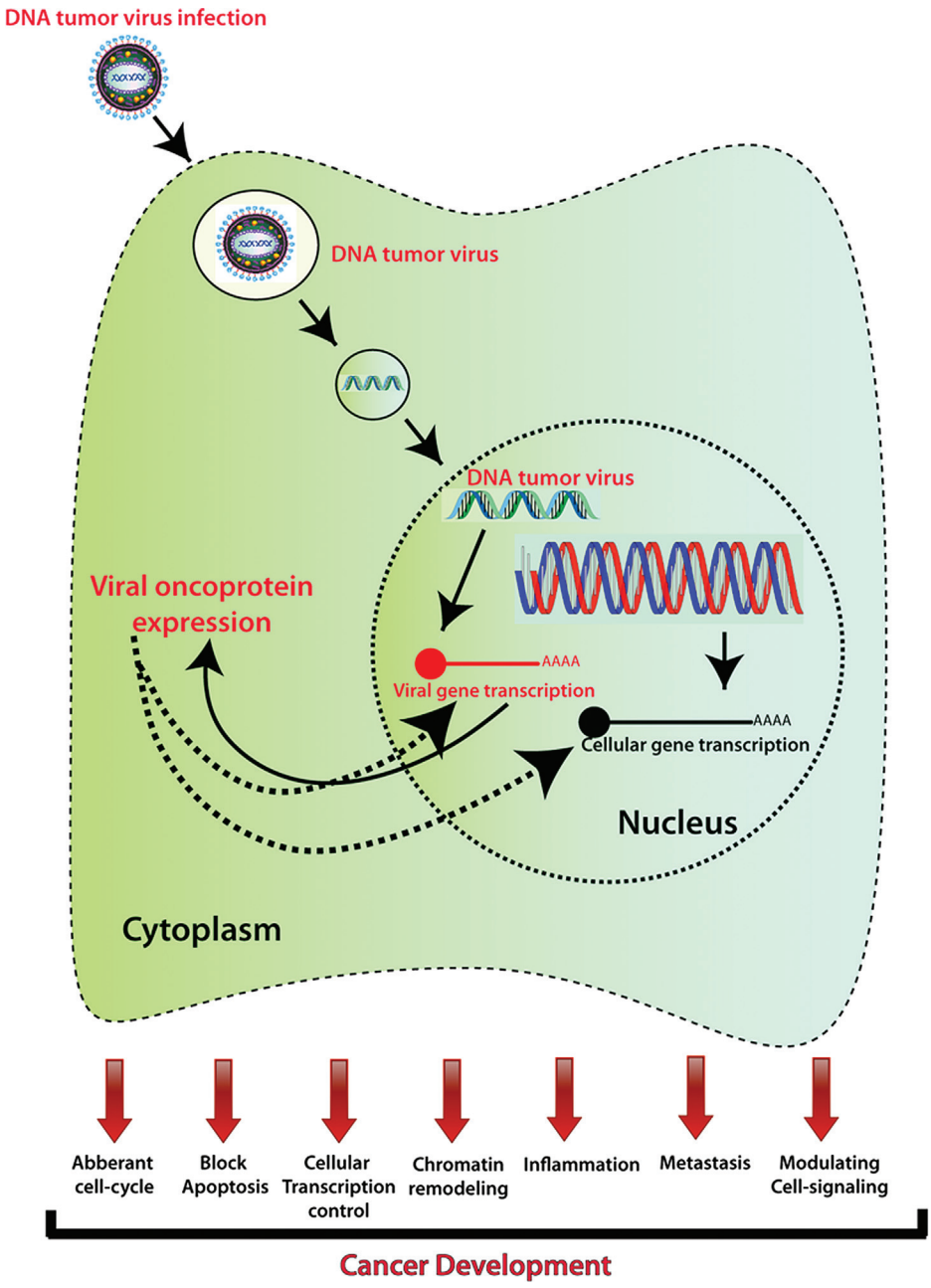

FIGURE 7.1 DNA tumor virus infection leads to immortalization of the infected cell through deregulation of multiple cellular pathways involved in cellular signaling, cell-cycle control, and defense system via expression of many potent oncoproteins.

TABLE 7.1 KEY VIRAL ONCOPROTEINS AND THEIR CELLULAR TARGETS.

Tumor virus	Viral oncoproteins	Important cellular binding partners	Deregulated signaling pathways
EBV	LMP1	p53, Mdm2, pRb, p300, Chk2, c-Myc, HDAC1, SUMO-1, SUMO-3, Cyclin A, E and D1, TRAFs, TRADD, JAK	Cellular transcription, cell cycle, metastasis, ub-proteasome, apoptosis, inflammation, chromatin remodeling, cellular signaling
KSHV	vGPCR, vIL-6, vBcl2, vCyclin, LANA and vFLIP	p53, pRb, c-Myc, core histones, apoptosis, TRAF2, Transcriptional activators- Sp1, AP-1, and transcriptional inhibitors HP1 and mSin3	Cellular transcription, cell cycle, apoptosis, ub-proteasome, chromatin remodeling, cellular signaling
HPV	E6	p53, p73, c-Myc, pRb, p21CIP1, p27KIP1,	Cell cycle, ub-proteasome
	E7	IRF-1, cyclin A and E	
MCPyV	LT	p53, pRb	Cell cycle
HBV	HBx	NFκB, p53, c-jun, c-fos, PKC, c-myc	Cell cycle, apoptosis, cellular transcription, Cellular signaling, metastasis.

between viral oncoproteins and cell-cycle regulatory factors, that is, cyclin/CDK complexes, also play a crucial role in viral transformation. The EBV-encoded latent antigen, EBV nuclear antigen 2 (EBNA2), transactivates cyclin D2 through the activation of c-Myc in EBV-associated lymphomas. Similarly, KSHV-encoded LANA stabilizes β-catenin, resulting in the increased expression of both β-catenin and cyclin D1 in KSHV tumors (Abhik Saha et al., 2010). Viral oncoproteins also deregulate various cellular signaling pathways that are directly linked to the development of oncogenesis, such as Notch signaling, MAPK, TLR, JAK/STAT, JNK, Wnt, interferon regulatory factors (IRFs), the ubiquitin-proteasome system, tumor necrosis factor (TNF), and nuclear factor (NF)-κB-signaling pathways, to evade host immune responses and facilitate their survival (Abhik Saha et al., 2010).

History of human DNA tumor viruses and cancer

The International Agency for Research on Cancer estimates that one-fifth of cancers worldwide are associated with viral infections (Ignatovich et al., 2002). Viruses have played a central role in the modern cancer research and have been providing profound insights into both infectious and noninfectious cancer cases. This diverse group of viruses revealed unexpected connections among innate immunity, immune sensors, and tumor-suppressor signaling that control both viral infection and cancer (Parkin, 2006). The first human tumor virus, EBV [also known as human herpesvirus-4 (HHV-4)], was identified by Anthony Epstein, Bert Achong, and Yvonne-Barr in 1964 in African pediatric patients with Burkitt's lymphoma. To date, seven viruses—EBV, KSHV [also known as human herpesvirus-8 (HHV-8)], high-risk HPV, HBV, HCV, HTLV-1, and MCPyV—have been classified as type-1 carcinogenic agents, linked to different types of human cancers (Table 7.2). Infectious cancer agents have been divided into two broad categories: direct-acting carcinogens, which are generally found in a monoclonal form within the tumor cells and express either viral or cellular oncogenes that directly contribute to cell transformation into cancer cell and indirect transform carcinogens, which are not conditioned to exist within the cell that forms the tumor. These agents presumably cause cancer by triggering chronic inflammation and oxidative stress or by producing immunosuppression that reduces or eliminates antitumor immune surveillance mechanisms, which in turn eventually leads to carcinogenic mutations in host cells (Moore and Chang, 2010). By definition, a direct viral carcinogen is present in each cancer cell and expresses at least one transcript to maintain the transformed tumor cell phenotype, as occurs with HPV, MCPyV, EBV, and KSHV-related cancers. Evidence supporting this comes from knockdown studies in which the loss of viral proteins results in the loss of host cancer viability. Even in these cases, external factors such as immunity and exposure to other infectious agents directly affect carcinogenesis. Indirect carcinogens could potentially also include "hit-and-run" viruses in which the viral genes are lost as the tumor begins to mature. Several agents such as HBV, HCV, and HTLV-I, which are involved in HCC, partly fit into this category (Moore and Chang, 2010). The oncogenic viruses are generally characterized by prolonged and often lifelong latency and evasion of the host immune surveillance as only a small subset of viral genes are normally expressed that may elicit an immune response

TABLE 7.2 HUMAN ONCOGENIC VIRUSES.

Virus	Genome	Notable cancers	Year identified
Epstein–Barr virus (EBV)	174 kb linear dsDNA	Burkitt's lymphoma, nasopharyngeal Carcinoma, Hodgkin's disease, infectious mononucleosis and X-linked lymphoproliferative disorders	1964
Kaposi's sarcoma herpesvirus (KSHV)	137 kb linear ds DNA	Kaposi's sarcoma, primary effusion lymphoma, and multicentric Castleman's disease	1994
Human papillomaviruses (HPV)	8 kb circular ds DNA	Most cervical cancer, penile cancers, anogenital, head and neck cancers	1983
Merkel cell polyomavirus (MCV)	5 kb circular ds DNA	Merkel cell carcinoma	2008
Hepatitis B virus (HBV)	3–3.3 kb of relaxed circular, partially duplex DNA	Hepatocellular carcinoma	1965

(Moore and Chang, 2010). Among DNA tumor viruses, latency is well studied on herpesvirus family members, EBV and KSHV. During latency, the virus is tumorigenic and persists as multiple copy, extrachromosomal circular episomes in a highly ordered chromatin structure whose propagation is dependent on their ability to hijack the replicative machinery of the host (Moore and Chang, 2010). The most likely explanation for the connection between virus latency and tumorigenesis is that productively replicating viruses initiate cell death, which has long been known to virologists as the cytopathic effect (Cai et al., 2010; Enrique et al., 2010; Ann Arvin et al., 2007). Small DNA tumor viruses, that is, HPV and MCPyV do not encode their own replication proteins, while large DNA tumor viruses, such as EBV, KSHV, and HBV, do encode their own viral DNA polymerase, but still require components of cellular replicative machinery for efficient viral DNA synthesis. A contrasting feature between small and large DNA tumor viruses is that small DNA tumor viruses can integrate into the host chromosomal DNA during or before cancer progression (Moore and Chang, 2010).

Epstein–Barr virus

EBV/HHV-4 is a double-stranded (ds) DNA virus that belongs to the genus *Lymphocryptovirus* of the human γ-herpesvirus family and is found in approximately 95% of the adult population worldwide. EBV is the primary cause of infectious mononucleosis (IM) and is associated with epithelial cell malignancies such as nasopharyngeal carcinoma and gastric carcinoma, as well as lymphoid malignancies including Hodgkin's disease, Burkitt's lymphoma, non-Hodgkin lymphoma, and posttransplant lymphoproliferative disorder (Moore and Chang, 2010; Damania, 2007). Immunocompromised patients, including AIDS or postorgan transplant patients, have a high probability of getting EBV-associated lymphomas. The virus only infects cells expressing the receptor for complement C3d component (CR2 or CD21); these cells include epithelial cells (mainly in the upper digestive tract) and B-lymphocytes. EBV has a powerful transforming potential for B-lymphocytes and establishes a lifelong latent infection in the B-cell of the infected host. The EBV genome is a linear, 175 kb dsDNA genome, which is maintained in the nucleus as an episome via tethering to the host chromosome (Ann Arvin et al., 2007). EBV encodes several viral proteins that have a transforming potential. EBV latency proteins (LMP1, EBNA2, EBNA3A, and EBNA3C) have been shown to express in AIDS-associated lymphoma, posttransplant lymphoma patients and lymphoblastoid cell lines (LCLs) generated from EBV infection of primary B-cells (Ann Arvin et al., 2007). LMP1 and EBNA2 are essential for the ability of EBV to immortalize B-cells because deletion of LMP1 from EBV renders the virus nontransforming (Abhik Saha et al., 2010). EBV-encoded latent protein EBNA-LP functions like a co-stimulator of EBNA2-mediated transactivation of many cellular and viral genes shown to be critical for B-cell immortalization (Damania, 2007). EBV nuclear antigen 1 (EBNA1) is essential for the maintenance and segregation of the EBV genome. EBNA3A and 3C are also critical for B-cell immortalization, while EBNA3B enhances the survival of cells. All three EBNA3 proteins are shown to bind with RBP-Jκ/CBF1 and regulate cellular gene transcription important for transforming B-cells into immortalized LCLs (Abhik Saha et al., 2010).

Kaposi's sarcoma–associated herpesvirus

KSHV/HHV-8 is a member of the γ-herpesvirus family (genus *Rhadinovirus*), which is tightly associated with human cancers, including Kaposi's sarcoma (KS), primary effusion lymphoma (PEL), and multicentric Castleman's disease (MCD) (Abhik Saha et al., 2010). More recently, KSHV-inflammatory cytokine syndrome (KICS) has been identified as a new inflammatory disorder associated with KSHV infection (Ann Arvin et al., 2007). KSHV remains asymptomatic in healthy individuals and establishes a lifelong persistence in B-lymphocytes after primary infection, similar to EBV (Uldrick et al., 2010). KS, originally called "idiopathic multiple pigmented sarcoma of the skin," was first described by Moritz Kaposi, a Hungarian dermatologist (Blake, 2010). KS was initially thought to be an uncommon tumor of Mediterranean populations until it was identified throughout sub-Saharan Africa and later became more widely known as one of the AIDS-defining illnesses in the 1980s (Ann Arvin et al., 2007). The viral cause for this cancer was found by Yuan Chang and Patrick Moore in 1994 by isolation of DNA fragments of a herpesvirus from a KS (Enrique et al., 2010) tumor in an AIDS patient (Hahn et al., 1999). Similar to EBV, KSHV has a linear dsDNA genome of approximately 165–170 kb that encodes for nearly 86 viral open reading frames (ORFs); however, only a small subset of these genes is expressed during latency, which includes LANA, vCyclin, vFLIP/K13, K12/Kaposin, and an micro RNA (miRNA) cluster (Chang et al., 1994). LANA, encoded by ORF73, is a multifunctional nuclear antigen and functional homolog to the EBV EBNA1 protein that plays a central role in deregulating various cellular functions, including maintenance of the viral episome

(Cai et al., 2010; Enrique et al., 2010), degradation of the p53 and pRb tumor suppressors, transactivation of the telomerase reverse transcriptase promoter, promotion of chromosome instability in KSHV-infected B-cells (Verma et al., 2007), and accumulation of the intracellular domain of Notch in KSHV-mediated tumorigenesis (Verma et al., 2007). LANA also plays a crucial role in maintaining latency by regulating the expression of RTA, another critical viral-encoded transcriptional activator required to switch from the latent to the lytic cycle (Abhik Saha et al., 2010; Moore and Chang, 2010). During latency, LANA tethers the viral episomal DNA to the host chromosomes, which helps in the efficient partitioning of the viral DNA in the daughter cells after cell division. Disruptions of LANA expression led to reduction in episomal copies, suggesting the importance of LANA in KSHV-mediated pathogenesis (Verma et al., 2007). Several KSHV genes have oncogenic potential, for instance, modulation of various cellular signaling pathways by K1 and K15, regulation of cell cycle by vCyclin and vIRF3, inhibition of cell death by K1, vFLIP, and vBcl-2, and immune modulation by vIRF3, K3, and K5 (Abhik Saha et al., 2010).

Kaposi's sarcoma

KS is a multifocal vascular tumor of mixed cellular composition that develops from the cells that line lymph or blood vessels and is most often seen as a cutaneous lesion (Hahn et al., 1999). The abnormal cells of KS form purple, red, or brown blotches or tumors on the skin called lesions (Hahn et al., 1999). KSHV is always found in the spindle cells of the lesion, which are thought to be of endothelial origin. In some cases, the disease causes painful swelling, especially in the legs, groin area, or skin around the eyes. KS can cause serious problems or even become life threatening when the lesions are in the lungs, liver, or digestive tract (Ann Arvin et al., 2007). KS in the digestive tract, for example, can cause bleeding, while tumors in the lungs can cause difficulty in breathing. There are four distinct clinical variants of KS, defined on the extent of immunosuppression and the severity of infection:

1. Classic KS is seen in HIV-negative elderly male patients of Mediterranean, Eastern European, and Middle Eastern heritage. Classic KS is more common in men than in women. Patients typically have one or more lesions on the legs, ankles, or the soles of the feet.
2. Endemic KS is a second type of KS, which is seen in Africa and affects HIV-positive and HIV-negative individuals and even children (Damania and Pipas, 2009). Rarely a more aggressive form of endemic KS is seen in children before puberty; it usually affects the lymph nodes and other organs and can lead to death within a year. There could be other factors in Africa (such as environmental cofactor and genetic predispositions in the population) that could contribute to the development of aggressive KS since the disease affects a broader group of people that includes children and women.
3. The third type of KS lesion is an iatrogenic form of KS that develops in posttransplant patients undergoing immunosuppressive therapy to prevent graft rejection after the organ transplantation (Enrique et al., 2010). Greater than 95% of all KS lesions, regardless of type, have been shown to contain KSHV viral DNA, thereby indicating a strong epidemiological link between KSHV infection and KS (Enrique et al., 2010; Damania, 2007).
4. AIDS-associated epidemic KS is a highly aggressive tumor and is primarily detected in HIV-infected individuals whose immune systems are severely damaged. In these individuals, the KS lesion is not restricted to the skin and often disseminates to the liver, spleen, gastrointestinal tract, and lungs. KS is the most frequently detected tumor in AIDS patients.

Primary effusion lymphoma

PEL (also called body cavity-based lymphoma) is a rare, rapidly fatal B-cell lymphoma commonly found in HIV-infected patients and is considered an AIDS-defining illness (Enrique et al., 2010; Damania, 2007). PEL is generally present as a pleural or pericardial effusion without a detectable tumor mass (Enrique et al., 2010; Yi-Bin Chen and Hochberg 2007). PEL cells are morphologically variable, with a null lymphocyte immunophenotype with evidence of HHV-8 infection. In PEL cells, KSHV presents as a single positive, indicating a strong epidemiological link between the presence of KSHV and the induction of PEL; however, 90% of these lymphoma cells often contain EBV as well. PELs are observed in both HIV-positive and HIV-negative individuals, with both types of PELs invariably containing KSHV viral DNA (Yi-Bin Chen and Hochberg, 2007).

Multicentric Castleman's disease

Castleman's disease, also called angiofollicular or giant lymph node hyperplasia, is a clinically heterogeneous entity that can be either localized (unicentric) or multicentric. MCD is an atypical lymphoproliferative disorder of a plasma cell type and is related to immune dysfunction (Damania, 2007; Yi-Bin Chen and Hochberg, 2007). There are two types of MCD, a hyaline vascular form, which presents as a solid mass, and a plasma-cell variant, which is associated with lymphadenopathy. Sometimes a mixture of both hyaline

vascular and plasma cell variants can also be found (Cai et al., 2010; Enrique et al., 2010). KSHV-MCD is characterized by intermittent flares of inflammatory symptoms, including fever, fatigue, cachexia, and edema, together with lymphadenopathy and/or splenomegaly. Nearly 100% of AIDS-associated MCD is positive for KSHV, whereas less than 50% of non−AIDS-associated MCD contains KSHV viral DNA (Damania, 2007). Patients with AIDS-associated MCD often develop malignancies like KS and non-Hodgkin's lymphoma (Damania, 2007).

KSHV inflammatory cytokine syndrome

KICS is a newly described clinical inflammatory condition that is characterized by systemic illness, poor prognosis, high KSHV viral loads, and elevated levels of interleukin 6 (IL-6) and interleukin 10 (IL-10) (Cai et al., 2010; Damania, 2007), comparable to those seen in KSHV-MCD. These features of KICS are comparable to the KSHV-MCD without the characteristic lymphadenopathy (Box 7.1).

Human papillomavirus

The main risk factor for the development of cervical cancer, the second leading cause of cancer death in women worldwide, is the persistent HPV infection (Uldrick et al., 2010). The first century of tumor virology research culminated with the Medicine Nobel prize granted to Harald zur Hausen for demonstrating an association between high-risk HPV infection and the development of cervical cancer (Damania and Pipas, 2009). The HPVs are small, nonenveloped, icosahedral DNA viruses, with a diameter of 52−55 nm. The virus particle contains a single dsDNA genome of approximately 8000 bps. The majority of the known types of HPV are asymptomatic, but some types may cause warts, while others can lead to cancers of the cervix, vulva, vagina, and anus (in women) or cancers of the anus and penis (in men) (Damania, 2007). Nearly, 50%−80% of sexually active population becomes HPV infected at some point in their lives. Most HPV infections in young females are temporary and have little long-term significance. Seventy percent of infections are gone in 1 year and 90% in 2 years. However, when the infection persists, in 5%−10% of infected women, there is high risk of developing precancerous lesions of the cervix, which can progress to invasive cervical cancer (Damania, 2007; Narisawa-Saito and Kiyono, 2007). Cervical screening using a Papanicolaou (Pap) test or liquid-based cytology is used to detect abnormal cells that may develop into cancer (Narisawa-Saito and Kiyono, 2007). Approximately 200 different HPVs have now been characterized and subsequently classified into low- or high-risk groups according to their potential for causing cervical cancer (Damania and Pipas, 2009). More than a dozen HPV types (including types 16, 18, 31, 33, 35, 45, 51, 52, 56, 58, 59, 62, 66, and 68) have been classified as "high-risk" types because they can lead to cervical cancer as well as anal, vulvar, vaginal, and penile cancers (Moore and Chang, 2010; Narisawa-Saito and Kiyono, 2007). HPV subtypes 16 and 18 are the most frequently detected HPV in tumors. Several types of HPV (type 16 in particular) have been found to be associated with HPV-positive oropharyngeal cancer (OSCC), a form of head and neck cancer (Abhik Saha et al., 2010; Moore and Chang, 2010; Damania, 2007; Narisawa-Saito and Kiyono, 2007). HPV-induced cancers often have viral sequences integrated into the cellular DNA (Damania, 2007; Narisawa-Saito and Kiyono, 2007). Low-risk viruses are associated with benign lesions such as condyloma accuminata or with basal cell and squamous-cell carcinomas of the skin (Damania, 2007). Another very rare inherited disease associated with HPV is epidermodysplasia verruciformis (EV), which is caused by an autosomal recessive mutation that leads to abnormal, uncontrolled papilloma virus replication (Damania, 2007). This results in the growth of scaly macules and papules on many parts of the body, but especially on the hands and feet. EV, which is associated with a high risk of skin carcinoma, is typically associated with HPV types 5 and 8

BOX 7.1

1. The Nobel Prize in Medicine (2008) was awarded to Dr. Harald zur Hausen for his discovery of HPV's association with cervical cancer, the second most common cancer among women. His discovery led to the development of two vaccines against cervical cancer. He shared this award with two other French virologists, Françoise Barré-Sinoussi, and Luc A. Montagnier, for discovering HIV.
2. The Nobel Prize in Medicine (2018) was awarded to Dr. James P. Allison and Dr. Tasuku Honjo for their discovery of cancer treatment by inhibition of negative immune recognition.

(other types may also be involved) (Damania, 2007; Narisawa-Saito and Kiyono, 2007).

Primary HPV infection occurs in the basal stem cells of the epithelium. The virus cannot bind to live tissue; instead, it infects epithelial tissues through microabrasions or other epithelial trauma as would occur during sexual intercourse or after minor skin abrasions that exposes segments of the basal membrane (Damania and Pipas, 2009). The virus then traverses upward and replicates in the terminally differentiated keratinocytes and is shed from the stratum corneum (Damania and Pipas, 2009; Narisawa-Saito and Kiyono, 2007). *HPV* genes encode proteins responsible for replication, cellular transformation, control of viral transcription, and those necessary for the generation of viral progeny (Damania, 2007). The main oncogenic proteins, E6 and E7 of high-risk HPV strains, are necessary for the maintenance of the malignant phenotype and have strong transforming abilities. HPV oncoproteins E6 and E7 have been shown to act synergistically to immortalize cells in vitro and induce skin tumors in transgenic animals (Abhik Saha et al., 2010; Damania, 2007). The HPV viral proteins target tumor suppressors, for instance, E6 binds to p53 and E7 binds to the Rb family of proteins, which induces their degradation through ubiquitin-proteasome-mediated degradation and leads to deregulation of the cell cycle and the inhibition of apoptosis (Damania, 2007).

Hepatitis B virus

HBV, a small-enveloped DNA virus, belongs to the hepadnaviruses family, which infects hepatic cells of humans causing inflammatory diseases of the liver (Abhik Saha et al., 2010; Cai et al., 2010; Narisawa-Saito and Kiyono, 2007). It is estimated that about 360 million are infected with the virus, and many of them are chronic carriers (Damania and Pipas, 2009; Martin and Gutkind, 2008) without any identifiable risk factor (Parkin, 2006; Kao, 2011). Most individuals with chronic hepatitis B remain asymptomatic for many years or decades. Patients with HIV infection or postorgan transplant patients taking immunosuppressive drugs are at higher risk of developing chronic infection (Colin et al., 2006). However, patients with chronic hepatitis B are at a risk of developing HCC, the fifth most common cancer and the third leading cause of cancer death worldwide (Damania, 2007; Colin et al., 2006). Infections with HBV and HCV are considered as the major contributors to HCC development, accounting for over 80% of all HCC globally (Damania, 2007; Colin et al., 2006; Kao, 2011).

Dr. Baruch Blumberg's laboratory discovered the viral cause of HBV in 1965. The genome of HBV is an enveloped virus with approximately 3.3 kb full-length negative-sense strand and a 2.8 kb short-length sense strand (Abhik Saha et al., 2010; Moore and Chang, 2010). One end of the full-length strand is covalently linked to the viral DNA polymerase, and the other short strand has an RNA oligonucleotide at its 5′ end. Thus, neither DNA strand is closed, and the circularity is maintained by cohesive ends (Moore and Chang, 2010; Damania, 2007). Viral DNA enters the nucleus and integrates into the host DNA immediately after infection. HBV replication initiates increased DNA synthesis and interferes with normal cellular detoxification and repair functions, causing chronic liver cell injury that leads to HCC (Damania, 2007). There are three different types of hepatitis B antigens encoded by the HBV genome. These include hepatitis B surface antigens (HBsAg; MHBsAg and LHBsAg), hepatitis B core antigen, and hepatitis B early antigen (HBeAg). HBsAg is most frequently used for diagnosis and is the first detectable viral antigen to appear during infection. There is a strong correlation between HBsAg chronic carriers and the incidence of HCC (Colin et al., 2006; Kao, 2011). It has been shown that HBsAg carriers have a risk of HCC, that is, 217 times than that of a noncarrier, and 51% of the deaths of HBsAg carriers are caused by liver cirrhosis or HCC compared to 2% of the general population (Colin et al., 2006; Kao, 2011). The virus is classified into four major serotypes (adr, adw, ayr, and ayw) based on antigenic epitopes presented on its envelope proteins and into eight genotypes (Kao, 2011) according to overall genomic variation (Vaughn and Elenitoba-Johnson, 2001). The genotypes show a distinct geographical distribution and disease severity and are used in tracing the evolution and transmission of the virus (Kao, 2011). HBV is transmitted by exposure to infectious blood or body fluids through sexual contact, blood transfusions, reuse of contaminated needles and syringes, and vertical transmission from mother to child during childbirth (Kao, 2011). Without intervention, a mother who is positive for HBsAg confers a 20% risk of passing the infection to her offspring at the time of birth (Damania, 2007; Colin et al., 2006; Kao, 2011). This risk is as high as 90% if the mother is also positive for HBeAg (Colin et al., 2006). However, holding hands, sharing utensils or glasses, kissing, hugging, coughing, sneezing, or breastfeeding cannot spread HBVs (Colin et al., 2006).

Approximately 80% of HBV-related HCC shows integrated HBV sequences; however, HBV can also be found integrated in non-HCC tissue (Colin et al., 2006). Integration of HBV leads to severe mutagenic consequences, such as large inverted duplications, deletions, amplifications, and translocations, that result in chromosomal instability (Abhik Saha et al., 2010;

Damania, 2007; Martin and Gutkind, 2008). HBV-encoded oncogene *HBx* is a viral replication protein that participates in transcription and DNA repair, through which it regulates cell cycle, apoptosis, and genomic instability. In addition, HBx transgenic mice develop liver carcinomas. HBx is most commonly found to be integrated in patients with HBV-related cirrhosis and dysplasia (Abhik Saha et al., 2010). HBx has been shown to interact with epidermal growth factor receptor, c-myc, c-jun, c-fos, p53, AP-1, NF-κB, and SP1 in multiple ways to contribute to the molecular pathogenesis of human HCC (Abhik Saha et al., 2010; Moore and Chang, 2010).

Human polyomaviruses

Polyomaviruses are nonenveloped, circular, dsDNA viruses of approximately 5000 bp. Ludwik Gross discovered the first murine polyomavirus in 1953 (Abhik Saha et al., 2010; Moore and Chang, 2010; Martin and Gutkind, 2008). Subsequently, many polyomaviruses have been found to infect birds and mammals. Polyomaviruses were so named because they are potentially oncogenic and cause a wide range of tumors in a number of animal species. Polyomaviruses are icosahedral in shape, with a small circular DNA genome (Damania and Pipas, 2009). For several years after the discovery of leukoencephalopathy [JC virus (JCV)] and nephropathy [BK virus (BKV)] in 1971, it was considered that only these two viruses infected humans, but next-generation sequencing techniques have enabled the identification of at least nine other members in humans, including MCPyV. By using digital transcriptome subtraction and high-throughput genome sequencing techniques, Chang and Moore along with their coworkers identified the presence of MCPyV in Merkel cell carcinoma (MCC) in 2008 (Damania, 2007). Polyomavirus infections via respiratory tract are highly common in childhood and young adult infections. Most of these infections are asymptomatic although the virus persists lifelong in almost all adults (Huichen Feng et al., 2008). Clinical evidence of disease caused by human polyomavirus infection is most common among persons who become immunosuppressed by AIDS, who are of old age or persons taking immunosuppressive drugs after organ transplantation. BKV was identified from the urine of a kidney transplant patient, and JCV was identified from the brain of a Hodgkin's lymphoma patient who developed progressive multifocal leukoencephalopathy (PML). The human JCV and BKV have been linked to several different human cancers. However, whether the role of the virus is causal or incidental has been the subject of much debate (Silva RLd, 2011). JCV DNA has been identified from brain tumors found in patients with or without PML and with glial tumors and pediatric medulloblastomas (Damania, 2007; Silva RLd, 2011). JCV has also been shown to be associated with colon cancer and central nervous system (CNS) lymphoma (Damania, 2007). Similarly, BKV is associated with polyomavirus nephropathy (PVN), a form of acute interstitial nephritis (Damania, 2007), and BKV DNA has also been found in pancreatic islet tumors and brain tumors (Silva RLd, 2011). This indicates that BKV is newly emerged as an opportunistic CNS infectious agent in AIDS and transplant patients, particularly those with a coexistent urologic disease and neurological decline (Damania, 2007).

Merkel cell polyomavirus

In 2008, MCPyV was discovered as the causative agent of MCC, a rare but aggressive skin malignancy, also termed as trabecular carcinoma of the skin (Silva RLd, 2011). Although MCPyV is similar to classical oncogenic polyomaviruses, subtle differences are beginning to emerge. It is found clonally integrated into the majority of MCC tumors. It develops in hair follicles or on (or beneath) the skin (Shailender Bhatia and Nghiem, 2011). MCC occurs most often on the sun-exposed face, head, and neck. MCC can also be found on the trunk and genitals but at a reduced frequency. Epidemiological studies indicate that older, light-skinned people or individuals taking immunosuppressive drugs after organ transplantation are at higher risk to develop MCC (Shailender Bhatia and Nghiem, 2011). MCPyV appears to be widely prevalent among healthy individuals. It has been shown that the prevalence of MCPyV seropositivity was 0% in infants, 43% among children aged 2–5 years, and increased to 80% among adults older than 50 years (Shailender Bhatia and Nghiem, 2011). In addition, MCPyV DNA was detected in cutaneous swabs from clinically healthy individuals with a prevalence of 40%–100%. Although widely prevalent, active MCPyV infection appears to be asymptomatic in healthy individuals, with the exception of MCC. This virus has not been yet convincingly linked with any other human disease (Shailender Bhatia and Nghiem, 2011). The exact mode of transmission remains to be elucidated and could involve cutaneous, fecal-oral, mucosal, or respiratory routes. MCPyV viral DNA has been detected in lower frequencies among respiratory secretions, on oral and anogenital mucosa, and in the digestive tract. Importantly, it appears that the virus is being shed chronically from clinically normal skin in the form of assembled virions (Moore and Chang, 2010; Shailender Bhatia and Nghiem, 2011). The MCPyV large T-antigen transcript has numerous functions in MCPyV infection, including initiation of viral replication and manipulation

of the host cell cycle. It appears to retain the major conserved features of other polyomavirus LT-antigens, such as Rb-binding motif and helicase/ATPase domains (Shailender Bhatia and Nghiem, 2011). MCPyV is the first polyomavirus that has been shown to integrate into human genomic DNA (Moore and Chang, 2010; Shailender Bhatia and Nghiem, 2011). The virus then undergoes at least two mutations (Shailender Bhatia and Nghiem, 2011), the first being a nonhomologous recombination with the host chromosome and then a sequential large T (4)-antigen truncation mutation that eliminates its viral replication functions but spares its Rb-targeting domain. These sequential mutational events result in persistent T-Ag expression, which play a key role in turning asymptomatic viral infection into an aggressive MCC (Moore and Chang, 2010).

Principle

DNA tumor viruses encode proteins with oncogenic potential, which alter the normal growth of cells by deregulating various cellular pathways. Here, we discuss the roles of viral proteins and RNA in inducing tumorigenesis by taking the example of EBV. A diagrammatic representation of the EBV life cycle is depicted in the flowchart **(**Fig. 7.2**)**: (1) The first step in EBV primary infection is the viral entry through the buccal cavity. (2) EBV shows higher affinity to B-cells in primary infection of naïve B-cells that occurs in the oropharyngeal mucosa. (3) Upon entry into the target cells, EBV enters into a short burst of lytic proliferation followed by a well-defined latency program. (4) EBV persists in the B-cells in the peripheral circulation and this latent infection of B-cells leads to various types of lymphomas. (5) Latently infected B-cells mature into plasma cells. (6) Plasma cells undergo lytic reactivation releasing infectious EBV particles. (7) EBV produced thus could reinfect fresh naïve B-cells or epithelial cells to cause nasopharyngeal carcinoma.

Epstein–Barr virus genome structure

A mature EBV viral particle is approximately 120–180 nm in diameter. It is composed of a linear dsDNA genome enclosed by a protein capsid surrounded by a protein tegument, which in turn is surrounded by a lipid envelope. The EBV genome is approximately 184 kb and contains 0.5 kb terminal repeats and internal repeat sequences (Shailender Bhatia and Nghiem, 2011). EBV strain B95-5 (derived from IM) was the first herpesvirus to have its genome completely cloned and sequenced. The virus encodes for approximately 80 proteins; however, not all of them have been fully characterized. The EBV genome was sequenced from a viral DNA BamHI-fragment cloned library; hence, ORFs, genes, and sites for transcription or RNA processing are frequently referenced to specific BamHI fragments, from A to Z, in a descending order of fragment size.

Entry into the cell

B-cells are the main target of EBV infection, although EBV can also infect epithelial cells, mainly in the upper digestive tract, which is thought to occur during viral reactivation process. During primary infection, EBV infects B-lymphocytes through interaction of the glycoprotein gp350/220 with the complement receptor CD21. Following primary infection, EBV persists in the infected host as an episomal DNA and causes lifelong asymptomatic infection (Ann Arvin et al., 2007). To achieve long-term persistence in vivo, EBV colonizes the memory B-cell pool where it establishes latent infection, which is characterized by the expression of a limited subset of virus latent genes (Ann Arvin et al., 2007). These genes affect the normal B-cell growth, leading to the immortalization of the cells. A low level of reactivation during the lytic cycle allows viral shedding into the saliva and transmission of the virus in vivo. In vitro, EBV can transform peripheral human B-lymphocytes into indefinitely proliferating LCLs that allows for genetic manipulation of the virus (Ann Arvin et al., 2007). The mechanisms of EBV entrance into epithelial cells are different from those of B-lymphocytes. To enter epithelial cells, viral protein BMRF-2 interacts with cellular β1 integrins, which in turn triggers fusion of the viral envelope with the epithelial cell membrane, thus allowing EBV to enter the cell. There are several well-described forms of EBV latency, each of which is utilized by the virus at different stages of its life cycle and are also reflected in various EBV-associated malignancies (Blake, 2010).

Epstein–Barr virus lytic replication

It has been shown that in newly infected cells before the establishment of latency, EBV undergoes a short burst of lytic DNA replication. EBV expresses a small set of viral genes that were previously classified as immediate-early or early genes of the lytic cycle. During this prelatent stage of infection, the immediate expression of these genes activates resting B-cells and protects them from immediate activation of apoptosis (Damania, 2007). The cascade of events in the lytic phase of the EBV life cycle is divided into three phases

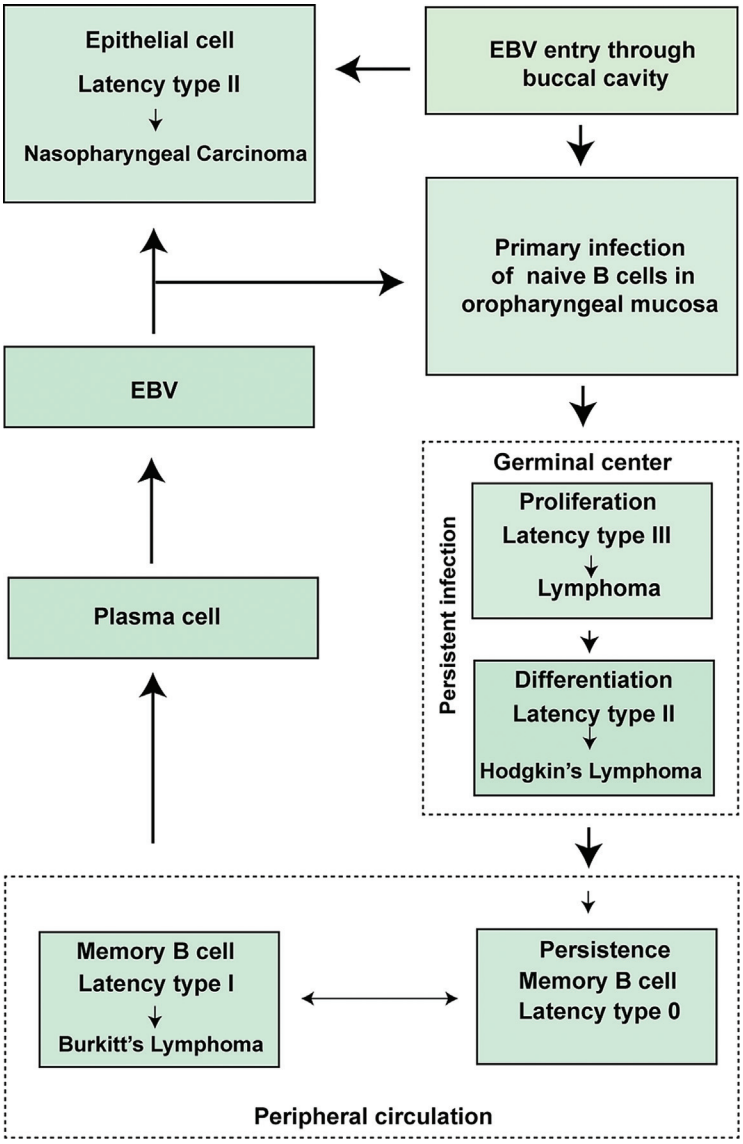

FIGURE 7.2 Flowchart of various stages of EBV life cycle.

of regulated gene expression: immediate-early, early, and late. The immediate-early gene products are transactivator proteins that trigger the expression of the early genes, the products of which include enzymes that are required for viral DNA replication (Blake, 2010). In turn, amplification of EBV DNA defines the boundary between early and late gene expression. During the late phase, viral structural proteins are expressed and assembled into virus particles into which the DNA is packaged before release of infectious virions. The principal switch from latency to productive infection involves activation of the immediate-early genes *BZLF1 (Zta)* and *BRLF1 (Rta)*. These two proteins can be expressed from a major 2.9 kb and a minor 3.8 kb bicistronic R-Z RNA transcribed from the R-promoter (Rp) (Blake, 2010). BZLF1, a viral transactivator protein, is involved in triggering the expression of the lytic genes and downregulation of latent genes, culminating in cell death and the release of infectious virions. These proteins upregulate the expression of other immediate early genes as well as their own expression. Both Zp and Rp are activated by ZEBRA, whereas Rta can upregulate Zp and autoactivate its own synthesis. However, synergistic effects of ZEBRA and Rta induce the maximum activation of the upstream Rp promoter. This synergism suggests that low levels of the two proteins are sufficient to trigger the lytic cascade. These immediate-early gene expressions in turn upregulate the expression of early genes such as viral DNA polymerase (BALF5) and thymidine kinase. The major proteins of the lytic phase are the EBV DNA polymerase, BALF5, and the late lytic cascade major capsid protein, BcLF1 (Ann Arvin et al., 2007). The lytic DNA replication of virion DNA start

from the lytic origin of replication (ori-lyt), which is distinct from the plasmid DNA replication origin (ori-P) that is used to maintain the episomal virus during latency. EBV ori-lyt lies within the BamHI H region of EBV DNA and contains two essential cis-acting regions, the BHLF1 promoter, and a 0.5 kb distant region required for replication (Ann Arvin et al., 2007). The ZEBRA response elements (ZREs) present within the BHLF1 promoter are essential for ori-lyt-directed replication. The six-core replication protein together with ZEBRA is absolutely required for the amplification and replication of the viral genome from ori-lyt. ZEBRA is a member of the basic zipper family of transcription factors and binds as a homodimer to ZREs within early gene promoters (Ann Arvin et al., 2007). Moreover, ZEBRA is an essential component of lytic DNA replication, and its association with the EBV helicase targets ZEBRA to the viral DNA replication compartments within the nucleus. Generally, herpesviruses replicate their DNA in G1-phase of the host cell cycle, which has been suggested to be advantageous to the virus due to the lack of competition with cellular DNA replication. As the key regulatory element, ZEBRA plays a critical role in the EBV lytic cycle by interacting with C/EBPα, which leads to an accumulation of p21CIP-1 and G1 cell-cycle arrest. Further studies suggest that apart from inhibiting cellular DNA synthesis, EBV induces an S-phase-like cellular environment during lytic replication. Latent EBV in B-cells spontaneously reactivates to switch to lytic replication, but the precise trigger for the induction is unknown. Many changes in physiological conditions or other nonrelated infection has been attributed to the triggering of spontaneous reactivation (Ann Arvin et al., 2007). In vitro, latent EBV in B-cells can be reactivated by stimulating the B-cell receptor (BCR) or by treating the cells with sodium butyrate or phorbol esters. EBV lytic replication does not inevitably lead to the lysis of the host cell because the virions are produced by budding from the infected cell.

Epstein–Barr virus latency

Latently infected B-cells maintain EBV genomes tethered to the host chromosome as 174-kb circular plasmids referred to as episomes and express only a limited number of viral latent gene products (such as EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C, and EBNA-LP) and three latency-associated membrane proteins (LMP1, LMP2A, and LMP2B) (Ann Arvin et al., 2007). Transcripts referred to as BARTs (BamHI A rightward transcripts) from the BamHI A region (Bam A) of the EBV genome, as well as small nonpolyadenylated RNAs, EBV-encoded RNAs (EBERs) 1 and 2, are abundantly expressed during latency (Blake, 2010). Four patterns of EBV latency programs are recognized at present. In type I latency, represented mainly in Burkitt lymphoma cells, viral gene expression is restricted to the two EBER genes, the BART transcripts, and EBNA1. In latency II, additional expression of three latent-membrane proteins (LMP-1, LMP-2A, and LMP-2B) is observed and is most frequently seen in Hodgkin's lymphoma. Latency III is seen in lymphoproliferative diseases (LPDs) developed in immunocompromised individuals and EBV-transformed LCLs. In this group, all six EBNAs, all three LMPs, and the two EBERs are expressed. Type IV latency is less strictly defined and pertains to IM patients and to posttransplant LPD. Some individuals also present putative latency program (latency 0), which shows no detectable level of latent gene. The principal mediators of EBV-induced growth and cellular transformation of B-lymphocytes in vitro include EBNA2, EBNA3A, 3 C, and LMP1 proteins (Blake, 2010). The *EBNA* genes are important for the transformation of primary B-lymphocytes, leading to transactivation and regulation of other cellular and viral genes. These proteins are involved in augmentation of the expression of genes coding for CD21, CD23, LMP1, and LMP2 proteins in B-lymphocytes. Growing evidence shows that EBV primarily persists in B-lymphocytes. Studies of EBV strains in donor–recipient pairs before and after bone marrow transplantation have shown that the recipient's strain disappears from the oropharynx and is replaced by the donor's strain, thus indicating that the bone marrow B-cells harbor EBV. Furthermore, patients with X-linked agammaglobulinemia, who are deficient in mature B-cells, are found to be free of EBV infection, suggesting that they are not able to maintain a persistent infection. Although much of the evidence described earlier implicates B-cells as the site of persistence, a role for infection of squamous epithelial cells is also suggested by the detection of EBV in oral hairy leukoplakia (Ann Arvin et al., 2007; Blake, 2010).

Epstein–Barr virus latent genes

An understanding of EBV latent gene function is significant both to the factors contributing to the establishment of persistent infection and to the role of the EBV oncogenesis. Recent research on EBV unraveled the essential roles of EBNA2 and LMP1 in an in vitro transformation of B-cells and highlighted roles for EBNA-LP, EBNA3A, EBNA3C, and LMP2A in this process. These studies confirm that EBV-induced B cell transformation requires the cooperative effect of several latent genes. A brief description of EBV latent gene function involved

in virus persistence and cellular transformation is as follows (Ann Arvin et al., 2007).

Epstein–Barr virus nuclear antigen 1

EBV-encoded multifunctional latent protein EBNA1 is a DNA-binding protein that plays an important role in the continued proliferation or survival of EBV-positive tumor cells. EBNA1 is expressed in all forms of latency and has been shown to be important for efficient EBV-mediated immortalization of B-cells in vitro. Several studies showed that EBNA1 is required for the replication and maintenance of the episomal EBV genome. EBNA1 binds to ori-P, the episomal origin of viral replication. EBNA1 is a transcriptional transactivator and upregulates Cp and the LMP1 promoter. The Gly–Gly–Ala repeat domain of EBNA1 is a cis-acting inhibitor of major histocompatibility complex class I-restricted presentation and regulates antigen processing via the ubiquitin-proteosome pathway. Targeting expression of EBNA1 to B-cells in transgenic mice results in B-cell lymphomas, suggesting that EBNA1 might also have a direct role in oncogenesis. EBNA1 acts as a transcriptional activator of several viral and cellular genes (Ann Arvin et al., 2007). In vivo, EBNA1 has been shown to lower p53 levels and apoptosis in response to DNA damage in U2OS cells.

Epstein–Barr virus nuclear antigen 2

EBNA2 is the earliest latent cycle protein of EBV and is essential for primary B-cell growth transformation, immortalization, proliferation, and survival. The role of EBNA2 in growth transformation was first revealed with studies in EBV-infected Burkitt's lymphoma cell line, P3HR-1, a nontransforming mutant form of EBV. EBNA2 is a transcriptional activator of both cellular and viral genes and upregulates the expression of various B-cell antigens, including CD21 and CD23, as well as LMP1 and LMP2. EBNA2 also transactivates the Cp promoter and thereby induces the switch from Wp to Cp detected early in B-cell infection. EBNA2 also transactivates *c-myc* oncogene, which is essential for EBV-induced B-cell proliferation and transformation (Ann Arvin et al., 2007).

Epstein–Barr virus nuclear antigen 3 family

Genetic studies have revealed that both EBNA3A and EBNA3C are essential latent antigens for efficient B-cell transformation in vitro, whereas EBNA3B is completely dispensable. Importantly, EBNA3B-mutated B-cell lymphomas were frequently found and evident that EBNA3B inactivation drives lymphomagenesis and immune evasion. Both EBNA3A and EBNA3C interact with a variety of cellular proteins that may mediate transcriptional activation, repression, or affect cell proliferation. Several evidences strongly suggest that EBNA3A and EBNA3C together inhibit the initiation of Bim transcripts. EBNA3C has been shown to induce the upregulation of both cellular (CD21) and viral (LMP1) gene expression. EBNA2 and the EBNA3 proteins work together to precisely control RBP-Jκ activity, thereby regulating the expression of cellular and viral promoters containing RBP-Jκ cognate sequence. EBNA3C has been shown to interact with human histone deacetylase 1, which contributes to the transcriptional repression of Cp promoter. In addition, EBNA3C has been shown to make stable complexes with several transcriptional cofactors, including mSin3A, prothymosin-expressing, and NCoR in EBNA3C-expressing B-cells. EBNA3C has been shown to repress the Cp promoter and interacts with the retinoblastoma protein, pRb, to promote cell transformation (Ann Arvin et al., 2007). EBNA3C can promote metastasis in EBV-positive tumors by modulating Nm23-H1 activities.

EBNA-LP

EBNA-LP, a critical regulator of EBV-induced B-cell immortalization, is encoded as a variable size protein depending on the number of BamHI W repeats contained in a particular EBV isolate. The role of EBNA-LP in an in vitro B-cell transformation is not clear, but EBNA-LP is required for an efficient outgrowth of LCLs. EBNA-LP binds with pRb in LCLs and with both pRb and p53 in in vitro assays, but its expression appears to have no effect on the regulation of the pRb and p53 pathways (Ann Arvin et al., 2007). EBNA-LP has also been observed to interact with several cellular proteins, including tumor suppressors (pRb, p53, p14ARF, and Fte1/S3a), heat shock proteins (hsp70 and hsp72/hsc73), cell-cycle regulatory molecules (DNA-PKcs and HA95), and anti-apoptotic (HAX-1) protein.

LMP1

LMP1, a latent-membrane protein, is the major transforming protein of EBV behaving as a classical oncogene in rodent fibroblast transformation and being essential for EBV-induced B-cell transformation in vitro. LMP1 has pleiotropic effects when expressed in cells resulting in induction of cell surface adhesion molecules and activation antigens, upregulation of antiapoptotic proteins (Bcl-2 and A20), and stimulation of cytokine production (IL-6 and interleukin 8). Studies have shown that the immortalization effect of LMP-1 on B-lymphomas is mediated by the possible cooperation between Bcl-2 and MCL-1. Recent studies have demonstrated that LMP1 can contribute to neoplastic transformation and tumor progression by modulation of the TNF receptor pathway through its

association with the CTAR1 and CTAR2 domains in a ligand-independent manner (Ann Arvin et al., 2007).

LMP2

The gene encoding LMP2 yields two distinct proteins, LMP2A and LMP2B. Neither LMP2A nor LMP2B is essential for B-cell transformation. The LMP2A amino-terminal domain contains eight tyrosine residues, two of which (Y74 and Y85) form an immunoreceptor tyrosine-based activation motif (ITAM) (Ann Arvin et al., 2007) (Matsuo and Itami, 2002). ITAM phosphorylation in the BCR plays a central role in mediating lymphocyte proliferation and differentiation by the recruitment and activation of the src family of protein tyrosine kinases. Expression of LMP2A in the B-cells of transgenic mice abrogates normal B-cell development, thus allowing immunoglobulin-negative cells to colonize peripherally to lymphoid organs. This indicates that LMP2A can drive the proliferation and survival of B-cells in the absence of signaling through the BCR. Together this suggests that LMP2 can modify the normal program of B-cell development to favor the maintenance of EBV latency and to prevent activation of the EBV lytic cycle (Matsuo and Itami, 2002). Other studies have demonstrated that LMP2A can bypass the entire p53 pathway in lymphomagenesis involving c-Myc.

EBERs

EBERs are EBV-encoded two small nonpolyadenylated (noncoding) RNAs, referred to as EBERs 1 and 2, and are most abundant viral transcripts expressed in all forms of latency. However, the EBERs are not essential for EBV-induced transformation of primary B-lymphocytes. The EBERs assemble into stable ribonucleoprotein particles and bind the interferon (IFN)-inducible, dsRNA-activated protein kinase, PKR. EBER-mediated inhibition of PKR function is important for viral persistence, perhaps by protecting cells from IFN-induced apoptosis (Ann Arvin et al., 2007). In addition, EBER expression can confer an apoptotic-resistant phenotype in immortalized nasopharyngeal epithelial cells.

BARTs

EBV is the first human virus in which the expression of miRNAs, such as MIR-BARTs, was identified. MIR-BARTs are derived from the BamHI A region (Bam A) of the EBV genome. BARTs were first identified in nasopharyngeal cancer (NPC) tissue and subsequently in other EBV-associated malignancies such as Burkett's Lymphoma and nasal T-cell lymphoma. Studies have demonstrated that MIR-BART5 promotes cell survival by targeting PUMA expression, leading to latent infection in NPC and germinal center B-cells. MIR-BARTs may play an important role in epithelial cells carcinogenesis as they are abundantly expressed in latently infected epithelial cells compared to B-cells. BARTs encode a number of potential ORFs, including BARF0, RK-BARF0, A73, and RPMS1; however, protein products of these ORFs have not been identified (Ann Arvin et al., 2007).

Micro RNAs

miRs are small, noncoding RNA molecules of only 21–24 nucleotides in length and have been shown to play a role in the posttranscriptional downregulation of target mRNAs. The EBV miRs are arranged in two clusters within the viral genome, that is, the BHRF1 cluster and the BART cluster, which comprises the remaining 20 miRs located in the introns of the BART transcripts. These two clusters of EBV-encoded miRs are differentially expressed in cells exhibiting different forms of EBV latency. The BART cluster is predominantly expressed during latency I or latency II, whereas the BHRF1 miRs are associated with latency III. The expression levels of several miRs from both clusters are enhanced following induction of lytic infection. However, the precise function of these miRs remains unclear (Ann Arvin et al., 2007).

Clinical correlation

Clinical symptoms and molecular diagnostic approaches differ according to the immune status of the patients. In healthy individuals, primary infection of EBV is most often asymptomatic. However, in immunocompromised individuals, EBV is associated with disorders with high rates of morbidity and mortality. The spectrum ranges from benign B-cell hyperplasia resembling IM to more classic malignant lymphomas. Molecular diagnostics is increasingly important for diagnosis and monitoring of patients affected by EBV-related diseases. As virus-specific treatments continue to be investigated, it becomes important to recognize these EBV-associated malignancies so that proper clinical management decisions can be made. New molecular tests [quantitative real-time polymerase chain reaction or EBER-RNA in situ hybridization] combined with traditional serological (heterophile antibody testing) or histochemical assays [immunofluorescence assay (IFA), in situ hybridization, or Southern blot] are helpful for diagnosis and monitoring of EBV-related diseases depending on the clinical setting and the types of samples available for testing. In situ hybridization for EBER-RNA on biopsy samples, and more recently EBV viral load testing of blood samples, provides an accurate measure of the clinical status in patients. So far, the methods of first choice in routine EBV diagnostics are

the IFA, still the gold-standard method, and different enzyme immunoassay (EIA) techniques, including solid-phase ELISAs and western blot analysis. While IFA or EIA is often used for screening, western blot analysis is mainly performed for confirmation. Today, a number of manufacturers provide commercially available EBV-specific IFAs and EIAs. Recently, in situ hybridization of EBER-RNA has become standard for EBV diagnosis in tumor cells, while quantitative polymerase chain reaction (qPCR) procedures are used for EBV typing. Investigations are underway to better define the utility of these assays across the full spectrum of EBV-associated diseases. In addition, gene expression profiling and array technology will likely improve our ability to subclassify these diseases and predict responses to therapy. Some of the most common EBV-associated malignancies are listed as follows.

Burkitt's lymphoma

The association between EBV and Burkitt's lymphoma (BL) has long been established (Ann Arvin et al., 2007). BL is an aggressive B-cell malignancy, classified in three forms, referred to as endemic-associated BL, sporadic-associated BL, and HIV-associated BL, on the basis of geographical distribution and EBV-association. BL is a tumor of the jaw and face found in children of equatorial Africa, which has rare occurrence elsewhere. The exact cause of this tumor is unclear, but there is probably a genetic factor as well as a malarial cofactor. Only 5% of BLs in the United States is associated with EBV, whereas in endemic areas such as eastern Brazil or Africa, nearly 90% of pediatric BLs harbor EBV. The BL cells show evidence of EBV DNA and tumor antigens, and the patients carry a much higher level of anti-EBV antibodies than other members of the population, although the exact role of EBV in BL pathologies remains to be elucidated. Tumor cells are monoclonal and show a characteristic translocation between chromosomes 8 and 14 that places the c-myc oncogene under the control of the immunoglobulin heavy or light chain promoters, resulting in the upregulation of c-myc oncogene in these cells (Bellan et al., 2003). BL is also associated with HIV infection or occurs in postorgan transplant patients undergoing immunosuppressive therapies to prevent graft rejection. BL may be one of the diseases associated with the initial manifestation of AIDS (Damania, 2007).

Nasopharyngeal cancer

NPC is a rare type of head and neck cancer of epithelial cells of the upper respiratory tract. The majority of nasopharyngeal carcinoma cases from individuals in Southern China, Southeast Asia, Mediterranean, Africa, and the United States are associated with EBV infection. The occurrence of NPC in the rest of the world is low, indicating that there may be a genetic predisposition for the development of EBV cancers in these populations, or there may be an involvement of environmental cofactors, such as dietary components (salted fish), creating low-grade, pre-invasive lesions that become susceptible to EBV infection (Damania, 2007; Bellan et al., 2003). As for the association of EBV with NPC, the contribution of the virus is less clear and appears to be a consequence of the aberrant establishment of virus latency in epithelial cells that have undergone premalignant genetic changes. The presence of monoclonal EBV episomes in NPC indicates that viral infection precedes the clonal expansion of malignant cells.

Hodgkin's lymphoma

Hodgkin's lymphoma, formerly known as Hodgkin's disease, is a B-cell LPD of the lymphatic system in which 1% of the tumor population is composed of Hodgkin/Reed-Sternberg (HRS) cells, which are derived from germinal center B-cells. The HRS cells are multinucleated giant cells that have distinct nucleoli (Damania and Pipas, 2009; Damania, 2007). The first sign of Hodgkin's lymphoma is often a swollen lymph node, which appears without a known cause. The disease can spread to the nearby lymph nodes and later may spread to the spleen, liver, bone marrow, and other organs. There are three types of Hodgkin's lymphoma: lymphocyte-depleted, nodular sclerosis, and mixed cellularity. Each of these differs in their association with EBV infection; 20% of nodular sclerosis Hodgkin's lymphomas are linked to EBV, whereas 100% and 70% of lymphocyte-depleted Hodgkin's lymphomas and mixed-cellularity Hodgkin's lymphomas, respectively, are associated with EBV infection (Damania, 2007; Ann Arvin et al., 2007). EBV is reported to play a direct or indirect role in the pathogenesis of Hodgkin's lymphoma, either by activating several pathogenic mechanisms or by regulating the process of immune recognition that supports the malignancy and reactivation of the virus. Immunosuppressed individuals, either due to HIV infection or immunosuppressive drugs in solid organ transplant patients, are at higher risk than the general population (Damania, 2007).

Infectious mononucleosis

IM (also called "the kissing disease") occurs through the exchange of saliva containing EBV from

infected individuals. Most people are exposed to the virus during their childhood, which is asymptomatic. However, the infected person sheds the virus from time to time throughout life. Infection acquired in adolescents and young adults leads to the development of IM after 1–2 months of infection. The disease is characterized by malaise, lymphadenopathy, fever, and enlarged spleen and liver (Damania, 2007). The severity of disease often depends on age, with younger patients resolving the disease more quickly. Although IM is usually benign, there can be complications. These include neurological disorders such as meningitis, encephalitis, myelitis, and Guillain-Barrè syndrome (Damania and Pipas, 2009; Ann Arvin et al., 2007). Secondary infections, autoimmune hemolytic anemia, thrombocytopenia, agranulocytosis, and aplastic anemia may also occur. In IM, infected B-cells are transformed, which proliferates and activates the suppressor CD8 T-cells. These T-cells differ from normal T-cells in appearance and are known as Downey cells (Ann Arvin et al., 2007). This T-cell response results in enlarged lymph nodes as well as an enlarged liver and spleen. The activation of T-cells limits the proliferation of B-cells, and the disease resolves. Uncontrolled viral replication can lead to a severe syndrome with B-cell lymphoproliferation, leukopenia, and lymphoma. In patients with T-cell deficiency, X-linked lymphoproliferative (XLP) disorder may occur. Patients with HIV infection or postorgan transplant patients who are under immunosuppressive therapies are at high risk to develop lymphoproliferative disorder (Damania and Pipas, 2009).

X-linked lymphoproliferative disease

XLP syndrome is a rare immunodeficiency disease that is characterized by a susceptibility for fatal or near-fatal EBV–induced IM in childhood and a markedly increased risk for lymphoma or other LPDs (Damania and Pipas, 2009). There is a mutation on the X-chromosome that has been found to be associated with a T- and natural killer (NK)-cell lymphoproliferative disorder. The mutation is denoted as Xq25 on the long arm of the X chromosome. This mutation creates a deletion in the *SH2D1A* gene that codes for signaling lymphocytic activation molecule–associated protein (SAP). The SAP protein is important in the signaling events that activate T- and NK-cells and result in the modulation of IFN-γ. Persons with XLP disorder have an impaired immune response to EBV infection, which often leads to death from bone marrow failure, irreversible hepatitis, and malignant lymphoma (Damania and Pipas, 2009).

Research methods and protocols

EBV infection during childhood is usually asymptomatic; however, it establishes a lifelong latent infection (Damania and Pipas, 2009). EBV transforms peripheral human B-lymphocytes into indefinitely proliferating LCLs that allow for genetic manipulation of the virus. LCLs are generated by EBV transformation of the B-lymphocytes within the peripheral blood lymphocyte (PBL) population as shown in the flow chart **(**Fig. 7.3**)**: (1) Latently, EBV-infected cells are grown in culture. (2) These cells are treated with 1 mM sodium butyrate and 20 ng/mL of 12-*O*-tetradecanoylphorbol 13-acetate to induce reactivation. (3) Culture supernatant is collected and filtered through a 0.4-μM filter to harvest the secreted virus followed by ultracentrifugation to concentrate the virus. (4) Purified EBV is then used for infecting human peripheral blood mononuclear cells (PBMCs). (5) Transformed B-cells presenting EBV antigens are then selected. (6) The transformed B-cells are further treated with interleukin 2 for clonal expansion. (7) The selected EBV-LCLs are analyzed for specificity and cryopreserved for further research. These LCLs, derived from B-lymphocytes, are extensively used for in vitro research on gene expression studies and surrogate models to study genotype–phenotype relationships in humans.

Conventionally, the bacterial artificial chromosome (BAC) system is efficiently used to generate herpesvirus mutants by homologous recombination. In the BAC system, the entire EBV genome is cloned as a plasmid and propagated in *Escherichia coli*, and any mutation can be rapidly and precisely introduced into viral genes. The EBV genome was first cloned as a BAC from EBV strain B95-5 derived from IM (Enrique et al., 2010). The establishment of this system enabled studies on epithelial cell background as well as on virus infection on other B-lymphocytes like Ramos and Raji (Ann Arvin et al., 2007). These virus-producing cell lines provided the required tools to study the latent and lytic virus replication, virus production, and regulatory role of viral and cellular proteins in establishing virus latency, transformation, and tumorigenesis (Bellan et al., 2003). In vitro molecular biology methods that reveal gene expression levels in LCLs are fairly comparable with the naturally occurring gene expression in primary B-cells.

Turning point: modeling Epstein–Barr virus infection and pathogenesis

Mouse models are considered primary in vivo tools and play a central role in biomedical researches, including infectious diseases, to identify molecular

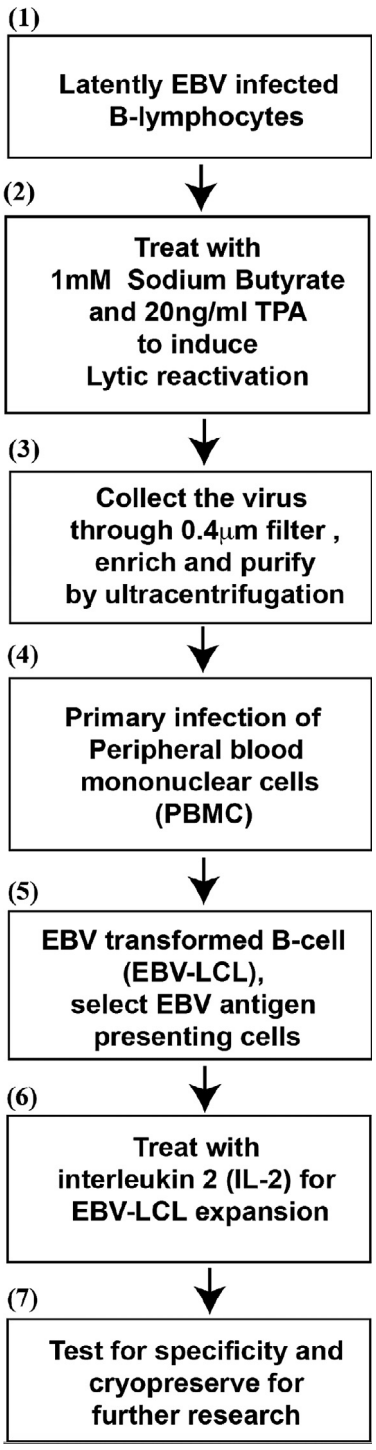

FIGURE 7.3 Flowchart of transformation by EBV.

targets and pathways implicated in tumor progression. In addition, mouse models help validate the efficacy and safety of antiviral therapies before they are tested in clinical trials.

A breakthrough in the generation of a highly immunodeficient mouse model of EBV infection was brought by the development of the *scid*-hu PBL mouse in 1988, based on the C.B-17 *scid* mouse. C.B-17 *scid* mice lack both B and T cells because of a mutation in the gene coding for a subunit of DNA-dependent protein kinase and do not have ability to reject human tissues and cells. Scid-hu PBL mice were prepared by intraperitoneal injection of PBMCs derived from healthy EBV carriers into C.B-17 *scid* mice, which resulted in the development of EBV-positive B-cell LPD. Analyses of the histology, marker expression, and EBV gene expression revealed LPD to be similar to the representative type of EBV-associated LPD in immunocompromised hosts. Scid-hu PBL mice were also used for modeling of HIV-1 and HTLV-1 infections (Enrique et al., 2010).

Since the *scid*-hu PBL mice lacked human immune responses to EBV, the new generation humanized mice were prepared based on novel immunodeficient mouse strains. Transplantation of human hematopoietic stem cells to highly immunodeficient mouse strains such as NOD/Shi-*scid* $Il2rg^{null}$ (Fujiwara et al., 2013) (Bouvard et al., 2009), BALB/c $Rag2^{-/-}Il2rg^{-/-}$, and NOD/LtSz-*scid* $Il2rg^{-/-}$ (NSG) resulted in the reconstitution of functional human immune system components, such as B cells, T cells, NK cells, dendritic cells, and macrophages. Thereafter, these new-generation humanized mice have been extensively used for studying the development and function of human immune system components in vivo and for modeling infections with EBV (Herrman et al., 2015), HIV-1, HTLV-1, dengue virus, HSV-2, and KSHV. In addition, immunodeficient mice carrying functional human hepatocytes were developed for modeling infections with HBV and HCV.

Current research perspectives

Understanding the different mechanisms by which DNA tumor viruses alter signal transduction pathways in malignant cells is important for detecting novel targets for cancer therapies. In addition to viral factors, host factors, genetic makeup, and geographical and environmental factors have also been found to interfere with the normal activities of signaling pathways leading to virus-induced cancers (Abhik Saha et al., 2010). Conventionally, for patients with AIDS-KS, highly active antiretroviral therapy is effectively used to reduce HIV infection together with up to a 90% reduction in KS occurrence (Damania, 2007). Furthermore, antiviral treatment, including acyclovir, ganciclovir, cidofovir, and foscarnet, has been shown to be moderately effective against EBV- and KSHV-associated infections in the lytic phase (Cai et al., 2010; Enrique et al., 2010). Although our knowledge on viral oncoproteins and their associated cellular factors that

are involved in carcinogenesis has greatly advanced, we are still lacking specific drugs that inhibit these viral proteins (Cai et al., 2010; Enrique et al., 2010). Today, most antitumor therapies against virus-induced cancers target cellular proteins that have a role in these processes rather than the viral proteins. Several companies have efforts underway to develop small molecule drugs that target host proteins involved in cell-cycle regulation, inflammatory response, proteasome, and signal transduction pathways (Damania, 2007). Small molecule drugs such as nutlin-3a that target the p53-Mdm2 interaction and bortezomib (Velcade), a proteasome inhibitor, can be used against EBV as well as KSHV-mediated cancer cell lines. Bortezomib has been shown to inhibit proliferation and to induce apoptosis in KSHV-infected PEL cells (Abhik Saha et al., 2010). In addition, PI3K inhibitor (LY294002), mTOR inhibitor (rapamycin), and interleukin-12 that negatively regulate the viral G-protein-coupled receptor pathway have shown promising results against KS (Abhik Saha et al., 2010). Other strategies like inhibition of matrix metalloproteinases and inhibitors for vascular endothelial growth factor are also found to be effective against KS and HBV-associated HCC (Abhik Saha et al., 2010). Interestingly, natural phenolic compounds like resveratrol (found in plants such as grapes) have been shown to be potent antiviral compounds against numerous viruses, including EBV, HSV, HIV, and influenza (Abhik Saha et al., 2010). The use of molecular drugs against cellular factors very often creates many undesirable off-target effects. Thus, developing vaccines or therapeutic drugs that specifically target DNA tumor viral proteins will be essential for effective protection against the virus with reduced drug cytotoxicity (Abhik Saha et al., 2010). Currently, promising vaccines are available only against HPV and HBV (Damania, 2007), and there are no vaccines available against EBV, KSHV, and human polyomaviruses (Lowy and Schiller, 2006; Chang, 2009). Significant efforts have been made to develop vaccines against EBV infection, and the current EBV vaccine development is focused on the most abundant envelope protein, EBV gp350, which is the major target of neutralizing antibodies in human sera (Moore and Chang, 2010; Damania, 2007). In a phase 2 trial, a gp350-based vaccine significantly reduced the incidence of IM but failed to decrease the overall infection rate. Other therapeutic vaccines have also targeted the LMP2 and EBNA-1 viral proteins. Efforts to develop KSHV vaccine are limited, but mouse infection with murine gammaherpesvirus-68 has been exploited as an experimental model for an effective vaccination strategy against the long-term viral latency. A vaccine to increase the immune control of KSHV lytic replication and to decrease the KSHV viral load in people already infected may reduce the risk of KSHV infection. For a therapeutic vaccine, incorporation of epitopes derived from KSHV latent proteins, LANA and Kaposin, will likely increase its efficacy (Herrman et al., 2015).

Ethical issues

EBV infection and tumorigenesis are a complex processes and heavily affected by genetic constitution, socioeconomic background, geographical locations, and subpopulation levels (Damania and Pipas, 2009). Generally, the prognosis and selection of the most appropriate treatment are assessed using both patient-related and standard tumor-related characteristics. The high mortality rate among patients with EBV-associated tumors is partly due to delays in diagnosis that result from the complexity of its initial clinical presentation. EBV-specific qPCR is mostly employed to detect EBV from infected PBLs or biopsy samples (Robertson, 2005). A better understanding of viral gene expression in the context of EBV infections from diverse populations will prove useful in diagnosis and treatment of EBV-associated malignancies. Clinical research on patient samples or animal models involves an array of ethical issues and should be in accordance with World Health Organization criteria. According to this, all clinical research involving patient samples should be approved and continuously monitored by a university or institutional ethical committee. In addition, EBV has strict host specificity, that is, it infects only humans; therefore, it is not practical to study the dynamics of protein expression during EBV infection and pathogenesis. However, an alternative method has been developed to study in vivo infection of EBV by generating a murine model known as "humanized mice." These mice have the potential to maintain human hematopoiesis, including human CD4 + leukocytes that can thereby support persistent EBV infection in vivo. Similar to clinical research, studies involving animal models also require the approval from the institutional ethical committee. Above all, participating patients or their guardians should provide informed consent according to the institutional guidelines. Institutional ethical committees annually review the protocols and progress made in clinical research and have the authority to disapprove of a study if it is deviating from the original guidelines (Robertson, 2005).

Translational significance

The effective early detection of tumor as well as efficient treatment methods could greatly reduce the incidence and mortality of EBV-associated cancers. Currently, there is no effective vaccine available to

prevent EBV infection. Newer formulations of phase 2 trial EBV gp350 that include additional glycoproteins, viral latent, and lytic proteins might improve the efficacy of the currently known EBVgp350 vaccine, making it a promising vaccine candidate against EBV-induced malignancies. Thus, a better understanding of mechanisms of oncogenesis by EBV is important for identifying novel therapeutic targets for the success of EBV-mediated cancer treatment and increase of patient's quality of life.

World Wide Web resources

http://www.virology.net/

All the virology on the worldwide web seeks to be the best single site for virology information on the Internet. It has a collection of all the virology-related web sites that might be of interest to our fellow virologists and others interested in learning more about viruses.

http://www.microbe.tv/twiv/

TWiV (This Week in Virology): This site is an independent podcast network for people who are interested in latest discoveries in the field of science, viruses, and microbes.

https://www.nature.com/subjects/virology

It is the world's foremost international weekly scientific journal that publishes peer-reviewed state-of-art research in all fields including virology.

http://www.ncbi.nlm.nih.gov/books

NCBI Bookshelf provides free access to books and documents in life sciences and healthcare. A vital node in the data-rich resource network at NCBI Bookshelf enables users to easily browse, retrieve, and read content, and spurs discovery of related information.

http://www.ncbi.nlm.nih.gov/pubmed/

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Glossary

Cytopathic effect Refers to degenerative changes in cells, especially in tissue culture.
Deregulation The act of freeing from regulation.
Hit-and-run viruses Viruses that can initiate cancers or play a role in their development, but then can disappear from host.
Immunesurveillance A process in which immune system continually recognizes and removes malignant cells.
Intravasation The invasion of cancer cells through the basal membrane into a blood or lymphatic vessel.
Knockdown Gene knockdown refers to techniques by which the expression of one or more of an organism's genes is reduced.
Oncogene A gene that in certain circumstances transforms a cell into a tumor cell.
Onco proteins A gene product that causes the transformation of normal cells into cancerous tumor cells.
Secondary tumor Metastasis is one of the hallmarks of malignancy. This new tumor is known as a metastatic or secondary tumor.
Oncogenesis The process of tumor formation/induction.

Abbreviations

AIDS	Acquired immunodeficiency syndrome
BAC	Bacterial artificial chromosome
BCR	B-cell receptor
BL	Burkitt's lLymphoma
CNS	Central nervous system
EBER	EBV-encoded small RNA
EBNA	Epstein–Barr nuclear antigen
EBV	Epstein–Barr virus
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
EV	Epidermodysplasia verruciformis
HAX-1	HCLS1-associated protein X-1
HBV	Hepatitis B virus
HBeAg	Hepatitis B early antigen
HBsAg	Hepatitis B surface antigen
HCC	Human hepatocellular carcinoma
HCV	Hepatitis C virus
HHV-4	Human herpesvirus-4
HHV-8	Human herpesvirus-8
HIV	Human immunodeficiency virus
HPV	Human papillomavirus
HPyV	Human polyomavirus
HRS	Hodgkin/Reed-Sternberg
HSC	Hematopoietic cells
HSV-2	Herpes simplex virus-2
HTLV-1	Human T-cell lymphotrophic virus
IFA	Immunofluorescence assay
IL-6	Interleukin 6
IL-10	Interleukin 10
IM	Infectious mononucleosis
IRF	Interferon regulatory factors
ITAM	Immunoreceptor tyrosine-based activation motif
JAK/STAT	Janus kinase-signal transducer and activator of transcription
JNK	c-Jun *N*-terminal kinase
KICS	KSHV-inflammatory cytokine syndrome
KS	Kaposi's sarcoma
KSHV	Kaposi's sarcoma–associated herpesvirus
LANA	Latency-associated nuclear antigen
LCL	Lymphoblastoid cell lines
LMP1	Latent membrane protein1
LPD	Lymphoproliferative disease
MAPK	Mitogen-activated protein kinases
MCC	Merkel cell carcinoma
MCD	Multicentric Castleman's disease
MCPyV	Merkel cell polyomavirus
MDM2	Mouse double minute 2 homolog
miRs	microRNAs
MTCT	Mother-to-child transmission
NPC	Nasopharyngeal cancer
ORFs	Open reading frames
PBMCs	Perinuclear blood mononuclear cells
PEL	Primary effusion lymphoma
PKR	Protein kinase RNA dependent
PML	Progressive multifocal leukoencephalopathy
PVN	Polyomavirus nephropathy
qPCR	Quantitative polymerase chain reaction
SAP	Signaling lymphocytic activation molecule–associated protein
TLR	Toll-like receptors
TNF	Tumor necrosis factor
vFLIP	Viral Fas-associated death domain-like interleukin-1β-converting enzyme-inhibitory protein
vIRF3	Viral interferon regulatory factor-3
XLP	X-linked lymphoproliferative
ZREs	ZEBRA response elements

Long answer questions

1. What is virus transformation? How do DNA tumor viruses transform cells?

2. How small DNA tumor viruses differ from large DNA tumor viruses?
3. Which are the different DNA tumor viruses infecting humans?
4. What are viral oncoproteins? How do they contribute to tumorigenesis?
5. A person with AIDS is diagnosed with acute B-cell lymphoma, what could be the causative agent and why?

Short answer questions

1. Which of the DNA tumor viruses can cause B-cell lymphoma in humans?
2. Which tumor virus has been associated with cervical carcinomas?
3. What is the necessary prerequisite for defining an infection as latent?
4. Which DNA tumor virus has been associated with hepatocellular carcinomas?
5. Why excessive exposure to sun can cause Merkel cell carcinoma?

Answers to short answer questions

1. DNA tumor virus belongs to the family gammaherpesvirus, Epstein–Barr virus (EBV) and Kaposi's sarcoma–associated herpesvirus (KSHV) primarily causes B-cell lymphoma in humans.
2. Infection by human papilloma virus is the most common sexually transmitted disease and can lead to the development of cervical cancer.
3. During latency, the virus remains dormant and persists in a highly ordered chromatin state as episome, whose propagation is dependent on their ability to hijack the replicative machinery of their host. During latency, virus encodes only a few latency-associated proteins that are known to be tumorigenic. Among DNA tumor viruses, latency is well studied on herpesvirus family members, EBV and KSHV.
4. HBV, a member of the Hepadnavirus family, causes an infectious inflammatory disease of the liver. Patients with chronic hepatitis B are at high risk of developing hepatocellular carcinoma.
5. Merkel cell carcinoma (MCC) occurs most often on the sun-exposed face, head, and neck. MCPyV is the first polyomavirus, which has been shown to integrate into human genomic DNA. Virus then undergoes two sequential mutational events resulting in persistent T-Ag expression, which plays a key role in turning asymptomatic viral infection into an aggressive Merkel cell carcinoma. Excessive exposure to sun can induce these mutational events leading to MCC.

Yes/no type questions

1. Do virus-induced tumors retain part of the viral genome?
2. Is HCV a RNA tumor virus?
3. Is coronavirus associated with cancer?
4. Can latent infections persist in an individual without causing any symptoms?
5. Is integration of viral genes required for cell transformation in Burkitt's lymphoma?
6. Does HPV belong to the gammaherpesvirus subfamily?
7. The Nobel Prize in Physiology or Medicine was granted to Harald zur Hausen for his discovery of HBV causing cervical cancer?
8. Is KSHV associated with nasopharyngeal carcinoma?
9. Are the tumor viruses primarily subclassified according to the type of life cycle in the infected host cell?
10. Are the viruses extremely specific to the host and type of cells they infect?

Answers to yes/no type questions

1. Yes
2. Yes
3. No—They are believed to cause common colds in human adults.
4. Yes
5. No
6. No—Only EBV and KSHV does.
7. No—He was awarded the Nobel Prize for discovering the association of HPV with cervical cancer.
8. No—EBV is known to cause nasopharyngeal carcinoma.
9. No—They are classified on the basis of their nucleic acid.
10. Yes.

CHAPTER

8

Animal models for human disease

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Summary

A useful animal model for disease must be similar in its pathology to disease conditions in humans. Experimental animal models of rheumatoid arthritis and multiple sclerosis are useful for a better understanding of disease mechanisms and for evaluating the therapeutic efficacy of new and emerging drugs.

Outline

The significance of animal modeling in biotechnology was described.
Different types of animal model to study the pathogenesis of rheumatoid arthritis were explained.
Various methods for the evaluation of experimental models of multiple sclerosis were discussed.
Ethical topics for using living animals in scientific research were noticed.

What you expect to know

This chapter introduces the subject of animal models both spontaneous and induced models, used in different human disease studies. The benefits of animal models are that one can study the mechanisms of diseases as well as test new and emerging drugs for their therapeutic efficacy. Rheumatoid arthritis (RA) is one of the autoimmune disorders for which different animal models are available, and each and every model has its merits and demerits. We will discuss the importance and induction of RA by collagen. Collagen-induced arthritis in animal models reflects characteristic features of RA patients. Multiple sclerosis (MS) is another debilitating disease that affects the central nervous system (CNS) of humans. The animal model used to study MS is known as Experimental Allergic Encephalomyelitis (EAE). We have given the details of how to induce EAE and also how to apply this animal model. Although various ethical issues are involved with the development and use of animal models to study human disease, the importance of animal models can neither be ignored nor be denied.

Introduction

The architecture of human body is comprised in such a manner that cells cannot be considered as a separate entity. Physiologically, homeostasis is the reason that these components live and perform their functions within that environment. Disruption of this process leads to fatal conditions and is considered a disease. To investigate the mechanism of disease and to find the means to reverse adverse conditions, various strategies are used including cell-based assays and tissue culture studies. Although these models can provide useful information, they fail to address various physiological conditions and the complex interactions among different cell types of tissues and organs. Ideally a useful animal model for any disease has to have pathology similar to the disease conditions in humans. Use of animals in research has a long history that dates

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back to the fourth century B.C. In the 1600s, William Harvey used animals to describe the blood circulatory system. Many scientists, such as Louis Pasteur and Emil von Behring, have used animal models for experimental purposes to prove their hypotheses. Animal models are good for understanding disease mechanisms and treatment and for overcoming the limitations of clinical trials that use human subjects. For example, experimental animal models for diseases like rheumatoid arthritis or multiple sclerosis have been successfully employed to screen new bioengineered, chemical, or herbal therapeutics that might have the potential for the treatment of human patients. So far, more than 550,000 studies have been reported in the NCBI database; they use animal models for different diseases. Animal model studies have been the main reason for a better understanding of disease mechanisms. Animal models of disease can be divided into two categories (Kurkó et al., 2013): spontaneous disease models and (van Heemst et al., 2014) induced disease models. In the case of induced disease models, induction can occur by various agents, both chemical and biological. This chapter discusses some of the most important animal models.

Rheumatoid arthritis

Rheumatoid arthritis (RA) is an autoimmune disorder with progressive occurrence that preferentially affects peripheral joints. In spite of the fact that RA is severe and crippling and affects large numbers of people, very little knowledge about its etiology and pathogenesis is available in the literature.

Epidemiology and etiology

Rheumatoid arthritis affects about 1% of the population. The ratio of the prevalence of RA in males and females is 1−2.5. RA can occur at any age, but it is mainly reported to affect the 40- to 70-year-old age group. No doubt the incidence has been reported to increase with age. The etiology of RA is unknown, but it has been predicted that genetic and environmental factors play an important role in the onset of RA. Recent advances have identified genetic susceptibility markers both within and outside of the major histocompatibility complex (MHC). Human leukocyte antigen (HLA) genes located on chromosome 6p have been found to have a strong association with rheumatoid arthritis. The contribution of HLA to heritability of RA has been estimated to be 11%−37%. Individuals carrying HLA-DR4 and HLA-DR1 alleles have been shown to have a higher risk of RA. Apart from the known shared epitope alleles (HLA-DRB1*01, DRB1*04), other HLA alleles, such as HLA-DRB1*13 and DRB1*15, have been linked to RA susceptibility (Kurkó et al., 2013). The HLA class II locus is the most important risk factor for anticitrullinated protein antibodies (ACPA)-positive RA (ACPA + RA) (van Heemst et al., 2014). A positive correlation has been suggested for the role of HLA in terms of the severity of RA rather than the onset of the disease. The most relevant non-HLA gene single nucleotide polymorphisms (SNPs) associated with RA include PTPN22, IL23R, TRAF1, CTLA4, IRF5, STAT4, CCR6, and PADI4 (Kurkó et al., 2013; Suzuki and Yamamoto, 2015; Stanford and Bottini, 2014). Although the data regarding this conclusion are inconsistent, some of the studies have shown associations between tumor necrosis factor (TNF) alleles and rheumatoid arthritis. Other genes like those for corticotrophin-releasing hormone, interferon (IFN)-γ, and interleukin-10 (IL-10) have also been implied for RA. It can be concluded that the role of genetic components in RA is modest at the best (Viatte et al., 2013).

Epigenetics is another important factor that contributes to RA. In the case of identical twins, RA has not been shown to have 100% concordance; therefore, the role of nongenetic factors has also been implicated in the etiology of RA (Meda et al., 2011). Throughout the world, rheumatoid arthritis is more common in women than in men. This indicates that hormones may play an important role in the development of the disease. Pregnancy has also been considered as a risk factor for rheumatoid arthritis. Studies show that the onset of RA is rare during pregnancy, but the risk increases after delivery. Smoking is associated with increased incidences of RA, especially in men. On the contrary, populations that consume a diet high in omega-3 fatty acids have been reported to be protected from rheumatoid arthritis. From experimental models in animals, a large number of infectious agents such as viruses and bacteria have also been suggested to trigger or contribute to the development of rheumatoid arthritis. However, no relationship between infectious agents and the development of RA has been found.

Pathogenesis

An inflamed synovium is central to the pathophysiology of rheumatoid arthritis. Histologically, RA shows pronounced angiogenesis, cellular hyperplasia, an influx of inflammatory leukocytes, and changes in the expression of cell-surface adhesion molecules, proteinases, proteinase inhibitors, and many cytokines. Synovial changes in rheumatoid arthritis vary with disease progression. In the first weeks of the disease,

tissue edema and fibrin deposition are prominent and can manifest clinically as joint swelling and pain. Within a short period, the synovial lining becomes hyperplastic, commonly becoming ten or more cells deep and consisting of type A (macrophage-like) and type B (fibroblast-like) synoviocytes that produce glycosaminoglycans (e.g., hyaluronan, as reported to be present in synovial tissue and synovial fluid). The sublining also undergoes alterations for its cellularity, both in cell type and in cell numbers, with prominent infiltration of mononuclear cells, including T cells, B cells, macrophages, and plasma cells.

The abundance and activation of macrophages at the inflamed synovial membrane correlates significantly with the severity of the disease. Activated macrophages over-express major histocompatibility complex (MHC) class II molecules and produce pro-inflammatory or regulatory cytokines and growth factors [IL-1, IL-6, IL-10, IL-13, IL-15, IL-18, TNF-α, and granulocyte macrophage colony stimulating factor (GM-CSF)], chemokines [IL-8, macrophage inflammatory protein 1 (MIP-1), monocyte chemoattractant protein 1 (MCP-1)], metalloproteinases, and neopterin. These biomolecules are routinely detected in inflamed joints.

Most of the T cells infiltrating the rheumatoid synovium express CD45RO and CD4, which is an indication that the T-cell subset present in the synovium is memory helper T cells. Surprisingly, 10%−15% of the T cells present in the case of the synovium have granzymes A and perforins. This 10%−15% of cells present in the synovium represents cytotoxic T-cell subsets. Therefore, it can be concluded that CD8-expressing cells are infrequent in the synovium. In the synovial fluid of rheumatoid arthritis patients, CD4 and CD8 T cells are equally represented. TCRα/TCRß is expressed on most of the T cells while only a minority of cells show TCRγ/TCRδ expression. It has, however, been found that the expression of TCRγ/TCRδ is increased in the synovium of patients with active RA. Synovial-vessel endothelial cells transform into high endothelial venules early during the course of disease. High endothelial venules are specialized post-capillary venules usually present in secondary lymphoid tissue or inflamed nonlymphoid tissues; these venules facilitate the transit of leukocytes from the bloodstream into tissues.

The cytokine-mediated events have conventionally been viewed in the milieu of the CD4 + Th1/Th2 paradigm. Nowadays, newer cytokines of the IL-17/IL-23 axis and others (IL-27, IL-33, and IL-35) have changed investigations into the immunopathogenesis of arthritis. Both the CD4 + Th17 and γδ-T cells secreted IL-17 which is a chemotactic for neutrophils and its response inhibited by IL-27 due to IFN-γ induction. The roles of other cytokines such as IL-18 and IL-33 in arthritis have been clarified further with inhibitors of them (Veenbergen et al., 2010; Palmer et al., 2009). Recent studies in arthritis models have revealed new aspects toward regulatory T cell (Treg) activity. In the CIA model, treatment with IL-35 induced the regression of arthritis via expansion of regulatory T cells(Kochetkova et al., 2010).

The formation of locally invasive synovial tissue (i.e. pannus) is a characteristic feature of rheumatoid arthritis. Pannus is involved in the erosion of joints in rheumatoid arthritis. Pannus is histologically distinct from other regions of the synovium and shows phases of progression. Initially, there is penetration of cartilage by synovial pannus, which is composed of mononuclear cells and fibroblasts, with a high-level expression of matrix metalloproteinases (MMPs) by synovial lining cells. In later phases of the disease, cellular pannus can be replaced by fibrous pannus comprised of a minimally vascularized layer of pannus cells and collagen overlying cartilage. The tissue derivation of pannus cells has not been fully elucidated, although they are thought to arise from fibroblast-like cells (type-B synoviocytes). *In vitro* work shows that these fibroblast-like synoviocytes have anchorage-independent proliferation and loss of contact inhibition, which a phenotype is usually found in transformed cells. However, the molecular pathogenic mechanisms driving pannus formation still remains poorly understood.

Clinical manifestations

The range of presentations of rheumatoid arthritis is broad, but the disease onset is insidious in most cases, and several months can elapse before a firm diagnosis can be ascertained. The predominant symptoms are pain, stiffness, and swelling of peripheral joints. Although articular symptoms are often dominant, rheumatoid arthritis is a systemic disease. Active rheumatoid arthritis is associated with a number of extra-articular manifestations, including fever, weight loss, malaise, anemia, osteoporosis, and lymphadenopathy.

The clinical course of the disorder is extremely variable, ranging from mild, self-limiting arthritis to rapidly progressive multisystem inflammation with a profound morbidity and mortality. Analyses of clinical course and laboratory and radiological abnormalities have been defined as negative prognostic factors for progressive joint destruction; unfortunately, none of these are reliable enough to allow therapeutic decision-making. Frequent assessment of disease symptoms and responses to therapy is crucial for a successful and long-term management of rheumatoid arthritis. Joint destruction from synovitis can occur rapidly and early in the course of the disorder; radiographic evidence is

present in more than 70% of patients within the initial 2 years. More sensitive techniques such as magnetic resonance imaging (MRI) can identify substantial synovial hypertrophy, bone edema, and early erosive changes as early as 4 months after the onset of disease. These radiographic changes predate misalignment and functional disability by years; by the time physical deformity is evident, substantial irreversible articular damage has commonly occurred. Furthermore, the biopsy analysis of clinically symptomless knee joints in patients with early rheumatoid arthritis shows active synovitis, highlighting the poor correlation between clinical assessment and disease progression, and the rapid development of polyarticular synovitis.

Treatment

The main goal of RA treatment is to stop inflammation, relieve symptoms, prevent joint damage, and reduce long-term complications. The past decade has seen a major transformation in the treatment of rheumatoid arthritis in terms of approach and choice of drugs. The previous therapeutic approach generally involved initial conservative management with nonsteroidal antiinflammatory drugs (NSAIDs) for several years; disease-modifying antirheumatic drugs (DMARDs) were withheld until a clear evidence of erosion was seen. DMARDs were then added individually in slow succession as the disease progressed. This form of treatment has been supplanted by early initiation of DMARDs and combination DMARD therapy in patients with the potential for progressive disease. The idea of early intervention with the conventional disease-modifying antirheumatic drugs (cDMARD) has been validated in several randomized trials. cDMARDs contain medications from different classes of drugs including methotrexate, gold salts, hydroxychloroquine, sulfasalazine, ciclosporin, and azathioprine. DMARDs are often partly effective and poorly tolerated for long-term therapy. In meta-analyses of dropout rates from clinical trials, 20%−40% of patients discontinued the use of DMARDs assessed as monotherapy during the duration of the trial; even in clinical practice, the median duration of DMARD monotherapy was less than 2 years for nonmethotrexate agents. Although there are many reasons for the lack of long-term adherence to treatment, poor efficacy, delayed onset of action, and toxic effects are major limitations. Additionally, DMARDs therapy requires patients to undergo frequent monitoring of blood and physical examinations for toxic effects of treatment protocol. Results from clinical trials showed that DMARD therapy decreased markers of inflammation such as erythrocyte sedimentation rate and swollen joint counts, and that improved symptoms in a selected subset of patients; however, most patients continued to show progression of irreversible joint destruction on radiography. cDMARDs is increasingly burdened by side effects or clinical inefficacy, so other immunosuppressive drugs such as tacrolimus that blocks T-cell activation by specifically inhibiting calcineurin pathway and leflunomide have been developed. A new synthetic DMARD, Iguratimod, which exerts its action by the inhibition of the inflammatory cytokines (TNF-α, interleukin (IL)-1 β, IL-6, IL-8, and IL-17), is recently developed.

The findings illustrate the consequences of progressive disease and have shown the need for the development of new and more effective therapies based on the therapeutic principles used for oncology; it means that treatment protocols for RA patients require the use of several therapeutic agents from different classes to be used in combination. Recent studies have shown that combination therapy of biological DMARDs like TNF-α inhibitors with methotrexate has clear-cut benefits with tolerable toxic effects. Treatment with agents that can block TNF-α function has proved to be highly effective against RA. Further studies reported downregulation of synovial GM-CSF, IL-6, and IL-8, suggesting that TNF-α supports the production of other pro-inflammatory cytokines. However, the mechanisms behind the clinical effect of the TNF-α-blocking treatment are not fully understood. In an animal model, TNF-α-blocking agents such as etanercept (a soluble TNF-α receptor) and infliximab (a monoclonal antibody) reduce the expression of vascular adhesion molecules and inhibit the spontaneous production of IL-1 and IL-6. Patients with a new onset of symptoms and those with diseases of several years' duration and who had failed previous DMARD therapy all benefited. These results suggest that patients in many stages of disease progression can benefit from combination therapy (Chiu et al., 2012). With the approval of TNF-α inhibitors (infliximab, etanercept, adalimumab, certolizumab, and golimumab), non-TNF biologic agents (rituximab, abatacept, tocilizumab, and anakinra), and other biologic agents, determining advances in treatment options of RA were made. Rituximab (chimeric monoclonal antibody targeted against CD 20) is a selective B-cell depleting agent for treating refractory rheumatoid arthritis. Abatacept selectively modulates T-cell co-stimulation and has shown efficacy in several clinical trials. Tocilizumab, a humanized monoclonal antiinterleukin-6 receptor antibody, has proven to be efficacious in patients who did not respond to methotrexate or other synthetic DMARDs.

Recently, several clinical trials have focused on a new class of drug: the Janus kinase (JAK) inhibitors. JAKs are a family of nonreceptor tyrosine kinases

(JAK1, JAK2, JAK3, and TYK2) involved in the intracellular signal transduction of many cytokines. Tofacitinib is a pan-JAK inhibitor that primarily inhibits JAK1 and JAK3. In addition to Tofacitinib, other JAK inhibitor molecules including baricitinib, peficitinib, and decernotinib have also been studied in RCTs. Finally, Filgotinib is a selective JAK1 inhibitor which is currently in clinical development for the treatment of RA (Calabrò et al., 2016).

As mentioned before, pro-inflammatory/regulatory cytokines and growth factors play important roles in the pathogenesis of RA. Therefore, each of them or their pathway represents an attractive therapeutic target for RA. Tocilizumab, a humanized monoclonal antibody targeting IL-6 receptor, has already been approved for the treatment of RA in patients who failed to achieve remission with cDMARDs. Another cytokine that plays an important role in the pathogenesis of RA is IL-17 in which ixekizumab and brodalumab as humanized monoclonal antibody were developed against IL17A and its receptor. A new possible therapeutic target for the treatment of RA is the GM-CSF pathway. The efficacy and safety of Mavrilimumab (an anti-GM-CSF receptor monoclonal antibody) in patients with moderate-to-severe RA has been investigated (Takeuchi et al., 2015). As an increased activation of osteoclasts contributes to bone erosions in RA, the inhibition of RANKL that is essential for the osteoclast activation by denosumab (human monoclonal antibody against RANKL) can reduce joint destruction in RA patients. Takeuchi et al. (2016), finally, do not forget that certain nutritional components interfere in the pathological inflammatory process, so that they should be considered as coadjuvant in the treatment of RA. It has been mentioned that flavonoids reduce cytokine expression and secretion. In this regard, flavonoids may have a therapeutical potential in the treatment of inflammation-related diseases as cytokine modulators (Rosillo et al., 2016; Leyva-López et al., 2016).

Experimental models

In order to study the pathogenesis of RA, one can use different animal models. There are many experimental models that resemble RA in different respects. Since RA is a heterogeneous disease, there is probably a need for different animal models that each reflect a characteristic feature of a particular subgroup of RA patients or illustrate a particular aspect of the disease.

Spontaneous models

Despite the fact that RA is not a spontaneously developing disease, spontaneously developing models for arthritis may be useful to study the role of genetics in the development of the disease. An activated immune response was reported in models such as the human tumor necrosis factor-α transgenic (hTNFtg), interleukin 1receptor-α (IL-1Ra) knockout, IL-6R-activating mutation knockin, or SKG mouse which bears the primary inflammatory response in joints (Keffer et al., 1991). In addition, arthritis can be rapidly induced with an adoptive transfer of T-helper 17 cells in the IL-6R knockin mouse. Transgenic mice expressing a TCR specific for bovine pancreas ribonuclease develop spontaneous arthritis that is mediated by antibodies (Korganow et al., 1999). This model is particularly interesting because it demonstrates that T cells specific for a ubiquitous antigen may induce an organ-specific autoimmune disease. The expression of the gene product causes an upregulation of several cytokines (IL-1, IL-6, TGF-β1, IFN-γ, and IL-2) and subsequent development of arthritis (Iwakura et al., 1995). There are some other spontaneous models for arthritis in nontransgenic mice (Bouvet et al., 1990).

Induced models

Arthritis can be induced by complete Freunds' adjuvant (CFA). Pearson (Pearson, 1956) described this model for the first time. Subsequently, it was demonstrated that other adjuvants, such as IFA, pristane, or squalene, could also induce arthritis (Carlson et al., 2000). Microbially derived products such as lipopolysaccharide (LPS), muramyle dipeptide (MDP), and trehalosedimycolate (TDM) can also induce arthritis when given with mineral oil (Lorentzen, 1999; Kohashi et al., 1980).

Collagen-induced arthritis (CIA) is normally induced by the immunization of susceptible mouse (e.g., DBA/1) or rat (DA, Lewis) strains at the base of the tail. The inoculum used for immunization contains both adjuvant and collagen type II. The adjuvant has to be sufficiently strong to cause tissue destruction as well as induction of a strong pro-inflammatory immune response (Holmdahl and Kvick, 1992; Kleinau et al., 1995). Susceptibility to CIA is dependent on both MHC (class II region) and non-MHC genes (Lorentzen and Klareskog, 1996). Antibodies against collagen II are essential for the development of CIA. This fact has been demonstrated by the passive transfer of anti-CII antibodies, which results in synovitis (Svensson et al., 1998). T cells are also important for CIA development during early stages of disease progression. The dependence of both T- and B-cell responses has also been demonstrated in the same model (Seki et al., 1988).

Pathology of collagen-induced arthritis

In CIA, an immune response is being directed against a joint collagen type II (CII) antigen. Inflamed

joints in CIA are infiltrated by inflammatory cells that accumulate in the synovial membrane and fluid, similar to RA. The most frequent cell type in the synovial fluid is granulocyte. There is also a great infiltration of leukocytes into the synovial membrane. These cells have signs of an activated phenotype of RA since MHC class II molecules are expressed (Klareskog and Johnell, 1988). In addition, there is an intense production of macrophage-derived cytokines in inflamed joints (e.g., TNF-α and IL-1β) (Mussener et al., 1997; Ulfgren et al., 2000). A small number of T cells are encountered, and some of these T cells have IL-2 receptor α chain upregulated. The disease shows a thickened synovial membrane that subsequently forms a pannus on the cartilage surface (Holmdahl et al., 1988; Holmdahl et al., 1991). In both CIA and RA, cartilage and bone destruction occurs mainly at the cartilage–pannus junction. There are some features of the pathology of CIA that differ from what is usually observed in RA (e.g., extra-articular manifestation). Although the compatibility of the CIA model to human RA has been argued, many pathological features of CIA are similar to those of rheumatoid arthritis. Currently, collagen type II-induced arthritis in mice and rats is one of the most widely used arthritis models in academia and industry.

Methodology and protocols

Experimental collagen-induced arthritis was initiated by injecting bovine collagen type II at the base portion of the tail of the animal (Saadat et al., 2005). Male Lewis rats weighing about 160–180 g were used. After the induction of CIA, animals were divided randomly into four or more groups based on the experimental design. At least four different groups were needed, including a control group without arthritis, animals with collagen-induced arthritis, CIA animals with treatment, and CIA animals treated with methotrexate as a positive control.

Sample preparation: bovine collagen type II (CII) was dissolved in 0.1 M acetic acid at a concentration of 2 mg/mL by stirring overnight at 4°C (the dissolved CII can be stored at −70°C if it has to be used at a later time). Before injecting the animals, CII was emulsified with an equal volume of complete Freund's adjuvant (CFA). For the induction of CIA, on Day 1 rats were injected intradermally at the base of the tail with 100 μL of emulsion (containing 100 μg of CII). After 12–16 days, animals showed the development of inflammation at peripheral joints (Fig. 8.1). On Day 21, a booster injection of CII in CFA was administered. This model was used to evaluate the anti-RA effect by giving intraperitoneally injections of test materials

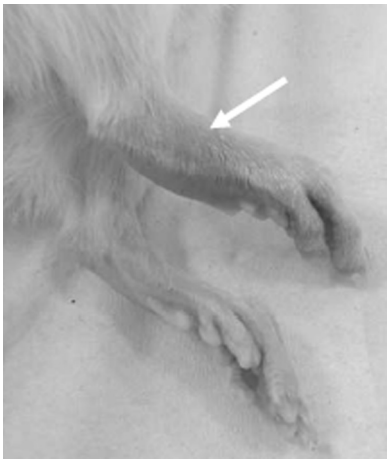


FIGURE 8.1 Inflammatory edema (*white arrow*).

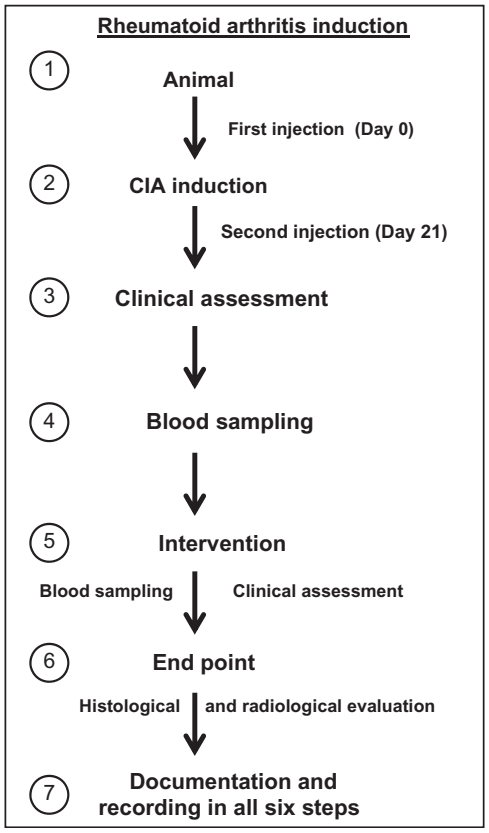
(e.g., chemical or herbal extracts). Methotrexate was a control used to evaluate the effect of the test compound and to compare the efficacy of the new compound with methotrexate. In this model, the test compound was given from Day 25, where the frequency, route of administration, and dose could be selected as needed. The end point and days for the evaluation of different parameters were selected; one of the most common points was Day 35 (Flow Chart 8.1). The paws and knees were then removed for the histopathological assay.

Clinical assessment of collagen-induced arthritis

The visual observation can be done by using the macroscopic system as given in Table 8.1. Moreover, rats immunized with CFA should be checked for weight gain from the first to the end of experiment at least every other day. The decline in body weight that followed on the onset of arthritis was proportional to the disease severity and, hence, can be used as a measure of disease activity. The scaling to record the observation should be from 0 to 4 for each paw (Szabó et al., 1998).

Histological assessment

On Day 35, animals were anesthetized with sodium pentobarbital (45 mg/kg intraperitoneally) and euthanized. Blood was collected by intracardiac puncture, and paws and knees were removed, trimmed, and fixed in 10% buffered formalin, decalcified, and then embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin for the histological examination. Joint damage was assessed based on synovial hypertrophy, pannus formation, inflammatory cell infiltration, and cartilage and subchondral bone destruction. Joint erosion was graded on a scale of 0–3 for each limb (Table 8.2), according to the severity of damage (Fig. 8.2).



FLOW CHART 8.1 CIA induction for rheumatoid athritis.

TABLE 8.1 Scaling to record the signs of collagen-induced arthritis (CIA).

Scale	Observed symptoms
0	No signs of arthritis
1	Swelling and/or redness of the paw or one digit
2	Involvement of two joints
3	Involvement of > two joints
4	Severe arthritis of the entire paw and digits

TABLE 8.2 Scoring for histological evaluation of collagen-induced arthritis (CIA).

Scale	Histological evaluations
0	No signs of inflammation
1	Infiltration of inflammatory cells in the joints
2	Swelling and tissue edema in the joints
3	Bone erosion in the joints

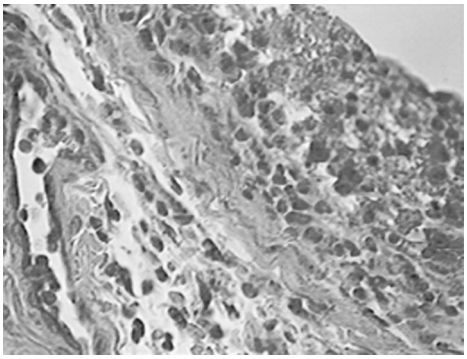


FIGURE 8.2 Representative histopathological slides of a hind limb joint of a healthy Lewis rat.

TABLE 8.3 Scoring for radiological evaluation of collagen-induced arthritis (CIA).

Scale	Radiological evaluations
0	No alteration of joint
1	Swelling of soft tissue
2	Joint space narrowing
3	New bone formation and bone destruction

Radiographic evaluation

Radiological scoring was performed by an investigator who was blind to the treatment protocol (on Day 35). Radiographical analysis of affected joints in control rats typically showed soft tissue swelling, joint space narrowing, reduced lucency due to demineralization, and areas of recalcification indicative of new bone formation. A score was assigned to each joint on the basis of the information as listed in Table 8.3. Scores were 0–3 per joint (0, normal; 3, maximum joint destruction).

Multiple sclerosis

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disorder of the central nervous system (CNS) that affects over 2.3 million individuals worldwide. Similar to the affected population in other autoimmune diseases, twice as many women as men have MS. Multiple sclerosis, like many other diseases, has existed as long as human life. In the 1860s, the first report by Dr. Jean-Martin Charcot certified MS as a disease. A patient of him who suffered an unusual symptom died. After dissection, brain lesions were discovered. He called the disease *scleroseen plaques*. Myelin was subsequently discovered, although its exact role was not recognized. About one century of research resulted in the discovery

of MS as an autoimmune disease. Since this finding, extensive studies on MS have revealed some aspects of disease pathogenesis and etiology. Steroids and disease-modifying agents were used. However, this debilitating disease is not completely understood and MS remains an incurable neurological disorder.

Epidemiology and etiology

Multiple sclerosis is the most common inflammatory demyelinating disease of the CNS in Europe and North America. The prevalence of MS in North America and Europe is ~ 80–100 per 100,000 people. However, the prevalence is not globally uniform, geographically decreases in latitudes, and has been observed in only ~1–2 per 100,000 individuals in Africa and Asia. The etiology of MS is unknown. Both genetic involvement and environmental factors have been indicated in MS. The only consistent correlation of involvement of the MHC locus is the MHC class II allele HLA-DR2, which reflects a linkage with MS. In addition to associations within the major histocompatibility complex (MHC) region, other non-MHC loci reached a genome-wide significance. They map to the genes *L3MBTL3*, *MAZ*, *ERG*, and *SHMT1*. Products of the genes *L3MBTL3*, *MAZ*, and *ERG* play important roles in immune cell regulation. *SHMT1* encodes a serine hydroxymethyl transferase catalyzing the transfer of a carbon unit to the folate cycle, which is important for establishment and maintenance of epigenetic signatures (Andlauer et al., 2016). Some other factors like dietary components (e.g., milk), pathogens like human herpes virus 6 (HHV-6), measles virus, Epstein–Barr virus, and chlamydia have been implied as etiological factors. However, the association between any of these agents with MS is debatable.

Pathogenesis

The presence of CNS inflammation is a hallmark of MS. This inflammatory process greatly increases in the CNS by the activation and deregulation of different cell types of the immune system. Activation and entry of myelin-specific lymphocytes into the CNS cause damage to oligodendrocytes, leading to demyelination. Most of the cells from the immune system can contribute toward demyelination, but the main process of demylation is mediated by antibody and complement. So far, it has been noticed that antibody and complement are responsible for lesions in 40%–50% of MS patients.

Myeloid cells may cause axonal damage by releasing molecules, such as glutamate, reactive oxy-gen species, and reactive nitrogen species. Besides, these cells decreased the expression of glutamate clearance. As a result of increased glutamate in the cerebrospinal fluid, MS patients would be vulnerable to degeneration (Yandamuri and Lane, 2016).

In addition, CD8 + T cells play an important role in MS pathogenesis. CD8 + T cells make up the largest percentage of lymphocytes found in the brain of MS patients. All neuroectodermal cells in MS lesions express MHC class I molecules, making them an excellent target for CD8 + T cells. In addition to their pro-inflammatory properties, CD8 + T cells can also suppress the immune system and down-regulate inflammation. However, experiments with perforin, an important regulator of cytotoxic damage to immune cells, have made it clear that CD8 + T cells present at MS lesions cause cytotoxicity, which could be the main source for demyelination and axonal damage (Sinha et al., 2015). Bystander CD4 + T cells do not contribute to the demyelinating process, but once CD4 + T cells move into the CNS and become activated against myelin antigen, these CD4 + T cells could be contributing directly toward the demyelination of CNS (Basdeo et al., 2016). Moreover, CD4 TH17 effector T cells are postulated to play a crucial role in the pathogenesis of MS (Bettelli et al., 2006). In addition to autoreactive immune cells against myelin and nerves, a progressive loss of the structure and function of neurons occurs. It has been reported that the alterations in the expression of miRNAs may play a crucial role in MS pathogenesis (Huang et al., 2016).

After demyelination, remyelination is possible, which could further damage the CNS. The ratio of demyelination to remyelination determines whether a patient will develop secondary progressive MS (SPMS) or relapse remitting MS (RRMS). If remyelination occurs before axonal damage, irreversible physiological damage can be prevented. None of the FDA-approved therapies target oligodendrocytes to stimulate remyelination, but it is a very interesting possibility for future therapeutic intervention (Huang et al., 2016).

Clinical manifestations

The majority of symptoms associated with MS can be directly attributed to inflammation, edema, demyelination, and/or axonal damage within the brain, spinal cord, and optic nerves. Clinical motor manifestations include weakness, stiffness, and/or pain in arms or legs, abnormal reflex activity, and spasticity. Often, the earliest symptoms of MS are somatosensory, including numbness and tingling. In MS, cerebral involvement is often accompanied by symptoms such as ataxia and intention tremor. Many individuals with

MS complain of increased urinary frequency, urgency, and incontinence. Bladder and bowel disturbances remain among the most disabling and embarrassing symptoms experienced by MS patients. Sexual symptoms are also very common among both men and women. Fatigue, sleep disturbances, depression, and deficits in cognitive functioning are also common. The clinical course of MS is often highly variable and is generally characterized by relapses or exacerbations and deterioration of neurologic function, which entitle relapsing remitting MS. The features of relapsing remitting MS are defined as "episodes of acute worsening of neurologic function followed by a variable degree of recovery, with a stable course between attacks." Approximately 80%−85% of patients are initially diagnosed with RRMS that evolves from an isolated demyelinating attack, which is characterized by multifocal inflammation along with varying degrees of axonal injury. A patient may experience disease progression with or without relapses and minor remissions; that clinical condition is defined as secondary progressive MS. It has been seen that 75% of RRMA patients will eventually develop the SPMS state. Primary progressive MS (PPMS) affects ∼10%−15% of MS patients. PPMS is defined as "disease progression from onset, with occasional plateaus and temporary minor improvements in clinical condition." The duration of MS varies significantly among MS patients. Some patients will live with MS for several decades, while about 10% will develop an acute, fulminant form of MS. Patients with an acute and fulminant form of MS show a rapid deterioration in their clinical signs and symptoms that have fatal consequences; these patients usually die within 1−3 years after the onset of disease. In general, the clinical spectrums among MS patients represent a benign disease and a low relapse rate, and these may never develop into secondary progressive disease. The heterogeneity of the clinical course of MS is shown to have a similar variation in its pathology.

Multiple Sclerosis lesions were recently segregated into four distinct subtypes. The general pathology of MS, the formation of demyelinating lesions in the CNS associated with infiltrating CD3+ T cells, activated macrophages, and microglia-containing myelin debris, and infiltrating B cells, is common to all forms of the disease. It is thought that MS lesions are mediated by soluble factors such as TNF-α and immunoglobulin deposition on the myelin sheath, and the local activation of the complement cascade. The diagnostic criteria for clinically definite MS (CDMS) include factors such as clinical history, MRI imaging, and CSF abnormalities. At present, there are no identifiable biomarkers that can predict the clinical subtype of MS. Similarly, there are no factors that can assist in predicting whether a patient diagnosed with MS will develop either a progressive or a benign version of the disease. The clinical and pathological heterogeneity in MS has made it important to either develop or identify reliable biomarkers. Several cytokines, immunoglobulins, MMPs, markers of axonal/neuronal injury, and apoptotic markers have been suggested to have potential as biomarkers, but these biomarkers need validation by rigorous durability trials.

Treatment

In MS, treatment strategies can be either acute or long term. During a relapse, the goal of acute treatment is to reverse neurological disability as well as to delay further neurological dysfunction, so that the normal function can be restored. This type of treatment for MS patients is in contrast to the goals of long-term treatments. The main objective of long-term treatment for MS patients is to decrease relapses (both severity and frequency), which could lend support to stopping the progression of disability. Patients experiencing a relapse, such as optic neuritis or transverse myelitis, are often administered high-dose corticosteroid first-line therapy. During progressive phases of the disease, patients may be prescribed immunosuppressive agents such as cyclophosphamide or mitoxantrone because the progressive phase is often accompanied by worsening inflammatory demyelination and axonal degeneration (Rommer et al., 2019).

In 1993, IFN-β was the first agent to demonstrate the significant clinical efficacy among patients suffering with RRMS. Although the exact disease-modifying effects of IFN-β in MS are unknown, several immunomodulatory mechanisms have been suggested. Presently, two forms of IFN-β, including IFN-βla (Avonex and Rebif) and IFN-βlb (Betaseron and Extavia), have been prescribed. Glatiramer acetate (Copaxone) is a synthetic mixture of polypeptides that has been approved to treat RRMS. Similar to IFN-β, glatiramer acetate is found to be not effective for progressive forms of MS. Natalizumab (Tysabri) is an alpha-4 integrin antagonist and is the first drug of an entirely new class of immune-directed therapies that has been approved by the FDA to treat relapsing MS. Natalizumab is a humanized recombinant monoclonal antibody that blocks leukocyte migration into the CNS by binding to α-4 integrins; these are components of the very late antigen-4 (VLA-4) complex constitutively expressed on the leukocyte surface. In monotherapy trials, natalizumab has been reported to reduce the risk for sustained progression of disability as well as decrease the frequency of relapses. Based on the current literature, natalizumab appears to be one of the most

effective agents to prevent relapses as well as to stop disease progression. Other monoclonal antibodies administered by intravenous (IV) infusion include lemtrada (alemtuzumab) and novantrone (mitoxantrone).

Currently, numerous other monoclonal antibodies are under investigation as potential therapies for MS; for example, anti-CD25 (daclizumab), anti-CD 20 (rituximab), and so on. A number of other agents are under investigation for possible future use in MS including secukinumab (a humanized monoclonal antibody to IL-17), RTL1000 (inhibitor of the activation of myelin-reactive T cells), firategrast (affect on the VLA-4 system) and Aimspro (neuropeptide stabilizer). Moreover, stem cell-based therapy might be considered as another approach for attenuating MS through regulating the immune system, although several challenges should be resolved. More investigations and clinical trials should be designed to assess the effectiveness of several drugs and approaches that can target both inflammatory and degenerative components of MS. These kinds of approaches may offer hope for individuals who are suffering from this debilitating disease (Mansoor et al., 2019; Agrawal and Yong, 2007; Hart and Bainbridge, 2016).

Experimental models

To gain ideas about MS mechanisms, a number of models have been developed. These experimental models fall into two categories: spontaneous models and induced models. Each model reflects characteristic features of MS patients and has its own merits and demerits.

Spontaneous models

Myelin basic protein mutant (taiep rat), proteolipid protein mutants (Rumpshaker and Jimpy mice), as well as gene-knockout animals (the myelin-associated glycoprotein (MAG) knockout, Thy1-EB3-YFP mice, and Thy1-XFP mice) show dysmyelination, altered neurotransmission and, in some instances, clinical disease. These models have frequently been used to study myelination.

Induced models

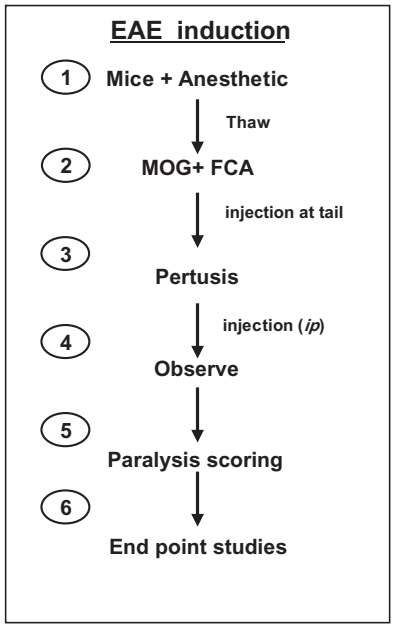
With chemically induced lesions, viral and autoimmune models are developed to show some evidence of demyelination, which is considered a pathological hallmark of MS. Direct injection of ethidium bromide or lysolecithin into the CNS produces demyelination. These induced models are usually effectively repaired once macrophages clear the myelin debris. For this reason, these models are rarely used at the present time. Besides, local administration of glutamate or nitric oxide donors induces axonopathy in mice and have also been used to understand mechanisms of axonal degeneration and regeneration (Luchtman et al., 2016). A number of viruses, including Semliki Forest Virus, Theiler's Murine Encephalomyelitis Virus, and a murine coronavirus have been found to induce disease by neurotrophic infection of the CNS, specifically oligodendrocytes (Lane and Hosking, 2010). Moreover, studies using immunodeficient RAG1 − / − mice have indicated that CD4 + and CD8 + T lymphocytes as well as macrophages are key contributors to demyelination in coronavirus-infected mice (Dandekar et al., 2001). Finally, experimental allergic encephalomyelitis (EAE) has received the most attention as a model for MS; this animal model is routinely used for testing different therapeutic strategies. Today, EAE as the most commonly used preclinical murine model of MS induced actively by the injection of defined encephalitogenic myelin protein epitopes plus CFA, or passively by the transfer of encephalitogenic myelin-sensitized T lymphocytes. Some of these EAE models also require the administration of the microbial-based immunologic adjuvant pertussis toxin (PT) (Yandamuri and Lane, 2016). EAE exhibits many clinical and histological features of MS and is caused by autoimmunity induced against antigens that are expressed either naturally or artificially in CNS (Denic et al., 2011).

Methodology and protocol

The method for EAE induction and preparation of antigens to induce EAE in C57BL/6 mice was adapted from the method described by Kafami et al. (2010). It is important for the successful induction of EAE to follow standard precautions for the use of animals. Female C57BL/6 mice that are 4- to 6-week old are used for the induction of EAE. Animals must adhere to the normal laboratory animal maintenance guide.

Protocol

Animals were immunized with the Hooke kits (Hooke labs, EK-0115, Lawrence, MA, USA). It is recommended to follow the manufacturer's instructions. A mesh was dampened in ether and put in a desiccator. The mouse was kept in the desiccator and observed until breathing slowed down to ascertain whether the mouse had been anesthetized. The mouse was removed from the anesthetic chamber and laid on its side. Two syringes were filled with 1 mL of myelin oligodendrocyte glycoprotein (MOG) emulsion with complete Freund's adjuvant. Each animal was given an injection of 200 μL. The needle was gently inserted into the subcutaneous space at the base of the tail, and 200 μL of emulsion was injected into the site. Since it



FLOW CHART 8.2 Important steps for the induction of experimental allergic encephalomyelitis (EAE).

was difficult to give the mouse a 200-μL injection, every mouse was given a 100-μL injection at two different sites on the same day. Immediately, and after 24 hours from the first injection, each mouse was given an intraperitoneal injection of pertussis toxin (100 μL/animal). The animal was observed until complete recovery, and it could move without a floppy gate. This procedure was repeated for all animals. After 2–3 days, the flanks were bulging in response to the subcutaneous injection (Flow Chart 8.2).

Clinical evaluation

One day before immunization, and from the 7th to the 35th day post-immunization, the animals were evaluated on a daily basis for signs of EAE following the 10-point score system (Table 8.4).

Three different clinical parameters were analyzed to compare the course of EAE (Fig. 8.3): (1) Severity of disease as the cumulative disease index (CDI) was the mean of the clinical scores of the animals; (2) disease onset, calculated as the mean of the first-day animals showed the signs of the disease in experimental animals; and (3) peak of disease score, which represented the mean of the highest clinical score of disease for all animals in each group. Tonicity of the tail and the distal part of the tail was ascertained by touching the tip of the tail. If the distal part of the tail was flaccid, the animal was removed from the base and observed to see if its tail remained erect or fell down (examined with the touch of the finger).

TABLE 8.4 Scoring criteria for paralysis in case of experimental allergic encephalomyelitis (EAE).

Scale	Clinical evaluations
0	No clinical disease
0.5	Partial tail paralysis
1.0	Complete tail paralysis
1.5	Complete tail paralysis and discrete hind limb weakness
2.0	Complete tail paralysis and strong hind limb weakness
2.5	Unilateral hind limb paralysis
3.0	Complete hind limb paralysis
3.5	Hind limb paralysis and forelimb weakness
4.0	Complete paralysis (tetraplegia)
5.0	Moribund or dead

After ascertaining tonicity of the tail, the gate of the animal was observed by keeping it in an open area (like a tabletop) and allowing it to walk. After checking the gate, the hind limb was observed by grabbing its tail. After that, the paralysis score was recorded for unilateral paralysis. By holding the animal in the palm of the hand, it was easy to evaluate the type of paralysis (unilateral or bilateral). It was noted whether the mouse rolled spontaneously in its cage or was dead with complete paralysis.

Histology

After 35 days, animals that had an EAE score of 5 and did not change for 3 more days were euthanized by Chloral hydrate injection (0.3 mL, ip). For histopathological evaluations, different tissues were harvested after dissecting the animals. Animals were placed appropriately in the dissection tray. A midline incision was made on the abdomen; the diaphragm was opened while ribbons were cut to expose the beating heart. The needle was inserted into the left ventricle of the heart while a phosphate-buffered saline (PBS) tap was allowed to fill the heart for 2 seconds. The right aorta was cut with small scissors to allow the PBS and PFA to circulate to exit. PBS allowed perfusion until the liver turned from red to yellow (~2–3 minutes). The best sign was when the liquid flowed out of the incised left aorta and turned from red to clear. Another indicator was when PBS entered the pulmonary system and emerged through the nose of the animal. Then, the PBS tap was closed and the tap was turned on for 4% paraformaldehyde (PFA, pH 7.4 at 37°C) to allow PFA to flow and perfuse the circulatory system for 3 minutes. Perfusion was evaluated by involuntary hind limb movement and tail shivering. When the mouse became stiff, it was time to stop PFA perfusion.

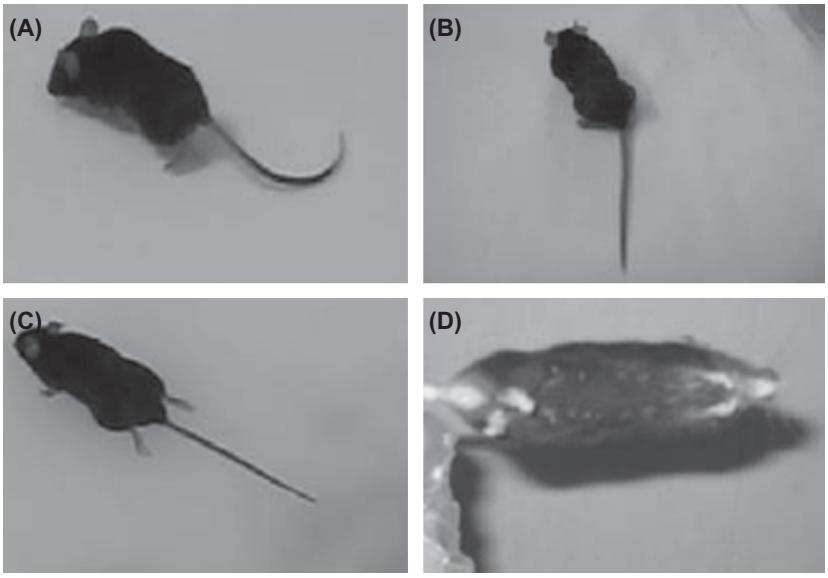


FIGURE 8.3 Clinical signs of EAE: (A) Loop tail; (B) flaccid tail and paralyzed limbs; (C) flaccid tail and paralyzed hind and front limbs; and (D) flaccid tail and weakness of hind limbs.

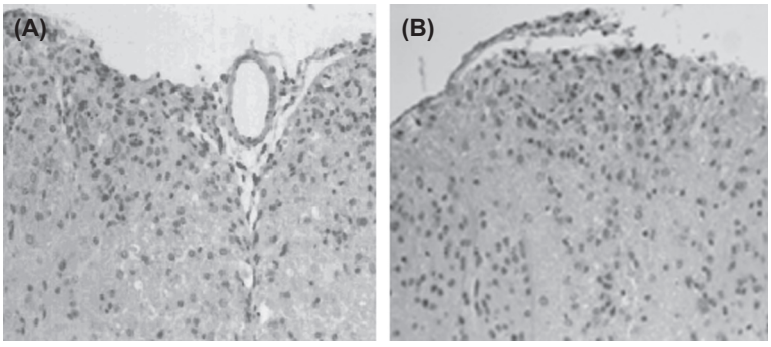


FIGURE 8.4 (A) Immunostaining for CD3 for T cells and (B) immunostaining for APP to assess acute axonal damage.

After the perfusion was complete with PFA, the system was washed with PBS to remove residual PFA. After perfusion, the various tissues of interest were harvested and stored in fresh 4% PFA for 3 days at 4°C. Then, these tissues were washed with PBS and the PFA-fixed tissue could be stored in PBS for a few months. These tissues were then available for sectioning and staining (Fig. 8.4).

Immunohistochemistry

For immunohistochemistry, the three sections showing the highest infiltrations were studied. An area $\geq 1.5 \times 107\ \mu m^2$ from the brain/spinal cord was selected and analyzed under $200\times$ magnification to assess the average number of positive cells per millimeter square and to quantify it on a computerized imaging system [BX51 microscope (Olympus, Hamburg, Germany) with AnalySIS software (Special SIS Docu; Soft ImagingSystem)] by planimetry. The inflammatory index had to be calculated as a percentage determined by dividing the number of visual fields with >10 CD3 T cells by the total number of visual fields examined. Detection of amyloid precursor protein (APP) was performed for acute axonal damage.

Enzyme-linked immunosorbent assay

To assess the content of circulating pro-inflammatory cytokines like IL-6, IL-4, IL-12, IL-10, TNF-α, and IFN-γ, enzyme-linked immunosorbent assay (ELISA) was employed. To evaluate the levels of different cytokines, blood was collected into tubes by a retro-orbital plexus method. The collected blood was kept in the tube to clot. After the clotting of the blood serum, it was separated and stored at -20°C. These serum samples were then used for the evaluation of different cytokines using the ELISA.

Real-time polymerase chain reaction

In order to quantify the mRNA of different pro-inflammatory cytokines such as TNF-α and IFN-γ, anti-inflammatory cytokines like IL-10, myelin-deteriorating matrix metalloproteinase MMP-9, and the content of

myelin basic protein (MBP 3–4), samples from animals had to be analyzed by real-time PCR. Animals were sacrificed with lethal injection and perfused with cold PBS. Then, the limbs and muscles were removed with scissors and the skin removed from these organs. A transverse cut was made at the base of the skull and vertebral column to separate them. The nasal bridge was broken with a small scalpel and the eyeballs removed. Very thin forceps were used under the skull bones to break it into pieces from the frontal to occipital lobes. The bony connection under the cerebellum was broken to expose the cerebellum. The broken bones of the skull needed to be removed. The nerve root connection with the brain was cut. The brain was removed and stored in liquid nitrogen. For the removal of the spinal cord, an oblique cut was made from the lateral side of the spinal cord (started from the cervical part) to the furthest part of the vertebral column (both sides). The spinal cord was then exposed by cutting the boney flap. The steps above were repeated to get to the coda aquina. The spinal cord was taken out by cutting its adhesion to the base. It was then stored in liquid nitrogen.

The frozen tissue sample was used for RNA extraction. First, the sample was homogenized by pushing and rotating it with a sterile glass homogenizer. Next, the homogenate sample was left on the bench top at room temperature (15°C–25°C) for 5 minutes to promote the dissociation of nucleoprotein complexes. Then, 200 μL of chloroform was added to the tube and the tube was shaken vigorously for 15 seconds. The tube containing the homogenate was placed on the bench top at room temperature for 2–3 minutes and then centrifuged again at 15,000 rpm for 15 minutes at 4°C. After centrifugation, the sample separated into three phases: an upper, colorless, aqueous phase containing RNA; a white interphase; and a lower, red, organic phase. The upper, aqueous phase was transferred to a new sterile Eppendorf tube. One volume (usually 600 μL) of 70% ethanol was added to the tube containing the aqueous phase and mixed thoroughly by vortexing. Visible precipitates after the addition of ethanol could then be noticed. Up to 700 μL of the sample was processed for total RNA extraction by using an RNeasy Mini spin column (Roche Germany) according to the kit instructions. After RNA extraction, RNA was quantified spectrophotometrically and the purity of RNA was ascertained by taking out a ration between the OD at 260 and 280 nm. A quantitative real-time reverse transcriptase PCR was performed to analyze the levels of mRNA of different cytokines using cytokine-specific primers. The first step was to perform cDNA synthesis by using a cDNA synthesis kit (TaKaRa, Japan), which was followed by a Syber Green I real-time PCR master mix kit (TaKaRa, Japan). A house-keeping gene (like the β-actin gene) was included in the study to compare the results.

Ethical issues

The use of laboratory animals in research is of major ethical concern. Much of the argument revolves around moral values. Today, there is a wide spectrum of views on animal rights. This has prompted the establishment of guidelines on the care and use of experimental animal models. The guidelines endorse some essential principles for the care and use of animals for scientific projects. The basis of these principles is to replace animals with other methods such as mathematical models, computer simulations, and in vitro biological systems, thus reducing the number of animals used in order to obtain valid results without unnecessary duplication, and finally, refining projects by selecting appropriate species and techniques to minimize pain or distress to animals using appropriate sedation or anesthesia.

As a researcher, one must always assume that procedures that cause pain to humans will cause pain in such situations in animals. Surgical procedures should be performed on anaesthetized animals. It should be kept in mind that if the animal would suffer severe pain during a procedure, or if at the end point cannot be alleviated swiftly, the animals must be killed humanely.

The transportation, housing, feeding, and handling of animals are also important. Housing facilities should be compatible with the needs of the species and equipped to achieve a high standard of animal care. The place should be designed to facilitate control of environmental factors. Cages should be comfortable and should fulfill behavioral requirements such as free movement and activity, bedding, contact with others of the same species, lighting, temperature, air quality, appropriate day/night cycles, and protection from excessive noise.

The population density of animals within cages should also be considered from an ethical standpoint. This statement refers to the need for the reader to operate in accordance with the guidelines at her/his academy.

Translational significance

The concept of translational research is to try to convert the results derived in animal models into a new understanding of disease mechanisms and therapeutics in human beings. It is a bridge from experimental models to clinical medicine. Over recent years, the

importance of this kind or research has progressively increased. Consequently, translational research is considered a key component to finding practical applications, especially within medicine.

With the improvement of technologies, significant progress has been made in producing various types of engineered experimental animal models based on a better understanding of the molecular and genetic principles of disease. As a result, any interventions in experimental models are more practical and repeatable when compared to patient-oriented research.

Various risk factors that are linked to, or even responsible for, differences in clinical results should also be considered as significant for the development of experimental models; this will enhance the translational value of experimental models. These risk factors can be categorized into genetic factors, acquired factors, and health conditions, which can be studied in models in a controlled manner. In medicine, the performance of successful translational research requires data from hospitals.

Clinical correlations

As we mentioned before, rheumatoid arthritis as a progressive debilitating disease is characterized by hyperplasia of synoviocytes leading to joint destruction and permanent deformity. Although the definite pathophysiology of RA is ambiguous, some evidence suggests that telomerase is also involved in the pathogenesis of this disease. Nobel laureates in physiology/medicine in 2009, Elizabeth Blackburn, Jack Szostak and Carol Greider, have solved a major problem of the chromosomal protection against degradation during cell divisions. They identified telomerase and a unique DNA sequence in the telomeres. Telomerase is a ribonucleoprotein enzyme that adds repeated units of TTAGGG to the ends of chromosomes. This enzyme is composed of an RNA component, called hTERT which serves as a template for addition of telomeric repeats. Although it is now known that the DNA sequence in the telomere attracts proteins that form a protective cap around the fragile ends of the DNA strands, a number of reports have mentioned a link between the increased telomerase activity of human tumor samples and degree of invasiveness. In patients with RA, an impaired telomerase enzyme and premature cellular ageing (senescence) of thymic naïve and memory T cells was reported. Moreover, transfection of rheumatoid arthritis synovial fibroblasts with vectors expressing antisense oligonucleotide against the hTERT component of telomerase enzyme has led to cytolysis of these cells that exhibit high telomerase activity. Taken together, their discoveries have shed light on disease mechanisms and stimulated the development of potential new therapies in experimental models.

There are many methods to evaluate telomerase activity, but we measured it by telomere repeat amplification protocol using TRAPeze telomerase detection kit (Intergen, Inc., USA) in animals treated with *Camellia sinensis* stew. In detailed, biopsies of synovial tissue were obtained aseptically from the knee joints of rat after the induction of CIA. Synovial tissue specimens were rinsed, minced, and digested with 0.2% collagenase in high-glucose DMEM containing 10% FBS and antibiotics. Following overnight incubation at 37°C, cells were collected, plated in culture flask, and allowed to reach confluency at 37°C in a humidified atmosphere of 5% CO2. After the lysis of equal number of cells which harvested from synovial tissue with the CHAPS lysis buffer, the telomerase was first extended for 30 minutes at 30ọC and then amplified by30 cycles of PCR. The products of PCR were detected by polyacrylamide gels and revealed by silver nitrate staining. Telomerase activity was calculated as the ratio of the intensity of telomerase ladders to the intensity of the 36-bp internal standard. In conclusion, we show that *C. sinensis* stew effectively suppresses collagen arthritis and a potent inhibitory effect on telomerase activity. So, natural products should continue to provide innovative lead compounds currently entering clinical trials. Recently, the circular plant peptide kalata B1 (cyclotide) was investigated by Thell et al. using the MS mouse model experimental autoimmune encephalomyelitis. According to their findings, treatment of mice with the cyclotide resulted in a significant delay and diminished symptoms of EAE by oral administration. Taken together, natural product should be considered as a candidate for the future investigations to possible implication for human health.

Conclusion

Using these above-mentioned models associated with other experimental models gives us such an opportunity to accomplish many findings in human medicine. Until now, many progresses in medical sciences have been achieved. The discovery of numerous types of antibiotics for controlling infectious disease and elimination some viral disease like smallpox might be considered as one of researcher and indeed experimental animals honor. Also, blood transfusions, open heart surgery, and other life-saving techniques have all been developed. Nevertheless, there are many unsolved subjects included cancer, aging, Alzheimer's disease, and acquired immunodeficiency syndrome in front of the society. Without no doubt, until to find another means for answering human beings dilemma,

the use of living animals in scientific research would be the best and applicable procedure. With all those valuable function, it is pivotal to consider ethical concerns over the quality of life of animals when you as a young researcher start to write a proposal.

World Wide Web resources

http://www.niams.nih.gov/

National Institutes of Health (NIH) site for the National Institute of Arthritis and Musculoskeletal and Skin Diseases.

http://www.ncbi.nlm.nih.gov/pubmed/

PubMed comprises over 22 million citations for biomedical literature from MEDLINE, life science journals, and online books. PubMed citations and abstracts include the fields of biomedicine and health, and cover portions of the life sciences, behavioral sciences, chemical sciences, and bioengineering. PubMed also provides access to additional relevant web sites and links to other NCBI molecular biology resources.

http://www.nlm.nih.gov/medlineplus/multiplesclerosis.html

MedlinePlus is the National Institutes of Health (NIH) site for patients and their families and friends. Produced by the National Library of Medicine, it brings you information about diseases, conditions, and wellness issues in easy-to-understand language. MedlinePlus offers reliable, up-to-date health information, anytime, anywhere, for free.

http://www.ebi.ac.uk/ipd/imgt/hla/

The IMGT/HLA Database provides a specialist database for sequences of the human major histocompatibility complex (HLA) and includes the official sequences for the WHO Nomenclature Committee for Factors of the HLA System. The IMGT/HLA Database is part of the international ImMunoGeneTics project.

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Glossary

Adhesion molecule A cell surface molecule (e.g., selectin, integrin, and member of the Ig superfamily) whose function is to promote adhesive interactions with other cells or the extracellular matrix. These molecules play crucial roles in cell migration and cellular activation in innate and adaptive immune responses.

Adjuvant A substance such as complete Freund's adjuvant (CFA) that enhances T- and B-cell activation, mainly by promoting the accumulation and activation of antigen-presenting cells at the site of antigen exposure. Adjuvants stimulate the expression of T-cell-activating co-stimulators and cytokines by antigen-presenting cells and may also prolong the expression of peptide–MHC complexes on the surface of these cells.

Autoimmune disease A disease caused by a breakdown of self-tolerance such that the adaptive immune system responds to self-antigens and mediates cell and tissue damage. Autoimmune diseases can be organ specific (e.g., thyroiditis or diabetes) or systemic (e.g., systemic lupus erythematosus).

CD molecules Cell surface molecules expressed on various cell types in the immune system that are designated by the "cluster of differentiation (CD) number."

Disease-modifying antirheumatic drugs (DMARDs) They contain medications from different classes including methotrexate, gold salts, hydroxychloroquine, sulfasalazine, ciclosporin, and azathioprine. DMARDs were often only partly effective and poorly tolerated in long-term therapy of autoimmune diseases.

Enzyme-linked immunosorbent assay (ELISA) A method of quantifying an antigen immobilized on a solid surface by use of a specific antibody with a covalently coupled enzyme. The amount of antibody that binds the antigen is proportional to the amount of antigen present and is determined by spectrophotometrically measuring the conversion of a clear substrate to a colored product by the coupled enzyme.

Experimental autoimmune encephalomyelitis (EAE) This is an animal model of multiple sclerosis, an autoimmune demyelinating disease of the central nervous system. EAE is induced in rodents by immunization with components of the myelin sheath (e.g., myelin basic protein) of nerves, mixed with an adjuvant. The disease is mediated in large part by cytokine-secreting CD4 + T cells specific for the myelin sheath proteins.

Granulocyte-monocyte colony-stimulating factor (GM-CSF) A cytokine made by activated T cells, macrophages, endothelial cells, and stromal fibroblasts that acts on bone marrow to increase the production of neutrophils and monocytes. GM-CSF is also a macrophage-activating factor and promotes the differentiation of Langerhans cells into mature dendritic cells.

Granuloma A nodule of inflammatory tissue composed of clusters of activated macrophages and T lymphocytes, often associated with necrosis and fibrosis. Granulomatous inflammation is a form of chronic delayed-type hypersensitivity, often in response to persistent microbes or to particulate antigens that are not readily phagocytosed.

Granzyme A serine protease enzyme found in the granules of CTLs and NK cells is released by exocytosis, enters target cells, and proteolytically cleaves and activates caspases and induces target cell apoptosis.

Homeostasis In the adaptive immune system, the maintenance of a constant number and diverse repertoire of lymphocytes, despite the emergence of new lymphocytes and the tremendous expansion of individual clones that may occur during responses to immunogenic antigens. Homeostasis is achieved by several regulated pathways of lymphocyte death and inactivation.

Human leukocyte antigens (HLA) MHC molecules expressed on the surface of human cells. Human MHC molecules were first identified as alloantigens on the surface of white blood cells (leukocytes) that bound serum antibodies from individuals previously exposed to other individuals' cells.

Interferons A subgroup of cytokines originally named for their ability to interfere with viral infections, but that have other important immunomodulatory functions. Type I interferons include interferon-α and interferon-β, whose main functions are antiviral; type II interferon, also called interferon-γ, activates macrophages and various other cell types.

Interleukins Any of a large number of cytokines named with a numerical suffix roughly sequentially in order of discovery or molecular characterization (e.g., interleukin-1 and interleukin-2). Some cytokines were originally named for their biological activities and do not have an interleukin designation.

Lipopolysaccharide (LPS) A component of the cell wall of gram-negative bacteria that is released from dying bacteria and stimulates many innate immune responses, including the secretion of cytokines, induction of microbicidal activities of macrophages, and expression of leukocyte adhesion molecules on endothelium. LPS contains both lipid components and carbohydrate moieties.

Major histocompatibility complex (MHC) molecule A heterodimeric membrane protein encoded in the MHC locus that serves as a peptide display molecule for recognition by T lymphocytes. Two structurally distinct types of MHC molecules exist. Class I MHC molecules are present on most nucleated cells, bind peptides derived from cytosolic proteins, and are recognized by CD8 + T cells. Class II MHC molecules are restricted largely to dendritic cells, macrophages, and B lymphocytes, bind peptides derived from endocytosed proteins, and are recognized by CD4 + T cells.

Matrix metalloproteinase (MMP) MMPs are a family of highly conserved endopeptidases dependent on Zn2 + ions for activity. MMPs can collectively cleave most extracellular matrix. At present, 25 vertebrate MMPs and 22 human homologs have been identified and characterized. MMPs participate in many physiological processes, such as embryonic development, organ morphogenesis, blastocyst implantation, ovulation, nerve growth, cervical dilatation, postpartum uterine involution, mammary development, endometrial cycling, hair follicle cycling, angiogenesis, inflammatory cell function, apoptosis, tooth eruption, bone remodeling, and wound healing.

Myelin oligodendrocyte glycoprotein (MOG) MOG is a CNS-specific type I membrane glycoprotein of the immunoglobulin superfamily expressed mainly on the outermost layer of the myelin sheath, making it an ideal target for antibody-mediated demyelination. It is highly immunogenic, and unlike other myelin proteins used to induce EAE, is unique in inducing both an encephalitogenic T-cell response and a demyelinating response in EAE.

Multiple sclerosis (MS) A chronic inflammatory demyelinating disorder of the central nervous system. The majority of symptoms associated with MS can be directly attributed to inflammation, edema, demyelination, and/or axonal damage within the brain, spinal cord, and optic nerves.

Nitric oxide (NO) A biologic effector molecule with a broad range of activities that in macrophages functions as a potent microbicidal agent to kill ingested organisms.

Pannus Formation of locally invasive synovial tissue is a characteristic feature of rheumatoid arthritis.

Perforin A protein that is homologous to the C9 complement protein and is present in the granules of CTLs and NK cells. When perforin is released from the granules of activated CTLs or NK cells, it promotes the entry of granzymes into the target cell, leading to apoptotic death of the cell.

Rheumatoid arthritis (RA) An autoimmune disease characterized primarily by inflammatory damage to joints and sometimes inflammation of blood vessels, lungs, and other tissues. CD4 + T cells, activated B lymphocytes, and plasma cells are found in the inflamed joint lining (synovium), and numerous pro-inflammatory cytokines, including IL-1 and TNF, are present in the synovial (joint) fluid.

Reverse transcriptase (RT) An enzyme encoded by retroviruses, such as HIV, that synthesizes a DNA copy of the viral genome from the RNA genomic template. Purified reverse transcriptase is

used widely in molecular biology research for purposes of cloning complementary DNAs encoding a gene of interest from messenger RNA.

TH1 cells Subset of CD4 + helper T cells whose principal function is to stimulate phagocyte-mediated defense against infections via secretion of a group of cytokines, including IFN-γ.

TH2 cells Subset of CD4 + helper T cells whose principal functions are to stimulate IgE and eosinophil/mast cell-mediated immune reactions via a particular set of cytokines, including IL-4 and IL-5.

TH17 cells Subset of CD4 + helper T cells that are protective against certain bacterial infections and also mediate pathogenic responses in autoimmune diseases.

Tumor necrosis factor (TNF) A cytokine produced mainly by activated mononuclear phagocytes that stimulates the recruitment of neutrophils to sites of inflammation.

TNF-α blocking agents A group of biological disease-modifying antirheumatic drugs such as Etanercept (a soluble TNF-α receptor) and infliximab (a monoclonal antibody).

Very late antigen (VLA) The set of integrins that shares a common beta-1 chain.

Abbreviations

μg	microgram
μL	microliter
μm	micrometer
ACPA + RA	anticitrullinated protein antibodies-positive RA
APP	amyloid precursor protein
CD	cluster of differentiation
CDI	cumulative disease index
cDMARD	conventional disease-modifying antirheumatic drugs
CDMS	clinically definite MS
CFA	complete Freund's adjuvant
CNS	central nervous system
CSF	cerebrospinal fluid
DMARDs	disease-modifying antirheumatic drugs
DNA	deoxy ribonucleic acid
EAE	experimental allergic encephalomyelitis
EDTA	ethylene diamide tetra acetic acid
ELISA	enzyme-linked immune sorbent assay
FDA	food and drug administration
Fig	figure
GM-CSF	granulocyte macrophage colony stimulating factor
H&E	hematoxyline and eosin
HHV-6	human herpes virus 6
HLA	human leukocyte antigen
IFN	interferon
IL	interleukin
LPS	lipopolysaccharide
MAG	myelin-associated glycoprotein
MBP	myelin basic protein
MCP-1	monocyte chemoattractant protein
MDP	muramyle dipeptide
mg	milligram
MHC	major histocompatibility complex
MIP-1	macrophage inflammatory
ml	milliliter
mm	millimeter
MMP	matrix metalloproteinase
MMPs	matrix metalloproteinases
MOG	myelin oligodendrocyte glycoprotein
MRI	magnetic resonance imaging
MS	multiple sclerosis
ng	nanogram
NO	nitric oxide
NSAIDs	nonsteroidal antiinflammatory drugs
OD	optical density
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PLP	proteolipid protein
PP-MS	primary progressive multiple sclerosis
PR-MS	progressive relapsing multiple sclerosis
RA	rheumatoid arthritis
RNA	ribonucleic acid
RPM	revolutions per minute
RR-MS	relapsing-remitting multiple sclerosis
RT	reverse transcriptase
RT-PCR	real-time polymerase chain reaction
SNPs	single nucleotide polymorphisms
SPMS	secondary progressive MS
TDM	trehalosedimycolate
TNF	tumor necrosis factor
Treg	regulatory T cell
VLA-4	very late antigen-4

Long-answer questions

1. Describe the significance of animal modeling in biotechnology?
2. How CIA is induced and how the ability of medications is evaluated in mice?
3. Discuss about different types of animal model to study the pathogenesis of rheumatoid arthritis?
4. Why the presence of inflammation in the CNS is considered as a hallmark of Multiple Sclerosis?
5. Explain the various methods for evaluation of experimental models of Multiple sclerosis?

Short answer questions

1. What is the reason of reportedly experiencing different animal models for studying the pathogenesis of Rheumatoid arthritis?
2. Give an example which shows the impact of the epigenetics in the initiation of RA?
3. Which types of evaluation should be performed after "collagen-induced arthritis" aroused?
4. What are the "intervening factors" in experimental model of multiple sclerosis?
5. After the activation of the immune system, which type of lymphocytes enters into central nervous system (CNS)?

Answers to short answer questions

1. There are many experimental models that resemble RA in different respects. Since RA is a heterogeneous disease there is probably a need for different animal models that each reflect a characteristic feature of a particular subgroup of RA patients or illustrate particular aspect of the disease.

2. The epigenetics of RA have also been responsible in the initiation of RA. Since the concordance of rheumatoid arthritis in identical twins is not 100% other nongenetic factors also play a role in the disease etiology.
3. Daily clinical assessment according to a macroscopic scoring system, histological processing and assessment of arthritis damage, radiographic evaluation by an investigator blinded to the treatment protocol on day 35.
4. Age, weight, and possible infectious disease in animals should be considered as the intervening factors.
5. Following activation, myelin-specific lymphocytes enter into the CNS and oligodendrocytes are damaged.

Yes/no type questions

1. The HLA class II locus is the most important risk factor for anticitrullinated protein antibodies (ACPA)-positive RA (ACPA + RA).
2. In the CIA model, treatment with IL-35 induced regression of arthritis via expansion of cytotoxic T cells
3. Tacrolimus induces T-cell activation by specifically inhibiting calcineurin pathway.
4. Adoptive transfer of T-helper 17 cells in the IL-6R knockin mouse induced arthritis.
5. The most widely used arthritis models in academia is CIA model in mice and rats.
6. Among genetic factor, both MHC region and non-MHC loci have been indicated in MS.
7. Increased glutamate in the cerebrospinal fluid of MS patients would protect them from the axonal degeneration.
8. Natalizumab blocks leukocyte migration into the CNS by binding to ICAM.
9. EAE induced actively by injection of the microbial-based immunologic adjuvant pertussis toxin.
10. Housing facilities should be compatible with the needs of the species and equipped to achieve a high standard of animal care.

Answers to yes/no type questions

1. Yes—The most important risk factor for ACPA + RA is the HLA class II locus.
2. No—IL-35 induced regression of arthritis via expansion of Treg cells.
3. No—Tacrolimus blocks T cell activation by specifically inhibiting calcineurin pathway.
4. Yes—Arthritis can be rapidly induced with adoptive transfer of T-helper 17 cells in the IL-6R knockin mouse.
5. Yes—currently, collagen type II-induced arthritis in mice is one of the most widely used arthritis models in academia and industry.
6. Yes—In addition to associations within the MHC region, other non-MHC loci reached genome-wide significance in MS.
7. No—As a result of increased glutamate in the cerebrospinal fluid, MS patients would be vulnerable to degeneration
8. No—Natalizumab blocks leukocyte migration into the CNS by binding to α-4 integrins
9. No—EAE model of MS induced actively by injection of defined encephalitogenic myelin protein epitopes plus CFA.
10. Yes—Housing facilities should be compatible with the needs of the species and equipped to achieve a high standard of animal care.

CHAPTER

9

HIV and antiretroviral drugs

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Summary

Acquired immune deficiency syndrome (AIDS) is the terminal stage of human immunodeficiency virus (HIV) infections. HIV is one of the dreaded infectious diseases of the late 20th century. This chapter provides information about the history, discovery, epidemiology, and replication of HIV. Anti-HIV drugs and novel anti-HIV drug targets for development of new drugs are also discussed.

What you can expect to know

Acquired immune deficiency syndrome (AIDS) is considered as the final chapter in the life of HIV-infected patients. Even though we have many choices for antiretroviral drugs, the fact is, "*HIV can be controlled but cannot be cured*." HIV is a retrovirus and its genome consists of nine genes along with a long-terminal repeat (LTR), which produces 15 different proteins during replication.

A better understanding of HIV replication has been the cause for the development of anti-HIV drugs that inhibit different stages of HIV replication. At present, we can block HIV replication by inhibiting enzymes such as reverse transcriptase (RT), protease, and integrase. Successful treatment strategies are ones that use a combination of different drugs to control HIV replication. Some other steps of HIV infections are being explored for the development of a new group of drugs. We have also discussed the classification of different clinical stages of HIV patients, which is useful and crucial to make decisions for the right choice of drugs. Lately, bone marrow transplantation is on the horizon and may open new avenues to treat HIV. The methods to evaluate drug toxicity as well as anti-HIV effects of drugs are provided to aid better understanding. There is no doubt that improved antiretrovirals have increased the life-span of HIV patients post-HIV infection, which has brought up another new health concern: NeuroAIDS.

History and methods

Introduction

Infectious diseases and humanity are closely intertwined with each other. Infections have been a part of human life since the inception of humanity. As human beings we are vulnerable to infections since the time of birth, and in certain circumstances, one can be infected even before birth (prenatal infections). Infections and human life are closely linked; therefore, the significance of infection in our lives can neither be denied nor ignored.

Vaccines and antibiotics have enormous implications for human welfare because they have decreased morbidity and mortality among the human population. The discovery of antibiotics and vaccines started to give us the idea that *we are winning the war against microbes/infections*. But is this true?

On one hand, the 20th century became famous for the eradication of some important diseases such as small pox, polio, etc., while on the other hand, we have seen a sudden eruption of entirely new diseases such as SARS, HIV, Ebola, etc. Out of these, HIV has received tremendous attention from various quarters of human society, including government organizations and nongovernment organizations, religious groups, clinicians, scientists, and even the media. HIV is an infectious disease that has received unexpectedly more than the desired attention. Nobody knows the answer

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to this intense public response seen for HIV infection, but it may be due to the shock that the initial cases reported were from one of the most resourceful nations, the United States.

Discovery and origin of HIV

It has been over 30 years since unique cases of infection were reported for the first time. In 1981, HIV infections were not known, and those initial patients were reported to be suffering from a devastation of the immune system. The compromised immune system led to a fatal clinical condition now known as acquired immune deficiency syndrome (AIDS). There is no doubt that AIDS is the ultimate chapter of life in HIV patients. AIDS is clinically defined as a condition with $CD4^+$ T-lymphocyte counts of less than 200 cells per milliliter of blood along with the presence of AIDS-defining illness such as HIV-associated dementia (HAD), HIV wasting syndrome, and AIDS-defining cancers such as Kaposi's sarcoma, etc. It is a general belief that HIV is a single type of virus; however, HIV is actually of two different types: HIV-1 and HIV-2. HIV-1 is more prevalent with a global presence and fatal consequences, while HIV-2 is geographically isolated, confined mostly to the African continent and is less pathogenic, which means patients who are infected with HIV-2 can survive a lot longer when compared with those infected with HIV-1. From this point, in the chapter we will use HIV to mean HIV-1.

History of HIV and AIDS

The initial cases of HIV infection were reported in five gay men from Los Angeles, California. These five patients were suffering from *Pneumocystis carinii* pneumonia (PCP). This report was published in *Morbidity and Mortality Weekly Report*, a publication from the Centers for Disease Control and Prevention (CDC) (Gottlieb et al., 1981). PCP is a disease that is normally not reported among the general population; PCP infections are commonly observed only in immunocompromised/immunodeficient patients. In 1981, these PCP patients were not reported to be infected with HIV or suffering from AIDS because until that time, medical science was neither aware of HIV and AIDS, nor had these words even been coined. As a matter of fact, the world community is indebted to a drug technician, Ms. Sandra Ford at the CDC, who brought this unusual observation to the attention of her bosses. She noticed an unusually high number of requests for a unique drug, pentamidine, which is used to treat PCP. She said in an interview to Newsweek.

> A doctor was treating a gay man in his 20s who had pneumonia. Two weeks later he called to ask for a refill of a rare drug that I handled. This was unusual – nobody ever asked for a refill. Patients usually were cured in one 10-day treatment or they died.

Realizing the gravity of the situation, the CDC formed a "Task Force on Kaposi's Sarcoma and Opportunistic Infections (KSOI)." By this time, very little information about this new deadly disease was available to anyone; therefore, people were skeptical about the mode of transmission. This disease did not have any official name, so initially, it was called lymphadenopathy or KSOI. It was observed that this disease was closely associated with the gay community; therefore people started calling it gay compromise syndrome (GCS), gay-related immune deficiency (GRID), acquired immunodeficiency disease, gay cancer, and community acquired immuno dysfunction. The growing epidemic led to the development of various organizations to deal with this deadly new disease both in the United States and United Kingdom. First and foremost, these organizations started advising gay men to follow safe sex practices. By the end of 1982, a 20-month old child who had received multiple blood transfusions died due to infections related to AIDS. The death of this child raised the alarm for blood banks: as blood banks needed a safe blood supply. With time, it was noticed that the disease was reported from all walks of life, including nonhomosexuals. Thus, names for the diseases such as GRID and GCS became completely irrelevant. Now the situation was more confusing among clinicians and doctors and they needed to find an appropriate name for this deadly disease. Ultimately, on July 24, 1982, a meeting was convened in Washington, D.C., and the name "AIDS" was coined.

KSOI, which was formed to look after this new disease, started following and monitoring cases throughout the United States. What was most surprising was that within a few months after the formation of KSOI, reports of cases of this disease started pouring in from all over the United States. Finally, the case of a hemophiliac from Haiti with AIDS was reported, which led to the speculation that the disease might have originated in Haiti. As time passed, it became clear that AIDS was affecting a wider group of people than was previously thought. By 1982, evidence started to accumulate that AIDS also existed among European nations and on the African continent. The number of cases reported in the United States was alarmingly high. By 1983, in the United States alone, 3064 cases of AIDS had been reported, out of which 1292 patients had died.

Active research was going on in France and the United States to identify the causative agent of AIDS. Dr. Luc Montagnier from the Pasteur Institute in Paris was

the first one to report the causative organism for AIDS. In 1983, his group named this virus as lymphadenopathy-associated virus (LAV). Montagnier's work was published in the May 20, 1983, issue of *Science* and was jointly authored by the 12 members of the team (Barre-Sinousi et al., 1983). In 2008, Luc Montagnier was finally awarded the Nobel Prize in Physiology or Medicine for his contribution to the discovery of HIV. Unfortunately, Montagnier's publication did not receive the required attention from the scientific community. Almost a year later, in the May 4, 1984, issue of *Science*, Dr. Robert Gallo from the National Cancer Institute, Bethesda, MD, published his work. His team named the virus as human T-cell lymphotrophic virus-1 (HTLV-I). Gallo's group consisted of 13 members (Gallo et al., 1984). Dr. Gallo's publication received a great deal of attention from the scientific community. Later, in 1985, both groups published genetic sequences of their samples, and they reported >90% similarity in sequences. The discovery of HIV was marred with numerous controversies. The Pasteur Institute in Paris, France, filed a patent 4 months before Dr. Gallo's group filed their patent. However, the US Patent and Trademark Office granted the patent to Dr. Gallo for blood testing of HIV. This chain of events led to a dispute between Dr. Gallo and Dr. Montagnier: who discovered HIV? This dispute received international attention and was finally resolved by a meeting between Ronald Regan, the president of the United States, and Jacques Chirac, the French prime minister. During this meeting, it was decided that Dr. Luc Montagnier and Dr. Robert Gallo had made equal contributions toward the discovery of HIV.

The first case of HIV transmission from mother to child by breastfeeding was reported in 1985. In the mean time, the two different names for the same virus (HTLV-I and LAV) started to create confusion; therefore, it became necessary to find a common name for this organism. In 1986, the International Committee on Taxonomy of Viruses decided to drop both names (LAV and HTLV-I) and adopted a new one: human immunodeficiency virus (HIV).

In 1987, World Health Organization (WHO) established special programs on AIDS, and AIDS earned the reputation as the only disease to be debated in the United Nations General Assembly. In 1987, azidothymidine (Azt) was approved by the Food and Drug Administration (FDA) for the treatment of HIV. In 1988, December 1st was declared as World AIDS day, and in 1991, a Red Ribbon was adopted as the symbol for AIDS awareness. In 1996, a joint United Nations Program on HIV became operational; it was called UNAIDS. In 1996, during the 11th International AIDS conference in Vancouver, Canada, a report on the efficacy of antiretroviral therapy was presented. In 2001, the UN Secretary General granted funds to fight HIV at the global level. Later, there was the G8 summit that has declared for Universal Access of Antiretroviral Treatment (2010).

Global disease burden

Since the constitution of UNAIDS by the United Nations, UNAIDS publishes annual reports about the status of HIV and AIDS. These reports provide comprehensive information about HIV and AIDS at both global and national levels. The UNAIDS annual report is considered to be the most authentic document for epidemiological facts. As per the UNAIDS Report (2012), ~34 million people are living with HIV with an expected range of ~31.4–35.9 million. Although there is no gender bias with regards to HIV and AIDS, the most saddening truth of the UNAIDS report is that ~3.3 million children are also infected with HIV; children in this category are up to the age of 15 years. Out of different regions in the world, the African subcontinent is the worst affected by HIV infections with ~23.5 million. The range is actually ~22.1–24.8 million or two-third of the total people infected with HIV in the world. African countries still have the highest incidences of new HIV infections. The highest number of deaths due to AIDS is in this continent. This is indicative of the fact that even though there are serious efforts to control HIV infection at global and international levels, these measures are not as effective as they should be. The Australian continent is least affected with HIV infections and AIDS. Globally, ~7400 new individuals get infected with HIV every day, while ~5500 people die every day due to HIV and AIDS-related causes. The difference in death and new infection rates leads to the accumulation of ~1900 new HIV seropositives to the existing pool of HIV-infected people. On an annual basis, these figures could add up to ~700,000 people per year, which is close to the population of any of the capital cities of an average European nation (barring a few large cities). Still, the majority of HIV seropositives and AIDS patients are from developing nations or underdeveloped nations. Such a high prevalence of HIV in these nations is indicative of multiple factors such as poor implementation of surveillance programs, poor health care, poor living conditions, lack of resources, social conditions, etc. There is no doubt that more serious efforts are required at local, national, and international levels to control this epidemic in those nations that are worst affected. Despite these devastating statistics, a closer look at new data offers new insights and hope:

1. Since 1996, the prevalence of HIV infection has not seen a significant increase in numbers.
2. There is no doubt that long-term HIV survivors and new infections are major contributing factors

toward the accumulation in the existing pool of HIV patients.

3. There are certain nations that have shown a decline in the number of HIV patients.
4. There is a decrease in AIDS-related deaths, which is attributable to improved antiretroviral drugs, an improvement in health care facilities, and access to better antiretroviral drugs for HIV-seropositive individuals.
5. There is no doubt that new HIV infections are declining on a global level, although the decline in HIV-seropositive individuals varies from one nation to the next.

Clinical stages of HIV

HIV infections are prevalent across the globe. For effective management of HIV patients and to apply appropriate treatment strategies, there is a need for classification of different clinical stages of HIV infection.

With time, various diagnostic tests were developed, along with improved drugs to treat HIV infection. A combination of drugs and diagnostic tests has been of great help in generating a reasonable amount of data regarding clinical conditions of HIV patients, as well as for the interpretation of laboratory data. Due to the nonavailability of any over-arching guidelines, every clinician, group of clinicians, and laboratory scientist was interpreting data to the best of their ability to treat patients. This ambiguous interpretation turned out to be a major impediment to develop the best possible treatments across the board. Nonavailability of coherent guidelines became a major concern for various issues related to HIV research, awareness, and treatment programs such as (1) HIV surveillance programs, (2) epidemiology, (3) health status of patients, (4) design of new diagnostic tools, (5) recommendation of different diagnostic tests, (6) appropriate treatment strategies, (7) maintenance of clinical data, (8) consultation and advice from other clinicians for the same HIV patient(s), and (9) comparison of prevalence from one geographical location to the next, etc.

The only answer to overcome this bottleneck was to classify clinical conditions into different categories based on the existing clinical and laboratory data. This task was taken up for the first time was by the CDC in Atlanta, GA. In 1986, they developed the first ever classification of various clinical conditions among HIV patients. This classification was tremendously useful toward the clinical management of HIV patients. The CDC keeps revising this classification on the basis of improved understanding of clinical conditions and the results of diagnostic data. Later, the WHO also developed a new classification for HIV infection that is considered to be a reference standard around the world. Although various other classification systems do exist in the literature, the most widely accepted and followed systems are those developed by the CDC and WHO. These two classification systems have the same purpose, but they cater to the needs of HIV patients under different circumstances.

The CDC initially developed a classification system to address the need for better understanding of HIV infections and to develop strategies to control it in the United States (Centers for Disease Control and Prevention, 1992). The criteria for the CDC's classification relies more on diagnostic end-points (i.e., CD4 counts and viral loads). It has been clearly demonstrated that these two clinical parameters are inversely related to each other. Undoubtedly, the CDC system has certain important end-points that are helpful to understand as well as useful for the improvement of treatment strategies for HIV patients on the basis of diagnostic tests.

It is unfortunate that all HIV-infected people do not have access to the resources available in countries such as the United States In developing countries, most HIV patients do not have access to either the necessary facilities to monitor disease progression via expensive diagnostic tests or the expensive and effective drugs necessary for the treatment. WHO gave consideration to both of these facts when addressing the problem for those individuals living in less fortunate conditions. This was the major motivating factor for WHO to develop a classification system in 1990 that was based on clinical signs and symptoms.

Classification of clinical stages

The WHO clinical staging system for HIV/AIDS was developed in 1990. The WHO system emphasizes clinical parameters as the guideline for clinical decision-making and is useful in resource-limited settings. This method of clinical staging can be effectively used even without information about CD4 counts or other laboratory diagnostic tests, and it is important to realize that CD4 counts are not the prerequisite for initiating antiretroviral therapy. In 2005, WHO revised its classification system for different clinical stages of HIV infection (WHO Guidelines, 2005). WHO classification has delineated four stages for HIV patients, designated Stages I–IV. A detailed description of these four different stages is given in Table 9.1.

Stage I: primary HIV infection or seroconversion stage

Within 2–4 weeks after initial exposure of HIV, primary infection represents acute HIV infection. During

TABLE 9.1 WHO classification of clinical stages of HIV infections.

Stage	Name	Signs and symptoms
Stage I	Primary HIV infection (seroconversion)	Asymptomatic
	Acute retroviral syndrome	
		CD4 >500 cells/mm^3
Stage II	Asymptomatic phase	Moderate weight loss
		Recurrent respiratory tract infections
		Herpes zoster
		Angular cheilitis
		Recurrent oral ulcerations
		Papular pruritic eruptions
		Seborrhoeic dermatitis
		Fungal nail infections of fingers
		CD4 >350−499 cells/mm^3
Stage III	Generalized lympadenopathy	Severe weight loss
		Unexplained chronic diarrhea
		Unexplained persistent fever
		Oral candidiasis
		Oral hairy leukoplakia
		Pulmonary tuberculosis (TB)
		Acute stomatitis, gingivitis, or periodontitis
		CD4 >200−349 cells/mm^3
Stage IV	Symptomatic phase	HIV wasting syndrome
		Pneumocystis pneumonia
		Chronic herpes simplex infection
		Esophageal candidiasis
		Extrapulmonary TB
		Kaposi's sarcoma
		CNS toxoplasmosis
		HIV encephalopathy, etc.
		CD4 <200 cells/mm^3

this stage, large numbers of viruses are produced in peripheral blood of the infected host, which leads to the activation of immune response. This causes production of protective antibodies as well as a cytotoxic T-cell response. This stage is known as "Seroconversion." During Stage I, the most common symptoms are quite similar to the symptoms of mild influenza (e.g., fever, diarrhea, sore throat, headaches); more than 65% of the cases of HIV infection are presented with these symptoms during early-stage infection. Diagnosis of Stage I for HIV infection is based on detection of either HIV antigens or anti-HIV antibodies in blood samples. Confirmatory tests are usually recommended to those individuals who are negative in preliminary tests at the seroconversion stage (Stage I) but considered to be high-risk cases as per their personal history.

Stage II: asymptomatic stage

The next stage of HIV infection is known as the asymptomatic stage. This stage usually lasts for ~10 years post-HIV exposure. During the asymptomatic phase, viral replication slows down significantly, but replication does not stop. At Stage II, HIV infection drops to low levels in peripheral blood, but antibodies against HIV can be detected on a regular basis. The CD4 T-cell counts are usually >500 cells/mm^3. Some patients can show CD4 T-cell counts <500 cells/mm^3.

Stage II can be further extended with the use of the right antiretroviral drugs. At present, antiretroviral drugs can prolong this stage up to 20 years or more post-HIV infection. The main objective of any antiretroviral treatment (ART) is to keep viral replication to a minimum so that immune-status deterioration can be minimized.

Stage III: persistent generalized lymphadenopathy

There are no specific signs or symptoms for this clinical stage. Stage III comes after the asymptomatic phase. In general, persistent generalized lymphadenopathy is observed which lasts for 3 months or more. Swollen lymph nodes are commonly observed, with a size of >1 cm in diameter. These patients otherwise look healthy, but nonspecific lymphadenopathy persists in these HIV seropositives, and lymph node biopsy is not routinely recommended for HIV patients showing Stage III.

Stage IV: symptomatic stage

Symptomatic HIV infections are mainly presented with higher incidences of various opportunistic infections and AIDS-associated cancers. This stage of HIV infection is often characterized with multisystem diseases and infections that affect various systems of the body. A rapid decline in immune status is a hallmark of this stage and is due to a significant increase in HIV replication. Increased HIV replication results in rapid disease progression. Some constitutional symptoms such as fever, malaise, etc., appear at this stage, which can be treated easily. It is difficult to control HIV replication at this stage. The choices for therapeutic interventions for these patients are limited and not very

helpful. These symptoms usually signal the terminal stages of illness. HIV patients at Stage IV require counseling about the final outcome of the disease; it is important that the patient be well informed. There is always the possibility of variations in the spectrum of opportunistic infections, which can vary from one geographical location to another (Walker, 2006).

WHO and the CDC update their classifications of the clinical stages of HIV infections on a routine basis. With time, new clinical symptoms are also emerging among long-term HIV seropositives. A new and emerging health concern among long-term HIV seropositives is NeuroAIDS, which is discussed later in this chapter.

Molecular biology of HIV

HIV is roughly spherical in shape and measures about 120 nm in diameter. Taxonomically, HIV belongs to the *Lentivirus* genus and the *Retroviridae* family. Being a member of the *Retroviridae* family, the genetic material in HIV is a single-stranded, positive-sense RNA. HIV does not have any DNA in its genome (Fig. 9.1). To utilize RNA as a genetic material, HIV has the RT enzyme, which helps to transfer genetic information to its new progeny. The HIV genome is ~9.8 kb with nine genes (Fig. 9.2), excluding LTRs, and produces 15 viral proteins (Abbas et al., 2008; Frankel and Young, 1998).

These 15 proteins can be divided into subcategories: (1) structural proteins (Gag, Env, Pol), (2) regulatory proteins (Tat, Rev), and (3) accessory proteins (Nef, Vif, Vpr, Vpu).

LTRs are present at both ends of the HIV genome and help in the integration of the provirus into the host-cell DNA. Synthesis of new virions starts from the LTR because transcriptional factors of host cells bind to the LTR region of the viral genome.

Envelope

"Env" is derived from "envelope." The genomic location of *env* is nucleotides 5771–8341; it is 2570 nucleotides in size. The total size of the Env precursor protein is 160 kDa. The envelope is a glycoprotein and is synthesized as a polyprotein precursor. The Env precursor is known as gp160 and is processed by a cellular protease that results in the production of two proteins: (1) surface Env protein also known as glycoprotein gp120 and (2) a

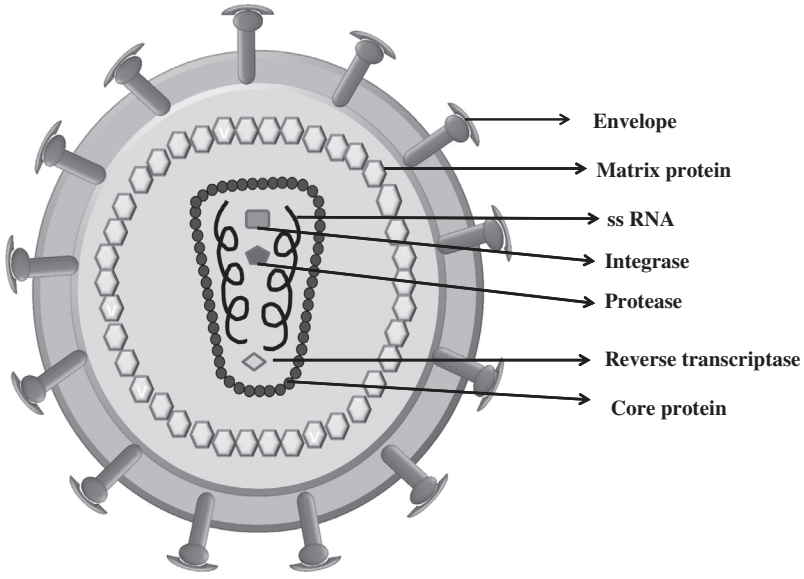


FIGURE 9.1 Structure of HIV. Graphical representation of cross-section of HIV. Envelope is the outermost layer; it consists of a lipid bilayer. The envelope layer is composed of gp120 and gp41. The layer next to the envelope is the MA. The matrix layer is followed by the core protein. At the center of the virion, two molecules of single-stranded RNA (ssRNA) and other enzymes are present. These enzymes are protease, integrase, and RT. RT also contains RNase H. The location of each individual protein and RNA is shown in the figure. (Polymerase is not shown; it contains integrase, protease, RT, and RNAse H).

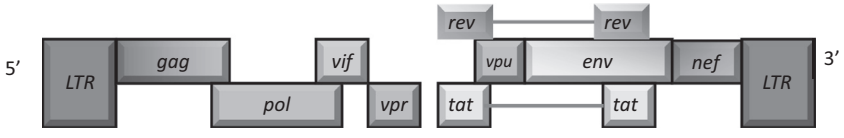


FIGURE 9.2 HIV genome. Schematic representation of the HIV genome. The genome is 9.8 kB in size, and consists of nine genes, which are flanked by LTRs on either side of the genome. These nine genes finally produce 15 proteins: *env*, envelope; *gag*, group-specific antigen; *LTR*, long terminal repeat; *nef*, negative factor; *pol*, polymerase; *rev*, regulator of expression of viral proteins; *tat*, transactivator of transcription; *vif*, viral infectivity factor; *vpr*, viral protein R; and *vpu*, viral protein U.

transmembrane glycoprotein called gp41. The gp120 glycoprotein contains determinants that interact with T-cell receptors and gp41 interacts with co-receptors; gp41 consists of three domains: (1) a domain essential for membrane fusion known as the ectodomain, (2) a transmembrane protein that serves as an anchor, and (3) a cytoplasmic tail. The gp120 glycoprotein primarily binds with the target cell due to co-receptor interaction, which induces conformational changes in gp41. Conformational changes in gp41 promote the formation of a gp120/gp41 glycoprotein complex, leading to fusion of the virus to the host cell membrane. At the heart of the fusion reaction is the ectodomain of gp41; this region contains a highly hydrophobic N-terminus ("fusion peptide") and two heptad repeat motifs referred as N-helix and C-helix.

Group-specific antigen

Group-specific antigen (*Gag*) produces a protein of 55 kDa that contains 500 AA. The *Gag* gene encodes for Pr55Gag, which is a polyprotein precursor. Pr55Gag is cleaved, leading to formation of four proteins: p6, p7, p17, and p24, as well as two spacer peptides, p1 and p2. p17 is a matrix protein (MA), p24 is a capsid protein (CA), p7 is a nucleocapsid, and p6 is known as Vpr-binding protein. The N-terminal of MA is mainly responsible for targeting and binding to plasma membrane.

Long-terminal repeats

LTR is present on either side of the viral genome. It harbors *cis*-acting elements, which are required for RNA synthesis, and is the initiation site for transcription of the viral genome. *LTR* consists of three regions: U3 (unique, 3′ end), R (repeated), and U5 (unique, 5′ end). Various elements present in U3 help in direct binding of RNA polymerase II (pol II) to DNA templates. Newly synthesized viral RNA falls into three major classes: (1) unspliced RNAs, which function as precursors for Gag and Gag-Pol polyprotein, (2) partially spliced mRNAs (5 Kb), which encode Env, Vif, Vpr, and Vpu proteins, and (3) small but multiple-spliced mRNAs (1.7–2.0 Kb), which encode for Rev, Tat, and Nef.

Negative factor

"Nef" evolved from "negative factor." Its genomic location is from 8343–8963 nucleotides, and the total length of the *nef* gene is 620 nucleotides. Nef is an accessory protein to HIV and contains about 206 AA with a molecular mass of 27 kDa. Nef is expressed during the early stages of replication in host cells. Nef downregulates expression of cell receptors such as CD4, CD8, CCR5, CXCR4, etc. These are the reasons why Nef is considered as an important protein for in vivo pathogenesis. The functions of the Nef protein, which have been observed in in vitro studies, are as follows: (1) it perturbs endocytosis; (2) it modulates signal transduction pathways in infected cells; (3) it enhances viral infectivity; and (4) it supports fusion of HIV-1 to target cells.

Nef seems to play a significant role in alterations of CNS functioning because Nef is present in higher levels in astrocytes, causing alteration in their growth. Nef can alter electrophysiology of neurons and induce inflammatory mediators from monocytes.

Polymerase

The name "Pol" is derived from "polymerase," and its genomic location is from 1839–4642 nucleotides; it consists of 2803 nucleotides. Pol protein is 112 kDa and consists of 935 AA. The *pol*-encoded enzymes are initially synthesized as part of a large polyprotein whose synthesis results from a rare frame-shifting event. The individual *pol*-encoded enzymes, viral proteases (PR or p10), RT (or p64), and integrase (IN or p32) are cleaved from the polyprotein precursor by viral proteases. Integrase protein promotes insertion of linear but double-stranded proviral DNA into the chromosome of the host cell. Integration is an absolutely necessary step for viral replication because integrase mutant viruses fail to spread infections.

Regulator of expression of viral proteins

The name "Rev" is derived from "regulator of expression of viral proteins." Rev is encoded by two exons, both of which are essential to producing functional proteins. The genomic location of *rev* is 5516–5591 and 7925–8199; it is composed of 75 and 274 nucleotides, respectively. Rev is a regulatory protein and is essential for regulation of viral replication. The molecular mass of Rev protein is 19 kDa, and it contains 116 AA. Rev downregulates post-transcriptional splicing of viral mRNAs. Rev contains two functional domains: (1) an arginine-rich domain that binds with viral RNA and supports nuclear localization and (2) a leucine-rich domain that is hydrophobic and mediates nuclear export of viral RNA.

Transactivator of transcription

The word "Tat" is derived from "transactivator of transcription." Tat is a regulatory protein essential for theregulation of viral replication. *tat* comprises 259 nucleotide sequences, and its genomic location is 5377–5591. Tat is an 86–110 AA long protein with a molecular mass of 16 kDa. Tat protein consists of several functional domains. Secreted Tat may be taken up by neighboring cells; therefore, Tat affects both

infected and noninfected cells. Tat induces apoptosis of neurons both in vivo and in vitro via oxidative stress pathways.

Viral infectivity factor

Vif is an accessory protein. The name is derived from "viral infectivity factor." The genomic location of *vif* is nucleotides 4587–5165, and it is composed of 578 nucleotides. Vif has a major role in the production of infection-competent new virions from infected cells. The molecular weight of the Vif protein is 23 kDa, and it is made up of 192 AA. Vif is expressed during late stages of HIV replication and is localized in the cytoplasm of infected cells. Vif appears to act during viral assembly in virus-producing cells, or subsequently in virion maturation to produce virions competent for reverse transcription in the target cell.

Viral protein U

Vpu produces a protein of 16 kDa that consists of 82 AA. Viral protein U is a type-1 integral membrane phosphoprotein unique to HIV. The genomic location of *vpu* is from nucleotide 5608–5856, and it consists of 248 nucleotides. Vpu enhances pathogenesis in vivo, even though it is not an essential protein. The Vpu protein usually gets expressed during replication in the host cell. Vpu cannot be detected in virions because it does not get packaged into viral particles. It performs two major functions during HIV replication: (1) enhances the release of viral particles and (2) promotes CD4 degradation. The outcome of Vpu-induced CD4 degradation is to liberate gp160 from Env/CD4 complexes in the endoplasmic reticulum.

Viral protein R

Vpr consists of 96 AA. Therefore, the predicted molecular mass of Vpr is 12.7 kDa. The genomic location of Vpr is 5105–5396, and it contains 296 nucleotides. Vpr is an accessory protein of HIV and is not essential for viral replication. Truncation of ORF from Vpr has resulted in the production of slow-replicating viral progeny. This is the rationale for the name Vpr: "viral protein, regulatory." Vpr is an accessory protein and is therefore not crucial for replication, but it has been implied in various biological functions such as transcription of new viral genomes, apoptosis induction, disruption of cell-cycle control, induction of defects in mitosis, nuclear transport of the preintegration complex, facilitation of reverse transcription, suppression of immune activation, as well as reduction of the HIV mutation rate. Vpr is capable of breaching cell membranes. This property supports entry of extracellular Vpr into uninfected cells.

Replication: steps and drug targets

Knowledge of HIV replication is essential to develop better antiretroviral drugs and effective treatment strategies. Unless we know the HIV replication steps, we will not be able to find the drug targets. In other words, we can say that a better understanding of HIV replication has given us new drug targets that can be studied and exploited for the development of new drugs. The latest concept that is under investigation is to explore the possibilities of blocking the entry of HIV into host cells. It is a reasonably good choice that can be used in combination with drugs rather than using it as the sole strategy to stop HIV infection (Male et al., 2006).

HIV, being a retrovirus, does not have DNA as a genetic material to pass its genetic information to the next viral generation. To overcome this problem, HIV has a unique enzyme called "reverse transcriptase." RNA can be reverse transcribed into DNA with the help of RT to complete the process of viral replication. HIV, like any other virus, is simply a bag of nucleoproteins. HIV cannot replicate by itself because it lacks the synthetic machinery for replication. For replication of HIV, it needs the synthetic machinery of a host or a host's cells. Replication of the virus will only start after the cells get infected with HIV; the process of viral replication starts with the attachment of viral particle until the time of release of new virions from infected cells (Freed, 2001). The process of HIV replication can be divided into the following main steps: (1) viral attachment and entry, (2) reverse transcription, (3) integration of proviral DNA, (4) transcription and translation, and (5) completion and release (Fig. 9.3).

1. Viral attachment and entry

   This is the first step of viral replication. In this step, HIV has to come closer to the cells that have specific receptors for HIV. CD4 is the main receptor for HIV attachment to the cells, and the target cells for HIV infection are T-lymphocytes. CD4 is a 58-kD glycoprotein present on the surface of a unique T-cell subpopulation known as T-helper cells. Apart from T cells, CD4 receptors are also expressed on other cells such as monocytes, macrophages, dendritic cells, microglial cells, and several other cell types. The first step for HIV infection is the attachment of HIV for its entry into cells.

   The process of HIV entry starts with the interaction of two envelope proteins, namely, gp120 and gp41. The gp120 viral protein binds with the CD4 receptor with high affinity. Binding of gp120

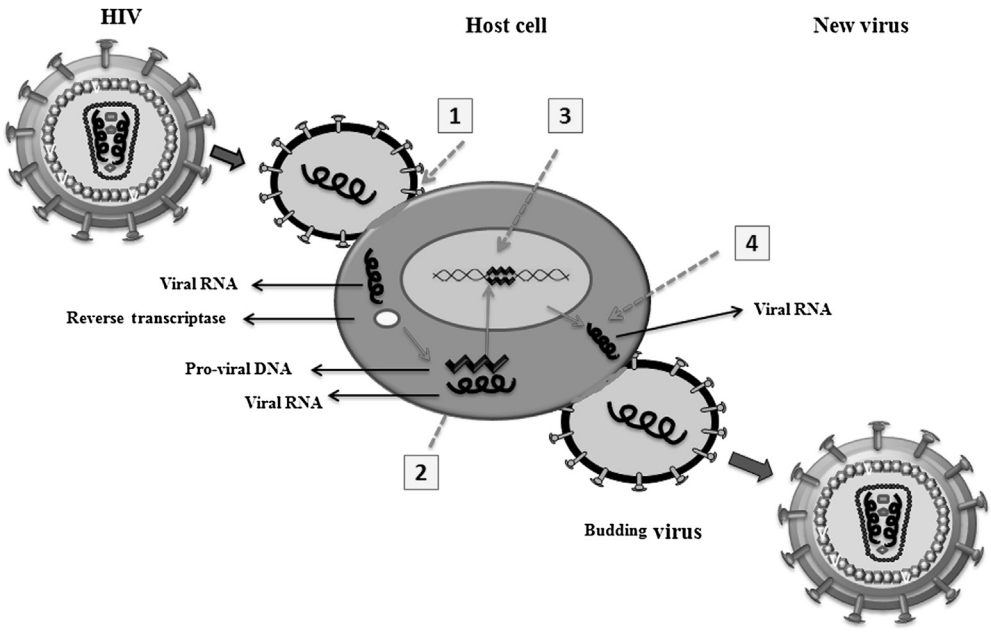


FIGURE 9.3 HIV regulation steps and drug targets.Schematic representation of HIV replication along with the stages where different groups of antiretrovirals work. During infections, HIV attaches to the cell surface of target cells and fuses with the cells to release viral RNA and other proteins. RT produces proviral RNA in the cytoplasm. Proviral RNA moves to the nucleus and integrates with the host cell genome with the activity of the integrase. After integration of proviral DNA into the host DNA, it gives rise to mRNA, which finally translates into different proteins required for the synthesis of new virions. These proteins get cleaved by proteases to get assembled into new virions; new virions are released into circulation due to budding from the cells. Steps for drug targets are mentioned in numerals in blocks: Step 1 is the target for FIs, Step 2 is the target for RT inhibitors, Step 3 is the target for integrase inhibitors, and Step 4 is the target for protease inhibitors: viral RNA and proviral DNA.

induces a conformational change in gp41, which unfolds and moves toward the cell surface. The gp41 protein binds with co-receptors for HIV (i.e., either CXCR4 or CCR5). Attachment of both gp120 and gp41 to their specific receptors leads to fusion of the viral envelop with the plasma membrane of the target cells. Fusion of the viral envelope to the cell membrane facilitates delivery of the viral genome and proteins into the host cells. This is the most common pathway for the entry of HIV into cells and is a lucrative target in designing drugs. This is an area of interest for drug development at the current time. Drugs that can block entry of HIV into cells are not the most effective means to stopping HIV infections. Therefore, initial research was focused on developing antiretroviral drugs targeting other crucial steps to inhibit HIV replication.

2. Reverse transcription

After fusion of the virus with cells, delivery of the viral genome into the host cell takes place. In the cytoplasm of host cells, RT starts working by copying viral RNA into complementary DNA (cDNA). This newly synthesized DNA is known as "proviral DNA." The major limitation of this process is that the RT enzyme does not have proofreading capabilities, making this process highly error prone. This is the reason that HIV develops resistance to drugs. For scientists, this turned out to be the most useful target to block HIV replication because it is the first step in producing new virions after viral entry. If one can block this step, then the production of new viral particles could be stopped. This is the reason why various classes of drugs have been developed to block this step (NRTI, NtRTI, NNRTI). These drugs are known to inhibit the activity of RT. Another benefit associated with these drugs is that alternative analogs can be used to control the development of resistance against any one of these drugs.

3. Integration of proviral DNA

Once the proviral DNA is synthesized, it has to be integrated into the host DNA. The newly synthesized proviral DNA integrates with the host's cell DNA; this process takes place due to the activity of integrase. Integrase is an enzyme of viral origin. The nine genes of HIV are flanked by LTRs. LTRs are essential for integration of proviral DNA into host DNA. If one can stop or block the integration of proviral DNA into the host, it can be a great strategy to stop viral replication. New drugs have been developed to block this stage of the replication cycle. Drugs that can block the integration

step are known as integrase inhibitors (e.g., Isentress, which was approved by the FDA in 2007).

4. Transcription and translation

After integration of viral DNA into the host cell, transcription and translation of viral genes starts. In some circumstances, the proviral DNA gets integrated but the virus does not undergo active replication because it remains latent. This phenomenon is known as "latent infection." The production of the infective virus requires activation of various cellular transcription factors of the host cells. Host cellular transcriptional factors, upon activation, lead to transcription of viral RNA with the help of cellular RNA polymerases. Splicing of transcripts takes place in a specific manner to produce mRNAs, which finally leads to the synthesis of different viral proteins. Tat and Rev are two proteins that are produced at this stage, and they support viral replication in activated T-cells. At a later stage, Env and Gag proteins are produced. The full-length RNA binds with gag protein and gets packaged into new virus particles.

5. Assembly and release

This is the last and final step of HIV replication. Env glycoprotein passes through the cytoplasmic endoplasmic reticulum and gets transported to the Golgi complex. In the Golgi complex, Env protein is cleaved by proteases and processed into two glycoproteins, gp120 and gp41. These glycoproteins move toward the host cell's plasma membrane, where gp41 anchors gp120 to the cell membrane of infected cells. Gag and gag-pol polyproteins help in budding of viruses. HIV protease cleaves polyproteins into a functional HIV protein and enzymes. Cleavage of these proteins completes the maturation of the newly infective HIV. Protease inhibitors (PIs) can block this step (e.g., Lopinavir, Ritonavir, etc.).

Antiretroviral drugs

Antiretroviral drugs are the most commonly known for treating HIV infections. The truth is that they can be used to treat any retrovirus infection. At the start of HIV, there was no drug available to treat HIV infection. Azt e was the first drug approved by the FDA (in 1985) to treat HIV infection. With time and a better understanding of the mechanism and various steps of HIV replication helped scientists to target important steps in HIV replication (which could be blocked by different drugs). Various drugs have been developed to block these steps of HIV replication and to treat HIV infection. Treatment with antiretroviral drugs is commonly known as ART (Olender et al., 2012; Boehringer-Ingelheim, 2005). In 1995, combinatory drugs were used to treat HIV called as "AIDS Cocktail", also called highly active ART (HAART). Various antiretroviral drugs are classified into the following categories: (1) nucleoside RT inhibitors (NRTIs), (2) nucleotide RT inhibitors (NtRTIs), (3) non-nucleoside RT inhibitors (NNRTIs), (4) PIs, and (5) integrase inhibitors (InIs) (Tables 9.2 and 9.3).

1. NRTIs

Drugs of this category block the activity of RT. Conceptually, these drugs are considered to be "False Building Blocks." They are called as false

TABLE 9.2 Antiretroviral drugs.

Approval	drugs	class of drugs	trade name
1987	Zidovudine	NRTI	Retrovir
1991	Didanosine	NRTI	Videx
1992	Zalcitabine	NRTI	Hivid
1994	Stavudine	NRTI	Zerit
1995	Lamivudine	NRTI	Epivir
	Saquinavir	PI	Invirase
1996	Indinavir	PI	Crixivan
	Nevirapine	NNRTI	Viramune
	Ritonavir	PI	Norvir
1997	Combivir	FDC	
	Delavirdine	NNRTI	Rescriptor
	Nelfinavir	(PI)	Viracept
1998	Abacavir	NRTI	Zigen
	Efavirenz	NNRTI	Sustiva
1999	Amprenavir	PI	Agenerase
2000	Didanosine EC	NRTI	Videx EC
	Lopinavir	FDC	Kaletra
	Trizivir	FDC	
2001	Tenofovir DF	NRTI	Viread
2003	Atazanavir	PI	Reyataz
	Emtricitabine	NRTI	Emtriva
	Enfuvirtide	FI	Fuzeon
	Forsamprenavir	PI	Lexiva
2004	Epzicom	FDC	
	Truvada	FDC	
2005	Tipranavir	FI	Aptivus
2006	Atripla	FDC	
	Darunavir	PI	Prezista

(*Continued*)

TABLE 9.2 (Continued)

Approval	drugs	class of drugs	trade name
2007	Maraviroc	CA	Selzentry
	Raltegravir	InI	Isentress
2008	Etravirine	NNRTI	Intelence
2011	Complera	FDC	
	Nevirapine XR	NNRTI	Viramune
	Rillpvirine	NNRTI	Edurant
2012	Stribild	FDC	
2013	Dolutegravir	InI	Tivicay
2014	Cobicistat	PE	Tybost
	Elvitegravir	InI	vitekta
	Triumeq	FDC	
2015	Evotaz	FDC	
	Genvoya	FDC	
	Prezcobix	FDC	
2016	Descovy	FDC	
	Odefsey	FDC	
2017	Juluca	FDC	
2018	Biktarvy	FDC	
	Climduo	FDC	
	Delstrigo	FDC	
	Doravirine	NNRTI	Pifeltro
	Ibalizumab	PAI	Trogarzo
	Symfi	FDC	
	Symfi Lo	FDC	
	Symtuza	FDC	

building blocks because they serve as alternative substrates for RT. During the synthesis of proviral DNA, these drugs compete with physiological nucleosides. These drugs have a minor modification of the azido group, which is attached to the ribose sugar. The azido group leads to the termination of DNA synthesis and blocks further HIV replication. Some of the common drugs of this group are AZT, ddI, ddC, d4T, 3TC, and FTC. These drugs are analogs of different nucleosides. AZT was the first drug used for HIV treatment, and the success of AZT led to the development of numerous other drugs of the same category. AZT and d4T are thymidine analogs, FTC and 3TC are cytidine analogs, ddI is an adenosine analog, and similarly, ABV is a guanosine analog.

As we know, RT does not have proofreading abilities, which is the reason that HIV resistance is commonly observed against the drugs of this group. During the course of treatment, the change in nucleoside analogs has proven to be effective in preventing drug resistance.

2. NtRTIs

This is another version of the NRTIs. The difference with NtRTIs is that these contain a nucleotide rather than a nucleoside. Nucleoside analogs must be converted to nucleotide analogs to show their inhibitory activity. NtRTIs have the added advantage that they skip the step of conversion of nucleoside into nucleotide; therefore, these drugs are less toxic when compared with nucleoside inhibitors. The mode of action of NtRTIs is similar to NRTIs, but NtRTIs have some serious side effects. TDF is an example of this category. A combination of TDF with ddI should be avoided for the treatment of HIV patients. NtRTIs are present in the market with various combinations.

TABLE 9.3 Antiretroviral drugs mechanisms of action.

Inhibitors	Abbreviations mechanism		Action
Nucleoside reverse			
Transcriptase inhibitors	NRTIs	Reverse transcription	Nucleoside analogues
Nucleotide reverse			
Transcriptase inhibitors	NtRTIs	Reverse transcription	Nucleotide analogues
Non-nucleoside reverse			
Transcriptase inhibitors	NNRTIs	Reverse transcription	Blocks catalytic site
Protease inhibitors	PIs	Protease inhibition	Inhibition final maturation stage
Integrase inhibitors	InIs	Integrase inhibition	Prevent integration of proviral DNA
Entry inhibitors	EIs	Entry inhibition	block receptors

FDC, fixed-dose combination; EI, entry inhibitors; FI, fusion inhibitors; InI, integrase inhibitors; NNRTI, non-nucleoside reverse transcriptase inhibitors; NRTI, nucleoside reverse transcriptase inhibitors; NtRTI, nucleotide reverse transcriptase inhibitors; PI, protease inhibitors.

3. NNRTIs
   This is another group of drugs that blocks or targets the activity of RT. The mode of action of NNRTIs is different than for NRTIs. NNRTIs bind directly to RT. Binding of NNRTIs with RT makes RT unable to catalyze proviral DNA synthesis. NNRTIs bind with RT at a site close to the binding site of the nucleoside; therefore, NNRTIs block the binding site and reduce or inhibit nucleoside binding. These do not require any further activation for their actions (e.g., Nevirapine, Delavirdine, Efavirenz, etc.).
4. PIs, entry inhibitors, and fusion inhibitors (FIs)
   These are a different group of antiretroviral drugs that are used to treat HIV infections. These drugs block different steps of HIV infections and replication. Examples of each drug is mentioned in Table 9.2.
5. InIs

As the name suggests these drugs inhibit the activity of integrase. The FDA approved a drug in this category in 2007 for HIV treatment (e.g., Raltegravir).

HIV resistance and antiretroviral treatment

Resistance against drugs is a very common problem with HIV infections. After a certain period of time, resistance becomes such an issue that different treatment modalities have to be tried to treat patients who fail to respond to a regular course of treatment (Tang and Shafer, 2012). Since the focus of this chapter is not on the clinical aspects, a brief of three different treatment modalities to treat HIV seropositive with drug resistance is discussed.

Highly active antiretroviral treatment

This is a new name for combinational therapy for HIV infections. Highly active antiretroviral treatment uses a combination of three to four antiretroviral drugs of different classes. Drugs selected for the HAART protocol are from the NRTI, NNRTI, PI, and InI categories. The strategy to treat HIV patients using a HAART regimen was decided by a panel from the National Institutes of Health (NIH) and other international organizations. HAART is designed to reduce the complexity of dose scheduling and problems related to the adherence of complex drug scheduling. A physician has to weigh the potential risks and benefits of drugs to a patient before the selection of drugs for a HAART regimen. HAART treatment is an effective way to deal with drug resistance. To overcome the problem of HIV mutation, HAART targets different stages of HIV replication simultaneously so that the development of resistance against drugs is reduced to a great extent.

Salvage therapy

Salvage therapy is also known as mega-HAART. This therapy is used when HAART does not give the expected results. In other words, salvage therapy is a last resort to treat HIV infection in those patients who are resistant or not responding to other treatment strategies. At present, up to nine drugs can be administered in different combinations under this therapy. This is a very expensive treatment for HIV patients and it also has serious side effects.

Drug holiday

A drug holiday is another treatment strategy for HIV patients. The rationale for this protocol is to stop treatment for a certain period of time so that HIV can exist without any selection pressure of a drug, and as a result, HIV will become more susceptible to anti-HIV drugs. If antiretroviral drugs are administered after the interruption, they may be effective in treating HIV that is resistant to drugs.

New types of antiretrovirals

Since the discovery of HIV and the miseries associated with HIV infections, the scientific community has worked to prevent HIV infections by finding newer anti-HIV drugs. Tireless researches have led to the identification of novel drug targets that can prevent HIV entry into cells.

The initial process for HIV infection is the attachment of the virus to target cells. During infection, the viral envelope glycoproteins (i.e., gp120 and gp41) interact with CD4, a primary receptor for HIV infection, and chemokines receptors (CCR5 or CXCR4), which are also present on target cells (Berger et al., 1999). During HIV infection, gp120 binds to CD4 and undergoes conformational changes. These conformational changes in gp120 are transmitted to gp41. These changes support gp120 interactions with co-receptors and gp41. Recently, one more host cell surface protein (i.e., protein disulfide isomerase, PDI) has been reported to play an important role in HIV entry. PDI belongs to a family of proteins that catalyzes formation, reduction, and isomerization of disulphide bonds. Intracellular PDI also assists in the folding of nascent polypeptides chains. PDI molecules that are attached to the cell surface are continuously shed and rapidly replaced by new PDIs.

Cell surface PDI has been shown to bind with CD4 receptor. Upon HIV infection, gp120–PDI–CD4 (a ternary complex) is formed. The conformational change that occurs in gp120 after binding to CD4 is because of reduction in some of its disulfide bonds, which is catalyzed by PDI of the gp120–PDI–CD4 complex (Fenouillet et al., 2001; Ryser and Fluckiger, 2005).

The knowledge gathered about gp120, gp41, CD4, and chemokine receptors suggests that this process can be divided into the following steps: (1) interaction of gp120 with CD4, (2) interaction of co-receptor (CXCR4/CCR5) with gp120, and (3) activation of gp41.

Targeting Steps 1 and 2 has led to the development of various new inhibitors of HIV entry that are under clinical investigation (Table 9.4).

1. Inhibitors of gp120 and CD4 interaction

   Targeting the first step of HIV infection (i.e., the interaction between gp120 and CD4) seems to be the most rationale step to prevent infection. BBS-378806 (BMS-806), an indole derivative developed by Bristol Meyers Squibb (BMS), inhibits the step mentioned above. BMS-806 binds at a site in gp120 that is in the vicinity to the site where the disulfide bonds (which undergo PDI-mediated reduction) are located. BMS-806 has been successfully tested against a large number of clinical isolates, even at nanomolar (nm) concentrations. However, BMS-806 did not turn out to be a very successful option because several HIV mutants were found to be replicating successfully, even in the presence of BMS-806. Another inhibitor of PDI, PRO542, is under phase II clinical trials; it binds to gp120. The peptide CD4-M33 acts as a mimic for the CD4 domain and binds to gp120 effectively, at least in in vitro conditions (Jacobson et al., 2004; Martin et al., 2003). TNX355, a monoclonal antibody known to bind with CD4, is also under investigation for its anti-HIV effects. The major limitation of these inhibitors is their bioavailability when administered via oral route.
2. Inhibitors of gp120 and co-receptor interaction

   CXCR4 and CCR5 are chemokine receptors that act as co-receptors for HIV entry into target cells. The natural ligands for CXCR4 are RANTES, MIP-1α, and MIP-1β, while the natural ligand for CCR5 is SDF-1α. The knowledge of availability of natural ligands for CCR5 and CXCR4 has provided a new direction for the development of ligands for chemokine receptors, the goal being to impair entry of HIV into target cells by blocking co-receptors. UK427,857 prevents binding of gp120 to CCR5; it is effective at low nanomolar concentrations. TAK-220 binds to CCR5 and CXCR4 and has been found to be effective to inhibit HIV entry. Both of these inhibitors are under further investigation.

   AMD-3100 (a bicyclam) has been identified to work against CXCR4. AMD-3100 has been effective to suppress HIV infections but has been reported to have serious side effects; so, it was discontinued and replaced by another analog (i.e., AMD-070). AMD-070 has been reported to be effective at low nanomolar concentrations, can be orally administered, and is under clinical investigation.
3. Inhibitors of gp41 activation

The third step is formation of the active state of gp41, which is targeted by T-20, a small 36 AA peptide. T-20 is reported to reduce viral RNA in the blood of HIV seropositives. It is FDA approved under the name Enfuvirtide, but it is not orally bio-available yet.

The role of PDI in HIV entry into target cells is an area of extensive research, with the goal of targeting PDI using drugs or inhibitors to prevent HIV infections. Various attempts to knockout PDI in mammalian

TABLE 9.4 Inhibitors of HIV entry.

Compound	Mechanism	Manufacturer	Identity
(A) gp-120–CD4 binding			
BMS-378806	gp120-binding	BMS	Indole derivative
BMS-488043	–do–	BMS	Second generation compound
Pro 542	–do–	Progenics	CD4–IgG4
TNX-355	CD4 binding	T/B	Anti CD4
(B) gp-120–co-receptor binding			
SCH-C[a]	CCR5 binding	SP	OPPA
SCH-D	–do–	SP	Not disclosed
Pro-140	–do–	Progenics	MoAb
Tak-779[a]	–do–	Takeda	Quaternary ammonium anilide
Tak-220	–do–	Takeda	Second generation compound
GW873140	–do–	GSK	Piperazine derivative
UK-427, 857	–do–	Pfizer	–
AMD3100[a]	CXCR4 binding	AnorMed	Bicyclam
KRH-2731	–do–	Kureha	Arginine derivative
(C) Fusion inhibitors			
Fuzeon	CHR mimic	Trimeris	Peptide

[a]*Discontinued.*

B, biogen; BMS, Bristol Myers Squibb; GSK, Glaxo Smith Kline; MoAB, monoclonal antibodies; OPPA, oxymino-piperidino-piperidine-amide; SP, Schering-Plough; T, Tanox.

cells have resulted in lethal mutations. Therefore, the only choice was to develop strategies that exclusively target the cell surface PDI. Unfortunately, there is no drug available on the market that can target cell surface PDI.

Another approach to control HIV infection could be by targeting the interaction between PDI and CD4. If an antibody can be raised that can block the interaction between PDI and CD4, then entry of HIV into its target cells can be blocked. Since PDI and CD4 both are proteins of host origin, it is easy to use them as drug targets as they are less prone to mutations. Binding between gp120 and CD4 can also be targeted for anti-HIV treatment by preventing PDI from reducing gp120 disulfide bonds. Under these circumstances, gp120 will not undergo conformational changes; so, HIV entry into target cells will be prevented.

The first disulfide reduction catalyzed by PDI may result in further oxidation and reduction in gp120 like a cascade reaction. It is also possible that in the near future several potential drug targets can be identified and better drugs could be designed to prevent HIV infections.

Methodology and principles

In addition to being highly infectious, HIV was also a totally new and unknown infection to the world. The credit for the discovery of HIV goes to Luc Montaignier. The most complicated problem associated with researching HIV was the risk related to work with it. In certain circumstances, risks for HIV infection are high. Clinicians as well as emergency responders such as policemen, firemen, and paramedical staff are at high-risk of exposure due to their workplace circumstances. Under laboratory conditions, the situation is a bit different as most of the time people are working under known circumstances and risk. Still, laboratory staff is always working with concentrated HIV stock or a very high virus titer. In both laboratory and clinical conditions, it is very important to follow safety procedures.

Laboratory personnel deal with HIV on a daily basis and should adhere to safety precautions whenever they perform experiments like growing HIV stocks, infecting new cells, quantifying viral infectivity in cultures, measuring end results like p24, viral load, etc. Safety precautions include the use of disposable lab ware, not using sharps unless essential, not using glassware, wearing masks while working with live HIV, using double gloves that are puncture proof, and wearing disposable lab coats. All procedures should be performed under Bio-Safety Level-3 (BSL-3) conditions. None of the materials infected with HIV should be taken outside of the laboratory without inactivating HIV and following specifically outlined safety procedures.

In case of antiretroviral drug testing, HIV stock is needed, so the virus has to be grown in the laboratory. A system for monitoring HIV infection is also needed. Testing viability of cells in the presence of HIV and after adding antiretroviral drugs is the most common method. There are various methods for testing antiretroviral drugs, but the choice of method depends upon cell type and HIV (Peters et al., 2013; Zhu, 2005). Apart from this, assays are also needed to monitor end points to evaluate a drug's anti-HIV effect. Some of the most commonly used end points are the determination of p24 levels, viral load (viral RNA), RT enzyme activity, etc. The following methods are explained here: growing HIV stock, monitoring antiretroviral drug toxicity, and evaluating anti-HIV effects of antiretroviral drugs. Also included is a brief overview of assays for antiretroviral drugs.

Growing HIV stock

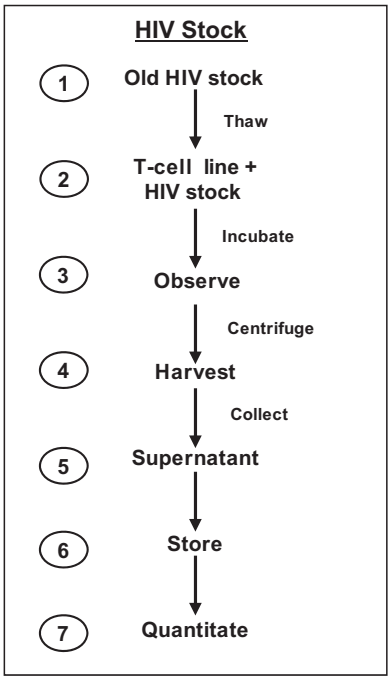
Two situations are possible to grow HIV stock. One can grow viral stock that has been obtained from patients for further study. In this case, larger volumes of HIV stock is needed; usually this stock of HIV is grown in primary cells such as peripheral blood mononuclear cells (PBMCs) from healthy donors.

The other situation is to grow viral stock from a laboratory strain of HIV. Laboratories involved with HIV research require HIV stock on a routine basis for various experiments. These viral stocks can be grown from previous stocks by culturing HIV with permissive cells. HIV-infected cells can be grown for a limited period of time, and HIV will replicate and be released into the culture supernatant. New stock of HIV can be harvested for further experiments. To make sure that viral stock has the same concentration every time, one has to adhere to endpoints. These endpoints could be multiplicity of infection (MOI), p24, RT enzymes, or viral load. Endpoints can be chosen on the basis of the facilities and expertise available in the laboratory, as well as by further application of HIV stock in the laboratory (Flowchart 9.1). To grow viral stock, one should be familiar with the principle and rationale of the procedures and steps involved. Before starting with an HIV culture, one must consider the following points:

Principles

1. Source of HIV

   This should be taken from the previous stock or the culture growing in the laboratory. It is always a good practice to use a fixed concentration of virus or known concentration of virus to develop a new



FLOWCHART 9.1 Schematic protocol to grow new HIV stock.

viral stock. This helps in growing stock of the same quality in terms of concentration of virus in the new stock.

2. HIV permissive cells
   Since HIV is known to grow only in $CD4^+$ cells, so it is always advisable to use cells that are $CD4^+$. In general, under laboratory conditions, HIV stock is grown using T-cell lines. The reason to use T-cell lines is that they are unlimited source of cells that can be grown as (and when) needed. Some of the common T-cell lines used to grow HIV stocks are MT-4, HUT_{78}, H-9, etc. Growth of HIV can be monitored with giant cell or syncytium formation in cell cultures after infecting with HIV.
3. Infection of cell
   It is always a good idea to use overnight grown cells for the initiation of new HIV stock. HIV stock can be added into a fixed number of cells. HIV infection can be checked for the syncytium formation at regular intervals. After a certain duration of incubation, one can harvest HIV from culture supernatant. If a more concentrated stock of HIV is needed, then new cells can be added to the same culture. Addition of new cells compensates for cells that have died due to HIV-induced cell death.
4. Harvesting of viral stock
   After a certain period of incubation of HIV and cells, a new stock of HIV can be harvested by centrifuging the culture. After centrifugation, cells will be pelletted at the bottom of the tube and new HIV will be present in the supernatant. The cell pellet has to be discarded while the supernatant must be collected. At this time, supernatant should be divided into small aliquots as needed. These aliquots can be frozen and thawed as per laboratory requirements.
5. Quantitation of viral stock

One can use any of the assays such as p24, RT assay, or MOI to quantify HIV titer. Detailed methods can be obtained from any standard laboratory manual regarding HIV.

Crucial steps

1. Old viral stock
   Works as the source of HIV to infect new cells.
2. Mixing of cells with HIV
   Since we used CD4 positive cells, they will be infected with HIV and allow HIV to replicate and to produce new virions.
3. Harvesting
   After incubation of HIV with cells, HIV will be released into the culture supernatant. HIV can be collected by centrifugation. Centrifugation removes the cells, and supernatant will have new HIV virions.
4. Quantitation

This is done to measure HIV particles in culture supernatant.

Assays for antiretroviral drugs

Antiretroviral drugs are drugs that can work against retroviruses. In general, the term "antiretroviral drug" is used for anti-HIV drugs. Since the discovery of HIV, there has been a desperate need to develop easy and convenient methods to evaluate antiretroviral drugs. There is no suitable animal model for HIV, so it is impossible to test the activity of antiretroviral drugs in animals. There are various assays that have been developed to test antiretroviral drugs in vitro. Before testing antiretroviral drugs for their anti-HIV effect, it is important to study the toxicity of the drug. Toxicity as well as the antiretroviral effect of a drug can be conveniently studied under in vitro conditions using different cell types. Protocols for these two considerations are discussed here.

Monitoring antiretroviral drug toxicity

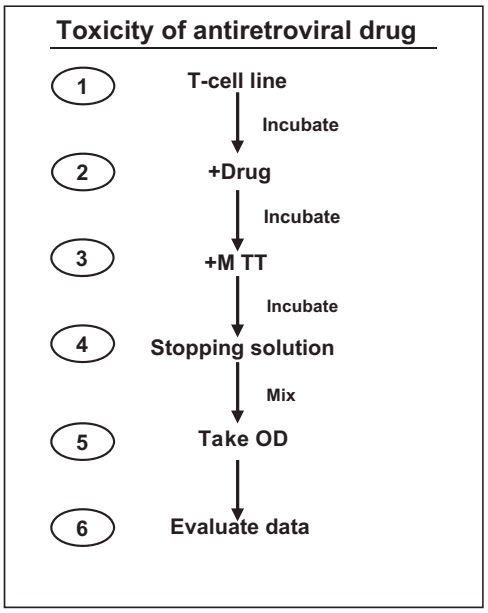
This assay is based on the application of MTT dye, which works with the action of mitochondrial enzyme. When MTT is added to cells, it gets converted into a blue-colored product. The blue-colored

product can be dissolved, and its optical density (OD) measured. OD is directly proportional to the viability of cells in a linear range. Only viable cells will convert MTT and give a blue color, whereas dead cells will not be able to convert MTT. These are the criteria for the application of MTT assay to evaluate drug toxicity. The MTT assay is versatile in its applicability; it can be easily used even in a 96-well format. The use of a 96-well format has the added advantage that a large number of drugs, and different concentrations of drugs with lesser numbers of cells, can be tested at the same time (Flowchart 9.2). To setup an experiment to study the drug's toxicity, the following procedure has to be performed: (1) culturing cells, (2) plating of cells, (3) preparation of drug dilution or concentrations, (4) addition of drugs, (5) addition of MTT, (6) stopping the MTT reaction, and (7) evaluation of data.

Principles

1. Culturing cells

   For this experiment, one can use primary cells such as PBMC or tumor cell lines. One should select cells that are targets for HIV infection. Toxicity of the drug may vary in different cell types. For anti-HIV effect, it is always useful to select T-cell lines because they can be infected with HIV; hence, results of drug toxicity and anti-HIV effects of the drug can be easily compared. If someone is using T-cell lines, then it is better to use overnight grown cells for this assay. As needed, cells can be grown either in large or small volumes.



FLOWCHART 9.2 Schematic protocol to test toxicity of antiretroviral drugs using MTT assay.

2. Plating of cells

   The next step is to plate cells in a 96-well plate. Before plating cells, it is essential to know the numbers of cells required for each well, so that they will give a reasonable OD that can be read. Then, the volume of cell culture media in the well has to be adjusted so that the wells can also accommodate other components such as drugs, stopping solution, etc. A fixed number of cells in each well will give a comparison of the toxic effect caused by the drug being tested.
3. Preparation of drug dilution

   One should check drug solubility before starting the experiment. Some drugs are water-soluble and some are soluble in organic solvent. When drugs are soluble in organic solvent, the solvent should be prepared with the minimal possible volume of organic solvent; otherwise, the organic solvent itself may contribute toward cell cytotoxicity. When using organic solvent to dissolve the drug, it is always advisable to use control wells for the solvent too, so that the effect of the solvent can be observed. The important point is to be careful when working with drugs which are not soluble in water.
4. Addition of drugs

   Determine the drug concentration in such a manner that after addition of the drug to the cells, each well will have the desired effective concentration of drug. The usual method for doing this is to use a 2×, 5×, 10×, or 20× concentration of drugs. After addition of drugs, the plate is incubated.
5. Addition of MTT

   After the desired time interval, drug toxicity can be evaluated by adding MTT solution to each well. Then, MTT has to be incubated with the cells so that live cells can metabolize MTT to give a blue color; this is done because it is an enzymatic reaction.
6. Stopping the reaction

   The MTT reaction is stopped by adding stopping solution after the desired period of incubation. Addition of stopping solution will prevent further reaction because stopping solution causes cell death or lysis of cells.
7. Data recording and calculation

Once the reaction is terminated, OD of the plate has to be read at specified a wavelength. On the basis of OD, one can find out the concentration of drugs that are toxic to the cells.

Special note

The concentration of drugs that is toxic to cells should usually be avoided when evaluating their anti-HIV effect.

Crucial steps

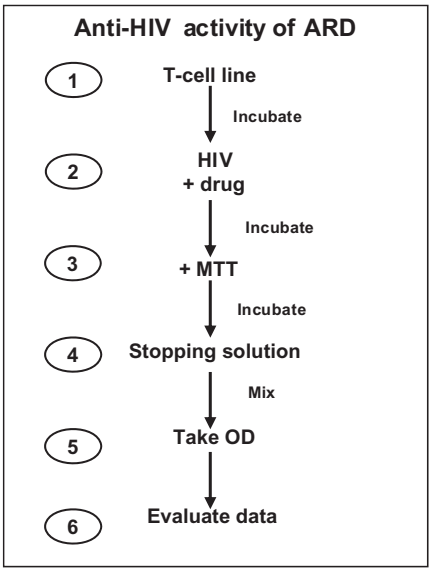
1. Culture of cells
   The desired number of cells must be grown for the experiment because they are the target cells.
2. Cell plating
   As per the experimental protocols, a fixed numbers of cells must be plated into the required number of wells with the required volume of culture medium.
3. Preparation of drug dilution
   The required concentration of drugs must be prepared by keeping in mind the effective concentration in their respective wells.
4. Addition of drugs
   At this stage, either different drugs or different concentrations of the same drug in specified volumes must be added to each well.
5. MTT reaction
   After incubation of the cells with the drug for the specified time, MTT dye must be added so that viable cells can give the blue color.
6. Stopping the reaction
   Stopping solution is added to stop the MTT reaction.
7. Data recording and calculation

Take an OD of the plate in an ELISA reader and calculate toxicity.

Evaluating anti-HIV effects of antiretroviral drugs

This assay is simply based on the principle of MTT assay for the evaluation of drug toxicity. One should select cells that can be productively infected with HIV, and HIV infection should induce cell death. So, the cell death caused by HIV infection can be evaluated using MTT. Once we add antiretroviral drugs and if these drugs are effective against HIV, then there is a decrease in cell death due to the anti-HIV effect of nontoxic concentrations of drug(s). This is one of the most convenient and cheapest assays to screen of antiretroviral drugs. Due to the convenience, this method can be easily employed for screening of large numbers of drugs and even to evaluate different analogs of same drugs to compare their efficacy (Flowchart 9.3).

It is advisable to follow all biosafety guidelines when working with infectious HIV. To setup an experiment to study anti-HIV effect of drug(s), following steps have to be performed: (1) growing cells of choice, (2) plating of cells, (3) thawing of HIV stock, (4) preparation of drug dilution or concentration, (5) addition of drugs, (6) addition of MTT, (7) stopping the MTT reaction, and (8) evaluation of data.



FLOWCHART 9.3 Schematic protocol to test anti-HIV activity of antiretroviral drugs using MTT assay.

Principles

1. Growing cells
   For this experiment, it is usually recommended that T-cell lines are used, which can be infected with HIV; cell death should occur after HIV infection. If a T-cell line is used, then it is better to use cells grown overnight for this assay.
2. Plating of cells
   The next step is to plate cells grown overnight in 96-well plates. Before plating cells, it is essential to know the number of cells required for each well so that a reasonable OD can be obtained during the measurement.
3. Thawing of HIV stock
   Take out HIV stock with a known titer. Thaw it and mix it into each well in an appropriate volume. Cells infected with HIV will show cell death.
4. Preparation of drug dilution
   After evaluation of drug toxicity, one should use only a nontoxic concentrations of drug to evaluate the anti-HIV effects of the drug that is being tested. It is advisable to try at least five different concentrations of the drug in order to test its anti-HIV effects.
5. Addition of drugs
   Adjust the drug concentration, so that after addition of other components, the final drug concentrations are reached in their respective wells. Usually, one can use 2×, 5×, 10×, and 20× concentrations of drugs. After addition, drug plates have to be incubated in CO_2 incubator.
6. Addition of MTT
   To find the effect of different concentrations of drug, the MTT solution is added to each well. Mixing can be

done with either a plate mixer or multichannel pipette. Then, incubate the cells for the required period of time, so that the live cells can metabolize MTT.

7. Stopping the reaction
   The MTT reaction can be stopped by adding the stopping solution. After adding the stopping solution, one can mix the crystals formed after MTT reaction by mixing the solution.
8. Data recording and calculation

When the reaction is stopped, the whole plate must be read for OD at the specified wavelength in the ELISA reader. On the basis of OD, one can find out the concentration of drug that is showing an anti-HIV effect.

Special note

Use an appropriate control for the blank, drug, and HIV only.

One can use XTT or other dyes for this assay as they more convenient.

Crucial steps

1. Growing of cells
   At this stage, a desired number of cells must be grown that is permissive to HIV infections.
2. Cell plating
   As per the experimental protocol, cells have to be plated in wells at a required volume of medium.
3. Thawing of HIV stock
   A known HIV viral stock has to be thawed to infect cells in culture.
4. Infection of culture
   A required volume of HIV stock has to be added into each well to infect cells.
5. Drug preparation
   The required concentrations of drug has to be prepared by keeping in mind the effective concentration in the respective wells.
6. Evaluation of drug
   At this stage, different drugs and their different concentrations at specified volumes must be added to respective wells.
7. MTT reaction
   After incubation of cells with drugs for a specified time, MTT dye must be added.
8. Stopping the reaction
   Add the stopping solution to stop the MTT reaction.
9. Observation

Take an OD of the plate and calculate toxicity.

Understanding HIV reservoir

HIV-1 can infect different immune cells of human body and produce more copies of itself. When adverse environment is encountered by the virus then it stops producing copy number. This is a latent state or a quiescent stage where minimal production of HIV-1 occurs. In this state, HIV-1 uses the host immune cells such as dendritic cells, resting $CD4^+$ T cells and macrophages as its personal sanctuary also known as reservoirs by HIV researchers (Fig. 9.4). Dendritic cells (DCs) serve as a connecting link between innate and adaptive immunity. DC is a kind of professional antigen-presenting cells, which presents processed antigens to $CD4^+$ T cells. DCs have three subtypes: (1) myeloid dendritic cell, (2) plasmacytoid dendritic cell, and (3) Langerhans cells. All of the above-mentioned subtypes of DCs can get infected by HIV-1, although Follicular dendritic cells are major HIV-1 reservoirs as they recruit resting $CD4^+$ T cells and aid in viral dissemination. When HIV infection becomes chronic at that time, large number of virions are released which get trapped by follicular dendritic cells present in the lymphoid organs. This entrapment protects HIV-1 virions from the action of antiretroviral drugs that might be prescribed to the patient (Clarke et al., 2000).

Macrophage is another key HIV-1 reservoir, as they are long-lived and present everywhere in the human body such as kidney, bone marrow, and brain. These places are not easily accessible to the antiretroviral drugs. Macrophages are susceptible to virus and actively produce virions in both ART treated as well as untreated patients. Macrophages engulf HIV-1-infected $CD4^+$ T cells, and in this process, they get themselves infected with HIV-1. Macrophages are professional antigen-presenting cell, and hence, they can easily infect other immune cells coming in contact with them. Macrophages are less susceptible to cytopathy post-HIV infection as HIV-1 induces NF-κB pathway (in infected macrophages) resulting in the activation of antiapoptotic genes and preventing cell death. Antiretroviral therapy is more effective in eliminating HIV-infected $CD4^+$ T cells and mucosal tissues when compared with macrophage elimination. Hence, infected macrophages remain a long-term source of HIV reservoir sustaining replicative viral particle and serving as an important contributor of HIV-1 reservoir. Brain cells such as astrocytes and microglial cells have CD4 receptors; hence, HIV-1 can infect them too, and it is reported that these cells serve as major HIV-1 reservoirs in infected individuals (Jordan et al., 1991). Antiretroviral drugs are unable to cross the blood–brain barrier (BBB), so brain is a kind of safe hiding place for the virus.

Mechanism of viral latency

Latency is a state in which production of viral particles is at a minimal or basal level. At the time of

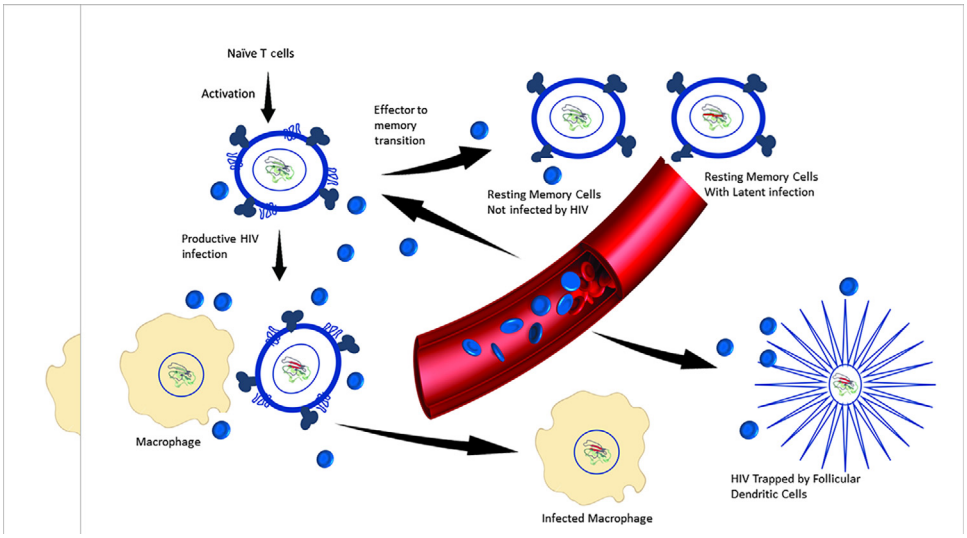


FIGURE 9.4 HIV reservoir. Development of HIV reservoir. HIV is circulating in blood stream, when Naïve T cells receive the signal, it become activated into activated T cells and the proliferation rate enhances. Activated T cells had CD4 and CCR5 expressed; it is the entry point to HIV virus. Activated T cells also transform into memory cells infected T cells that are responsible for latently memory cells formation. Infected T cells also get phagocytosed by macrophage, and macrophages get converted into another form of HIV reservoir. Circulating viral particle also get trapped by follicular dendritic cells and lead to the development of dendritic cell HIV reservoir. Memory T cells are not infected by HIV due to the lower expression of CCR5 on the membrane of memory T cells, although infected T cells lead to the generation of HIV reservoir. Macrophage, DCs, and $CD4^+$ T cells are main cells for HIV reservoir; these cells mainly resides in lymphoid tissues and endotheloreticulo system where antiretroviral drugs accessibility is minimal. Further, antiretroviral drugs have ineffective against latent virus.

integration into cellular genome, viral cDNA may get inverted, deleted, or mutated resulting in defects in the viral phenotype. Some of the host factors that may contribute to latency are repressive chromatin state such as obstructive nucleosome positioning, DNA methylation, and post-translational modification of histone and nonhistone proteins. Other HIV factors that contribute to latency are tat protein, RNAi, and defect in RNA splicing and export (Sengupta and Siliciano, 2018).

HIV-1 latency is categorized into two types: first is preintegration latency and second is postintegration latency. Preintegration latency was first determined in $CD4^+$ T cells, and later on, it was reported in monocytes too. In preintegration latency, HIV-1 cDNA is synthesized, but it is unable to integrate in the cellular genome and remains as extra chromosomal element known as episome. In postintegration latency, HIV-1 can integrate in the host genome but viral genome is not expressed fully; therefore, functional viral antigens cannot be produced.

There is always a possibility of latency reversal, which could be brought about by cytokines, latency reversal agents, and upon interruption of antiretroviral drugs. However, it has been studied that only 10% of all viral DNA present in latent form is intact and out of that only one tenth has the potential for conversion to active form. The site of integration of viral genome is also important as it determines whether viral genes will be expressed or not; studies have shown that cDNA integration at heterochromatin region remains silent, due to low accessibility of transcription factors to the regulatory regions.

Latency is a hallmark of reservoir where infected cells transcriptionally silent HIV-1 provirus. HIV latency and its reversal is still a mysterious problem faced by HIV researchers, and we expect unraveling of the answer in the near future. Till now, it can be said that maintaining and establishment of latency is a complex process and is governed by different factors; some suppress latency, for example, cytokines, toll-like receptors, effectors of NF-κB, inhibitors of histone-deacetylase and pleiotropic mammalian target of rapamycin effectors. The role of proviral DNA in latency has been studied too, and it is known that 5′LTR contains regulatory regions that control viral RNA synthesis by several independent mechanisms. Latency can be induced by inhibitors of viral transcription factor, histone modifiers, and cytokines that induce memory T-cell differentiation. Transcription factors too play a key role in the establishment of latency, as in the absence of these factors, HIV-1 transcription declines substantially in resting $CD4^+$ T cells. Transcription factors such as NF-κB, NFAT, and sp1 play an important role to determine the expression of viral gene (Gonzales et al., 2018). NF-κB is a key player in the establishment and maintenance of latency. NF-κB monomer, p50, and Rel (p65) dimerize and are translocated into the nucleus

upon pathogenic infections or in response to proinflammatory cytokines. Under such conditions, they interact with NF-κB regulatory elements as well as HIV-1 proviral DNA, which leads to an increase in the HIV-1 transcription.

Epigenetic modifications are also important in maintaining the latency of HIV-1. Chromatin structure and packing can determine expression conditions; condensed chromatin structure promotes latency; and relaxed chromatin structure promotes active viral pro-DNA transcription. Histone acetyltransferase (HAT) relaxes chromatin, increasing the transcriptional activity, whereas histone deacetylase promotes chromatin condensation, and viral expression is repressed. Cellular micro-RNA (miRNA) present in $CD4^+$ T cells have been reported to regulate HIV-1 gene expression by RNA-induced silencing complex formation and promoting transcript degradation. Computational studies have shown that miR-29a, miR-149, miR324-5b, and miR-378 can regulate HIV-1 transcript.

When the infected individual is treated with ART or HAART, then plasma load of virus decreases up to a level where it cannot be detected or quantified by the available methods. Following ART or HAART, virus persists in the form of integrated provirus in resting $CD4^+$ T cells. If regular drug intake is disrupted by any means, then provirus can proliferate and active transcription of viral gene begins. Till date, no method has been devised which can distinguish between latently infected and uninfected healthy T-cells. To overcome this drawback, new techniques have to be developed, which can make use of some biomarkers present on latently infected T-cells or other immune cells for the estimation of HIV-1 reservoirs in infected cases.

NeuroAIDS: an emerging health concern

Since 1981, HIV researchers have seen enormous development toward the diagnosis of infection and improvement in antiretroviral drugs. Azidothymidine (AZT) was the only drug approved in 1985. At present, HIV patients have numerous drug options and treatment modalities. An improvement in antiretroviral drugs and treatment strategies has made it possible to prolong the asymptomatic stage of HIV patients (Stage II) to ~20 years or more post-HIV exposure. No doubt, this has improved the quality of life in patients.

Early last decade, a sudden increase in neuropsychiatric complications was observed among long-term HIV seropositives. In the general population, the prevalence of neuropsychiatric diseases is 10%–15%. Therefore, it was surprising to observe a prevalence of 50% or more neuropsychiatric complications among HIV seropositives (McCombe et al., 2009; Power et al., 2009). Initially, various names were given to these neuropsychiatric complications, such as HAD, HIV-associated encelopathy, HIV-associated minor cognitive and motor disorders, etc. (McArthur et al., 2005). Now all of these neuropsychiatric disorders have been grouped under the name "NeuroAIDS." In the past 10 years or more, NeuroAIDS has turned out to be another health issue among long-term HIV seropositives. Some of the common neuropsychiatric complications are listed in Table 9.5.

TABLE 9.5 NeuroAIDS: common neuropsychiatric disorders.

Addiction
Anxiety
Depression
Epilepsy
Mania
Mood disorders
Neurocognitive impairment
Neuropathic pain
Physical disability
Seizures

These neuropsychiatric complications in HIV-seropositives affect both the central nervous system (CNS) and peripheral nervous system. The CNS is considered to be one of the most protected organ systems in the body. It is protected by the BBB. The main function of the BBB is to regulate the entry of any biomolecules or agents into the CNS. It is intriguing to note that most of the cells present in the CNS do not get infected with HIV; this is due to the fact that they do not express the receptors required for HIV infection. All the evidences suggest that neurons are nonpermissible to HIV infections, as they too do not have receptors for HIV infectivity. The question then becomes: how are these cells affected by HIV infection? One of the best rationales for HIV infection of the CNS is the "Trojan Horse Hypothesis." According to this hypothesis, HIV enters the brain along with the infected cells from the systemic circulation (e.g., monocytes or T cells). Once HIV enters the brain, it can remain latent for a long time, and at some point in time, it can start active replication, which leads to the production of various biomolecules. In turn, these viral biomolecules can induce numerous proinflammatory and inflammatory cytokines in the local milieu. No doubt, these cytokines are produced as a part of the protective immune response, but in CNS, they can cause neuronal death via apoptosis. Neurons do not regenerate, so neuronal death results in permanent damage.

Neuronal damage/death could be responsible for neuropsychiatric complications among HIV seropositives. NeuroAIDS is expected to become a major health concern for HIV patients for the following reasons: (1) the number of patients is increasing on a daily basis, (2) symptoms of NeuroAIDS hit right at the prime age of a human life (i.e., between the ages of 35–45 years), (3) a continuous supply of antiretroviral medications is costly, (4) NeuroAIDS patients need additional medication, (5) NeuroAIDS symptoms usually render these patients less productive, which can result in a substantial loss of individual and/or family income, and (6) the eventual need for a caretaker or caregiver can (again) translate into a serious financial burden.

Although the exact reason for the onset of NeuroAIDS is not yet known, there are various possibilities under active consideration: (1) Is HIV itself responsible for NeuroAIDS? (2) Is NeuroAIDS a secondary complication which has emerged due to long-term ART? (3) Is there low or negligible penetrance of ART into the brain? (4) Are low levels of persistent and chronic HIV infection contributing toward the development of NeuroAIDS? or (5) Is it a combined effect of all these possibilities? The present understanding of NeuroAIDS and its causes remains unclear.

Unfortunately, there is no good model either for in vitro or in vivo studies to understand the mechanism of NeuroAIDS. It is difficult to develop and test the efficacy of drugs to treat NeuroAIDS due to the nonavailability of suitable animal models. Because of these circumstances and by realizing the quantum of expected problems in near future, it is important to focus on a better understanding of the pathogenesis and drug targets for NeuroAIDS (Verma et al., 2010, 2012).

Bone marrow transplantation: a probable cure for HIV

There is no doubt that a cure for HIV must still to be found, so that this dreaded disease can be brought under control. Although there is not yet any single strategy to cure HIV, some clues from HIV patients themselves have offered possibilities for possible treatments in future. There is only one patient who has been cured using this approach. This man is famously known as Berlin Man or Berlin Patient.

During HIV epidemic, it was noticed that certain individuals were classified as high-risk individuals as per the criteria for HIV infectivity. These individuals had been exposed to HIV several times, and surprisingly, they did not get infected, even though they were never on any ART. This observation led to the conclusion that these individuals must have "natural resistance" against HIV infections (Shearer and Clerici, 1996). Further studies have revealed that the major reason for natural resistance against HIV infection is the requirement of co-receptors for infection; under normal circumstances, these co-receptors serve as receptors for chemokines. Initially, it was believed that CD4 receptors, which are present on T cells and monocytes, are required for HIV infection. The co-receptors that have been identified as essential for HIV infection are CXCR4 and CCR5. On the basis of infectivity through these receptors, HIV has been classified into two different strains. One is known as the X-4 strain (which shows a preference for infectivity through CXCR-4) and the other is known as the R5 strain (which shows a preference for CCR5 receptors).

Extensive research showed that people who are naturally resistant to HIV infection have a mutation in the CCR5 gene. The mutation is a deletion of a 32-bp sequence from the gene sequences of CCR5, which is the reason it is known as a Δ32 deletion. The story got more complicated with more detailed studies when it was found that these mutations could be either homozygous or heterozygous. Homozygous mutants (i.e., CCR5Δ32/Δ32) are fully resistant to HIV infections, while individuals carrying heterozygous deletions have shown very slow progression of the disease after exposure to HIV. So far, it has been observed that this deletion exists only in Caucasians. Even among Caucasians, the frequency of this mutation is only about 1%–3%. Hence, the possibility that it could be used as a common therapeutic approach is not a viable option. Nevertheless, this fact has not discouraged scientists and clinicians from making use of this natural phenomenon.

Berlin Patient earned a reputation as being the first case to test the above-mentioned fact that bone marrow transplantation with a resistant mutation could cure HIV infection. "Berlin Patient" is a 40-year old male who was HIV positive for more than 10 years. He was under a HAART regimen, and his HIV infection was under proper control due to the right choice of ART. This man developed acute myelogenous leukemia (AML), and he failed to respond to the AML treatment. The only option for clinicians was to treat this patient by allogeneic transplantation (Hütter et al., 2009). The surgeon treating this patient took an extra precaution to transplant this patient with bone marrow from a donor carrying CCR5Δ32/Δ32 mutations. When the patient was given this allogeneic transplantation, he did not show any signs of HIV positivity, even though ART was stopped. The probable reason for HIV negativity in this patient was due to the absence of the co-receptors required for HIV infection. This appears to be a new hope for curing HIV, but it has two major limitations: (1) this mutation is rare and (2) one has to weigh the substantial risks and benefits of bone marrow transplantation and ART among HIV seropositives.

Ethical issues

HIV does not have an ideal animal model, which is a major handicap for HIV research. The majority of data for HIV has been generated either by in vitro studies or directly from human patients. It is next to impossible to get human volunteers for a disease that does not have a cure. This leaves so many unanswered questions. Due to the unavailability of a suitable animal model for HIV, the only option for drug testing is to rely on in vitro assays or HIV patients. If a patient is already undergoing ART, is it ethical to change a treatment that is already working for the patient? Similar is the case for HIV vaccine trials.

A few major ethical issues related to HIV infection are discussed below:

1. What are the legal repercussions of an HIV-infected individual knowingly infecting someone else? How can it be prevented?
2. What if a person is infected with HIV, and due to social stigmas, he/she does not inform his/her spouse? This kind of irresponsible behavior can be disastrous to a partner and raises many ethical and legal concerns. What if a pregnancy is involved? Where does it stop?
3. Transmission of HIV due to unethical professional practices is also of concern. These negligent practices can be the mistakes by dentists, surgeons, phlebotomist, blood banks, etc., and their mistakes can make their patients sick for life.
4. Another concern is data obtained from partially suitable animal models such as monkeys and chimpanzees that could not be applied with full confidence to human subjects too.
5. Sometimes, HIV infection is transferred from victims of crimes to emergency responders. This can lead to serious ethical and legal issues.
6. From a workplace standpoint, what if someone is HIV seropositive and is denied employment? This raises questions regarding the ethical practices of the employer.

Unless a cure is found for HIV, these ethical concerns will remain. In addition, various new ethical issues are bound to arise with time, and they will need to be addressed in order to resolve any such dilemmas.

Translational significance

HIV turned out to be one of the most dreaded diseases of the 20th century. HIV is not only dangerous when contracted, but extremely infectious. The ultimate outcome of HIV infection is AIDS, which is considered to be the final chapter in the life of an HIV patient. Because most of the initial reports of HIV infection came from homosexual populations, much of the early media attention erroneously focused on socioreligious and political issues rather than the disease itself. The major obstacle in HIV research is that there is still no ideal animal model for HIV infection. Various animal models have been tried, including mice, rats, monkeys, chimpanzees, etc., but all of them have one limitation or another. This is the reason that all data about HIV infection and treatment is either based on in vitro studies or data from clinics.

Most of the time in vitro data cannot be applied as such for in vivo models, and certainly not for human studies. Clinical data often suggest important aspects of a disease, but individual variations can make it difficult to apply the same conclusions from one set of individuals to another. The HIV story got another twist when it was found that certain individuals do not get infected with HIV at all due to genetic variation in the receptor expression on cells of the immune system. This variation is attributed to a mutation that can be either homozygous or heterozygous. All these observations have translational significance, which must be explored vigorously to make them practical and useful. As far as the translational value for HIV research is concerned, it is just beyond imagination. Anything that is done with reference to HIV infections is important for its translational value. Even a better understanding of the mechanisms of replication and infection will help in developing a better animal model. An animal model will help in understanding the natural history of HIV, which in turn will be helpful in designing better strategies to treat HIV infections.

In fact, understanding of the steps involved in viral replication has been used to develop new antiretroviral drugs that target different steps/stages of HIV replication. This is the reason that at present we have various types of antiretrovirals such as NRTIs, NNRTIs, PIs, InIs, etc. Progress in understanding the mode of action of different antiretroviral drugs has led to the development of HAART and Drug Holiday regimens. Next-generation HIV drugs are under development which will provide better treatment for HIV infections by preventing viral attachment to host cells. No doubt in-depth information about HIV replication and infection, and better treatment strategies, can bring a halt to HIV infections. This information will also be helpful in developing effective prophylactic vaccines.

It will be because of translational research that one day we may be able to say with confidence that like small pox and polio, *HIV is now eradicated*.

Clinical correlation

Several monoclonal antibodies have been tried and tested for their anti-HIV effects till date. Monoclonal antibody VRCO1LS dedicated against CD4 binding site of envelope glycoprotein was evaluated in a Phase-I trial. The study was conducted by vaccine research centre at NIH, USA. VRCO1LS is a modified version of VRCO1, which is a broadly neutralizing monoclonal antibody currently being evaluated in a Phase-IIb adult HIV-1 prevention efficacy trial. This study concluded that VRCO1LS monoclonal antibody has fourfold greater half-life when compared with its earlier version VRCO1. This study showed that reduced clearance and less frequent and lower dose administration of VRCO1LS may be helpful in the prevention of HIV-1 infection in adult humans (Gaudinski et al., 2018).

HIV-1 envelope specific IgA monoclonal antibody produced by memory B-cell cultures isolated from PBMCs of a patient who received RV-144 HIV vaccine. This person had circulating IgA response to the vaccine immunogen and did not have HLA-II alleles that are involved in modulating vaccine induced antibody response. Two IgA-1 clonal cell lines from this person were isolated and selected for sequencing of variable heavy and light chains and characterization of antiviral functions. Their result showed potential antiviral function of vaccine elicited IgA directed against HIV-1 envelope. These monoclonal antibodies were shown to block the binding of viral infected cells to epithelial cells or HIV-1 transcytosis in vivo (Wills et al., 2018).

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World Wide Web resources

The World Wide Web has become an important resource for instant and easily accessible information, 24/7. Google searches (http://www.google.com) using three different keywords returned the following results: "HIV," 176 million hits; "AIDS," 332 million hits; and a combination of "HIV" and "AIDS," 308 million hits. The WWW is an excellent place to look for information about different aspects of HIV and AIDS, including history, discovery, clinical stages, clinical parameters, life cycle, routes of infection, myths, treatment types, prevalence, epidemiology, etc. Different search engines will undoubtedly return different depths of information.

Another source of information is the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov). This site is maintained by the National Institutes of Health, Bethesda, MD. It is an excellent source for the compilation and maintenance of scientifically authenticated information. "HIV" as a keyword returned more than 250,000 records, while "AIDS" gave more than 191,000 records; a combination of the two showed more than 105,000 records. This is also a good source for retrieving protein sequences, nucleotide sequences, sequence alignments, primers already in use, as well as tools for designing primers and models to predict signal transduction pathways.

Apart from these sources, Los Alamos National Laboratory, USA (www.hiv.lanl.gov), is a good source for data regarding HIV databases.

There are also various hospitals and clinics that have websites dedicated to HIV information for the general public, along with listings of clinicians treating HIV infections.

For recent developments and scientific information, one can check the websites of different journals published in this area (e.g., JAMA, NEJM, Science, Nature, JAIDS, etc.).

UNAIDS gives valuable information about HIV and AIDS (www.unaids.org). It is one of the most authenticated and updated sources of information about epidemiological data at global levels and includes information from many different nations.

Another important source for HIV information is The Body (http://www.thebody.com), a resource with easy-to-understand explanations.

There are various other good resources available on the web, including books on HIV and AIDS. Some of them are available for free, like *HIV-2011* (http://www.hivbook.com), while others require a subscription.

Apart from these helpful sites that provide statistical and clinical prevalence of HIV are https://www.cdc.gov/hiv, https://www.healthline.com, https://www.who.int, https://aidsinfo.nih.gov, https://www.avert.org, https://www.hiv.gov.

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Glossary

Proviral DNA Reverse transcriptase synthesizes DNA from viral RNA. This viral DNA is known as proviral DNA.

Antiretroviral drugs Drugs used to treat HIV infection. These drugs can also be used to treat other retroviral infections (e.g., Azt).

NeuroAIDS Neuropsychiatric complications in HIV patients are grouped under the term NeuroAIDS (e.g., anxiety, mood disorders, etc.).

Opportunistic infection An infection by a microorganism that normally does not cause disease but becomes pathogenic when the body's immune system is impaired and unable to fight off infection (e.g., *P. carinii*).

Protease inhibitors Drugs that inhibit the activity of proteases. These drugs are also used to treat HIV infection (e.g., Indinavir).
Integrase inhibitors Drugs that inhibit the activity of integrase in the case of HIV infections (e.g., Raltegravir).
Reverse transcriptase An enzyme that reverse transcribes RNA into DNA. It is found in retroviruses (e.g., HIV).
Asymptomatic stage One of the clinical stages of HIV infection that does not show any significant signs and/or symptoms of HIV infection. HIV replication is low and controlled during this stage.
Latency HIV integrated to host DNA without causing any symptoms and last after longer period
Reservoir A group of cells that is infected with HIV for longer period
ART Antiretroviral therapy used to treat patient who have infected with HIV.

Abbreviations

AIDS	Acquired immune deficiency syndrome
AML	Acute myelogenous leukemia
ART	Antiretroviral therapy
ART	Antiretroviral treatment
BBB	Blood–brain barrier
CNS	Central nervous system
Env	Envelope
FDC	Fixed-dose combination
Gag	Group-specific antigen
HAART	Highly active antiretroviral therapy
HAD	HIV-associated dementia
HAT	Histone acetyl transferase
HIV	Human immunodeficiency virus
InI	Integrase inhibitor
LTR	Long terminal repeat
Nef	Negative factor
NNRTI	Non-nucleoside reverse transcriptase inhibitor
NRTI	Nucleoside reverse transcriptase inhibition
NtRTI	Nucleotide reverse transcriptase inhibitor
PDI	Protein disulfide isomerase
PI	Protease inhibitors
PNS	Peripheral nervous system
Pol	Polymerase
Rev	Regulator of viral expression
RISC	RNA induced silencing complex
Tat	Transactivator of transcription
TAT	Trans-activator of transcription
Vif	Viral infectivity factor
Vpr	Viral protein R
Vpu	Viral protein U

Long answer questions

1. Explain HIV replication in detail.
2. What is NeuroAIDS? What are the implications of NeuroAIDS?
3. Discuss different classes of antiretroviral drugs and their mode of action, giving at least one example of each.
4. What is PDI? What is the mechanism by which PDI can inhibit HIV infection?
5. How can antiretroviral drugs be tested in vitro?
6. Discuss different genes of HIV and their role in HIV replication.
7. Give a brief account of HIV and AIDS history.
8. Discuss different routes for transmission of HIV infection.
9. Discuss the WHO system for classification of clinical stages of HIV infections.
10. Describe how to grow HIV stock.

Short answer questions

1. Name different genes of HIV.
2. Define proviral DNA.
3. Define NtRTI drugs and how they are different from NRTIs?
4. What is HAART?
5. What are opportunistic infections?
6. What are some other names that were once used for AIDS?
7. Name the receptor and co-receptors essential for HIV infections.
8. What is salvage therapy for HIV patients?
9. What is the asymptomatic stage of HIV infection?
10. What is the seroconversion stage?

Answers to short answer questions

1. The HIV genome has nine genes, which are flanked by long terminal repeats (LTRs). These genes are *gag*, *env*, *nef*, *pol*, *rev*, *tat*, *vif*, *vpr*, and *vpu*.
2. During reverse transcription, viral RNA gets transcribed into complementary DNA (cDNA) due to the action of reverse transcriptase enzyme. This cDNA is known as proviral DNA.
3. The full name of NtRTI is nucleotide reverse transcriptase inhibitor. It is a group of antiretroviral drugs that consists of nucleotide analogs instead of nucleosides. Their action does not require activation; this is the difference between NtRTIs and NRTIs.
4. The full name of HAART is highly active antiretroviral treatment. It is a kind of combinational therapy used to treat HIV infections. This treatment contains three to four drugs from different groups of antiretroviral drugs.
5. Opportunistic infections are infections that are rare in the general population. Opportunistic infections commonly occur in severely immunocompromised hosts. AIDS patients show opportunistic infections like *Pneumocystis carinii*, which usually causes pneumonia.
6. The term "AIDS" was coined by the Centers for Disease Control and Prevention (CDC), Atlanta,

GA, in 1982. Before the term AIDS was coined, this disease had different names like gay compromise syndrome (GCS), gay related immuno-deficiency (GRID), acquired immunodeficiency disease (AID), and gay cancer.

7. For HIV, CD4 is the receptor, which is found mainly on a subset of T cells known as T-helper cells. HIV also needs a co-receptor for infection, which can be either CXCR4 or CCR5.
8. As the name suggests, salvage therapy is designed to salvage the HIV-infected patient. Salvage therapy is also known as mega-HAART therapy. This therapy is used among those HIV patients who do not respond to (or are resistant to) various antiretroviral treatments. It is an expensive treatment for HIV patients and has serious side effects.
9. Stage II of HIV infection is known as the asymptomatic phase. In this stage, HIV replication is low and the patient does not show any significant signs and symptoms of HIV infection. A patient can remain in this stage for more than 10 years. This stage can be extended for more than 20 years with proper antiretroviral treatment.
10. Initial HIV infection (i.e., Stage I) is classified as the "Seroconversion Stage." During this stage, the patient shows mild symptoms of the disease. Patients also produce antibodies against HIV in their serum. It can last up to 6 months.

Yes/no type questions

1. Is HIV a single type of virus?
2. HTLV and LAV is two different Virus?
3. In Stage II, the CD4 T cells counts are less than 200 cells/mm^3?
4. In HIV virus, the genetic material is negative sense RNA?
5. Nef does not play any role in CNS functioning?
6. The first step of HIV replication is integration of the proviral DNA.
7. In some circumstances, the proviral DNA gets integrated but virus does not go active replication.
8. Is T-20 targeted against binding of HIV to CCR5?
9. Does MTT dye work with the action of mitochondrial enzyme?
10. Is the Tat protein-enhanced viral transcriptional rate?
11. Vpr protein of HIV is essential for replication?

Answers to yes/no type questions

1. No—HIV is not a single virus; it is of two different types, that is, HIV-1 and HIV-2.
2. No—Both these names were given around 1985 for the infection caused by HIV.
3. No—During Stage II, T cells count are very close to around 500 cells/mm^3.
4. No—the genetic material of HIV virus is single-stranded positive sense RNA.
5. No—Nef plays a significant role in CNS functioning because Nef is present in higher level in astrocytes, causing alteration in their growth.
6. No—The first step of HIV infection is viral attachment and entry inside the cells.
7. Yes—this is the case in which latent infection by HIV virus occurs.
8. No—T-20 is targeted against active state of gp41.
9. Yes—MTT dye works with the action of mitochondrial enzyme, when MTT is added to cells, it get converted into a blue-colored products.
10. Yes—Tat protein enhances viral transcriptional rate up to the 1000 fold.
11. No—Vpr is accessory protein of HIV, and it is not essential for viral replication.

CHAPTER

10

Animal models in advancement of research in enteric diseases

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Summary

Animal models are extremely imperative because they permit researchers to illuminate complex biological mechanisms that trigger cellular processes and molecular functions in infection and diseases (Fig. 10.1). To understand the complex molecular mechanism of disease and development of therapeutic strategies, direct human studies cannot be conducted because explaining it requires carefully controlled experimental manipulations, such as genetic knock-out/knock-ins, administration of pharmacological agents, or exposure to controlled environmental factors such as bacteria and bacterial-derived products. In this chapter we focus on the characteristics, current challenges, and limitations of various animal models for enteric bacterial pathogens that have been developed and can be used to monitor the microbial colonization, disease, pathology, host immune response, or combinations of these features.

What you can expect to know

Worldwide enteric infections rank second among all causes of disease burden, which result in significant childhood morbidity and mortality. Enteric infections are caused by a gamut of bacterial, viral, and parasitic pathogens. Due to the constant emergence of multidrug-resistant enteric strains, World Health Organization (WHO) has given highest priority to the development of new or improved vaccines as well as to identify the in-depth cellular and molecular mechanism of pathogenesis. Despite large investments in vaccine research and drug development, the overall success rate during clinical development remains low. One prominent explanation is flawed preclinical research, in which the use and outcome of animal models are pivotal to bridge the translational gap to the clinic. Therefore to facilitate development of novel therapeutic strategies and vaccines for humans against these pathogens, animal models that mimic one or more aspects of infection and disease are essential to address the clinical question (Hotinger and May, 2019).

Introduction

Enteric bacterial infections causing diarrhea, dysentery, and enteric fevers are major public health concerns worldwide. These bacterial infections represent a significant burden, cause more than a billion disease episodes per year worldwide, and claim nearly two million lives each year, especially affecting children under the age of 5 years in the less-developed regions of the world. Enteric infections also pose a high risk for travelers from industrialized countries who visit less-developed areas. The principal enteric bacterial pathogens recognized are as follows: (1) enterotoxigenic *Escherichia coli* (ETEC), a predominant etiological agent causing diarrhea in travelers from industrialized countries and in infants and adults in the developing world; (2) *Vibrio cholerae*, responsible for endemic and epidemic cholera; (3) *Campylobacter jejuni*, an important cause of diarrhea in young children worldwide, as well as an agent of diarrhea in travelers; (4) *Shigella* species, as a cause of both watery diarrhea and dysentery, particularly in developing countries; (5) *Salmonella typhi*, the etiological

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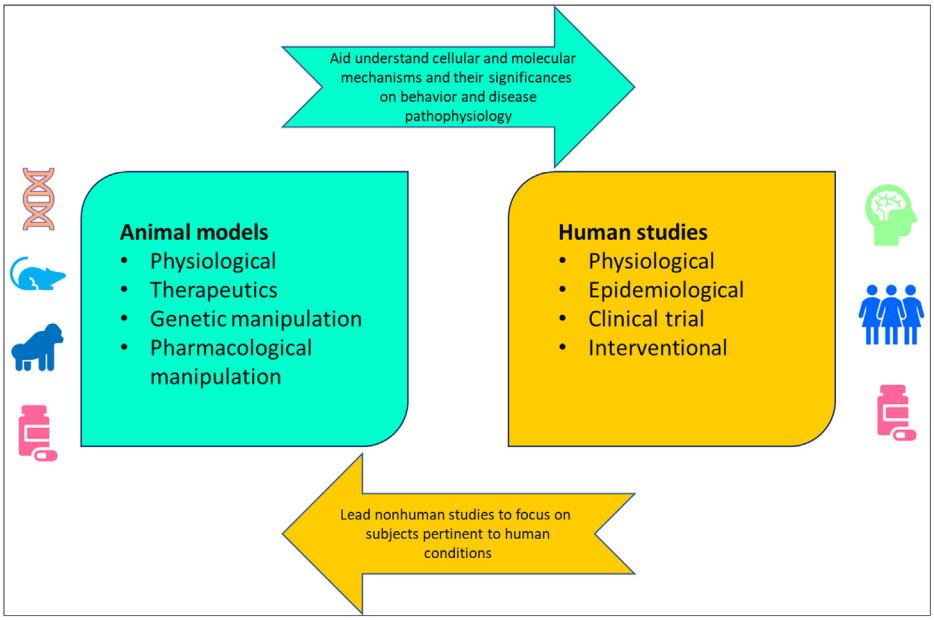


FIGURE 10.1 The association between animal models and human studies is reciprocal: both offer key insights into disease biology, mediators of disease pathogenesis, and identification of potential therapeutic target, drive the direction of one another's research, and complement each other's findings.

agent of typhoid fever, the most common enteric fever (a generalized infection of the reticuloendothelial system and intestinal lymphoid tissue accompanied by sustained fever and bacteremia); and (6) nontyphoidal *Salmonella* sp., an important cause of acute gastroenteritis in individuals in both developing and industrialized countries.

Vaccination offers the greatest hope as an effective and sustainable strategy against enteric illness. Development of successful enteric bacterial vaccines requires a more detailed understanding of cellular and molecular mechanisms of the infection process and how the host responds to infection, which also could ultimately lead to discover novel therapeutic strategies. Currently there is a substantial deficiency in the development of reliable and reproducible animal models that effectively portray immunological responses toward these pathogenic organisms at the gut mucosa. However the most currently used animal models are described in detail here.

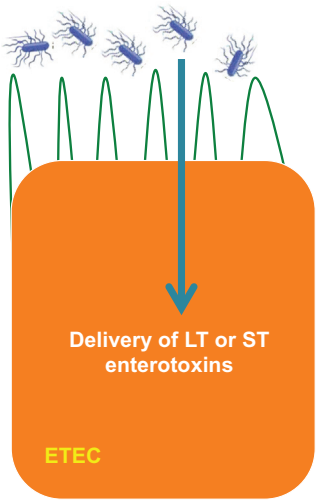


FIGURE 10.2 Enterotoxigenic *E. coli* (ETEC) adheres to the small bowel mucosa and delivers secretory enterotoxins, resulting in secretory diarrhea.

Animal models for enterotoxigenic *Escherichia coli*

ETEC is one of the most common causes of diarrhea in children in developing countries and in travelers to these areas. In the currently accepted theory for ETEC pathogenesis, fimbrial colonization factors (CFs) mediate colonization of the small intestine, where organisms elaborate enterotoxins, a heat-labile (LT) enterotoxin, and/or a heat-stable enterotoxin (ST), resulting in diarrheal disease (Fig. 10.2). However the actual process of infection in the gut remains mystery. The kinetics of colonization and toxin production, the distribution of organisms in the intestine during infection, and the mechanisms by which naive and immune hosts manage with colonizing bacteria and enterotoxin remain unclear. Several approaches have been adopted to investigate the pathogenesis of the disease and to evaluate anti-diarrheal therapies that is identification of potential vaccine candidates. Animal models that have been used in the study of ETEC include suckling, infant and adult mice, rats, and rabbits.

Suckling mouse model

The critical ETEC secretogenic virulence factors are the ST and LT enterotoxins. Both elicit net secretion of

ions and water, resulting in watery diarrhea, in the most serious cases it produces a profuse cholera-like condition. Numerous convenient and sensitive assay systems are available for the detection of LT, most laboratories dealing with ST depend on the suckling mouse model originally described by Dean et al. (1972). Briefly, newborn suckling mice (1−3 days old) are separated from their mothers immediately before the experiment. Each mouse is inoculated (intragastric, percutaneous injection) with 0.1 mL of crude bacterial culture filtrate containing 2 drops of 2% Evans blue. At various times after inoculation the mice are killed by cervical dislocation, the abdomen is opened, and the entire intestine is removed. The intestines from each mouse mice is pooled and weighed, and the ratio of gut weight to remaining carcass weight is calculated. Mice with no dye in the intestine or with dye within the peritoneal cavity at autopsy are discarded. The suckling mouse model aids understand the mechanisms of the pathogenesis of ETEC diarrhea due to the immaturity of the bacterial flora in the intestines of the newborn and the susceptibility of the infant mouse to the STa enterotoxin. This assay is a simple, fast, and reproducible assay for *E. coli* ST.

Infant mouse model

The infant mouse model described by Goldhar et al. (1986) is suitable for the study of bacterial properties responsible for various stages of intestinal colonization by human ETEC. Concisely 1 or 2 days after delivery, mother and her infants are caged separately and feed with normal diet, except that the drinking water of the mice to be used in experiments contain chloramphenicol. After 48 hours, the litters are separated from their mothers; 3 hours later, the infant mice are feed orally with 10 μL of a bacterial suspension containing 5×10^1 to 5×10^5 bacteria. Subsequently the infant mice are returned to their mothers and sacrificed after 3, 24, and 72 hours. The bowel segments are removed and opened longitudinally, placed in 5% saponin solution, incubated for 10 minutes at room temperature, and agitated on a vortex shaker for an additional 1 minute to release the adherent bacteria. The number of colony-forming unit (CFU) in the saponin wash of each intestine is determined by plating on MacConkey agar with or without chloramphenicol. The infant mouse model allows us to thoroughly monitor different stages of the colonization process. Furthermore this model permits isolation of isogenic mutants or variants with enhanced colonizing ability as well as identification of the factors involved in adherence and colonization of intestinal mucosa. Using this model demonstrated that colonization surface antigen 21 (CS21) is required for ETEC gut colonization, the first step of the diarrheal disease process, suggesting a relevant role of CS21 in ETEC pathogenesis.

Adult mouse model

The intranasal (i.n.) adult mouse model is suitable for better understanding the immunogenicity, pathogenicity of ETEC, and identification of virulence factors (Byrd et al., 2003). This model also provides valuable information on selection criteria for vaccine candidates for use in primate and human trials. Mice are lightly anesthetized with Metofane (methoxyfluorane) in a glass desiccator, followed by challenge with various doses (1×10^8 to 1×10^9 bacteria) of ETEC to measure morbidity and mortality. Fifty microliters of each preparation are administered drop wise to the external nares of each mouse with a pipette and the control mice are administered with 50 μL PBS. The challenged mice are euthanized, lungs are aseptically removed, and placed in sterile PBS. The lungs are subsequently homogenized to free bacteria into suspension, and a CFU procedure is performed to determine the number of bacteria present in the lungs at a given time postchallenge. CFA plates are used to determine the total number of bacterial cells present in the lungs, and MacConkey plates are used to ascertain that only lactose-fermentation-positive bacteria indicative of ETEC are present in the lungs of the mice. Furthermore the level of pathogenicity of ETEC strains or protective efficacy can be assessed by histopathology of the lung sections to determine the number of inflammatory infiltrates. The lungs are examined microscopically, and the number of inflammatory cells is noted. "Moderate-to-severe" indicates that the section of lung affected is from 11% to >41% with high numbers of inflammatory cells (neutrophils, macrophages, lymphocytes, and plasma cells), "minimal-to-mild" indicates that the section of lung affected is from 1% to 10% with low numbers of inflammatory cells, and "none" indicates that no histopathological changes are observed. This model showed that following ETEC challenge detectable levels of IgM and IgG antibodies to CF antigen and LPS are observed in sera as well as the pathogen evokes a T-helper cell response. Researchers frequently use the intranasal mouse model since the mouse intestinal epithelium shares similarity to the bronchus of the lung, as the lymphoid follicles present in the bronchial wall are similar to the Peyer's patches of the intestine. Mucosal immunity is an important protective mechanism, as attachment to mucosal surfaces is often the first step in establishing infection. However the major drawback of this model is that lack of the normal target cells of ETEC in the lungs of mice, and as a result of deficiency

of the normal receptors to which the CFs and enterotoxins adhere, along with the absence of the natural flora present in the intestines of humans, and these might influence the general outcome of an infection. Importantly another key point should be considered, it may be highly possible that the ETEC does not release enterotoxins in the lungs or the level is too low compared with the bacteria do in human intestines owing to the lack of appropriate physiological stimuli, such as acidic pH and bile salts.

Rat model

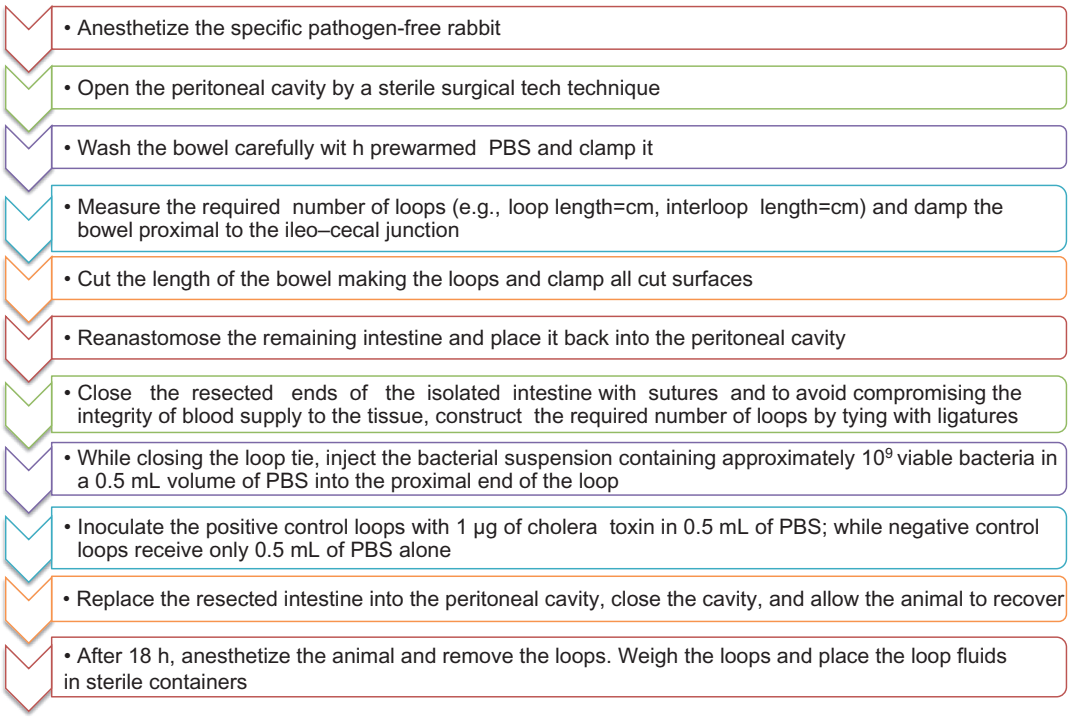
The use of rats as experimental model has been established by Klipstein et al. (1981) to evaluate whether active immunization with ST or LT yields protection against direct challenge with toxin-producing strains. Briefly rats are immunized by parenteral and/or intestinal routes. The priming dose is 100 μg and the boosting dose is 50 μg. For parenteral immunization the priming dose is dissolved in saline, emulsified with an equal volume of Freund's complete adjuvant (CFA), and administered intraperitoneally; for boosting doses, the toxin is given either in saline alone or mixed with Freund's incomplete adjuvant (FIA). For intestinal administration, the toxin is dissolved in saturated sodium bicarbonate and delivered by one of the three methods: (1) directly into the duodenum by means of a laparotomy, (2) perorally at 10 minutes after the peroral administration of the bicarbonate solution, or (3) perorally at 2 hours after the peroral administration of cimetidine at a dosage sufficient to ablate gastric secretions in the rat. All administrations referred as peroral are given directly into the stomach via an orogastric tube. The animals who received parenteral or intraduodenal boosts are boosted twice, at 2 and 4 weeks after priming; rats that are boosted perorally received doses at weekly intervals for 1 month after priming. Afterward rats are challenged at 1 week after the final boost by instillation of toxin or viable organisms, each at a concentration that yield 50% maximum secretary response or the maximum secretary response in unimmunized animals, into a single 10-cm ligated loop of distal ileum for 18 hours. The toxin is administered in sterile saline and for challenge with viable cultures, the bacteria are cultured, centrifuged, the bacterial pellet is resuspended in sterile saline, and instilled into ligated loops. This model established that ETEC ST enterotoxin conjugated with a protein carrier are protected against challenge with semipurified or purified ST and viable ETEC strains, hence proved that immunological protection is achievable in an experimental animal model against ETEC, which produces LT or ST, suggesting development of a feasible effective immunization approach against the pathogens in humans.

Rat offers many advantages as a model of human disease, the physiology is easier to monitor in the rat and, over time, the volume of data has developed that will take years to be replicated in the mouse. Moreover in many cases the physiology is more similar to the corresponding human condition. However the production of specific mutations, conditional mutations and marker manipulations, such as with knock-in models, are problematic in the rat.

Adult rabbit ligated ileal loop model

The removable intestinal tie adult rabbit diarrhea (RITARD) model described by De and Chatterje (1953) is employed extensively to study the mechanisms of action of cholera toxin (CT), *E. coli* LT toxin, and other enterotoxins and to investigate the efficacy of chemotherapeutic agents and antitoxic immunity in modifying the diarrhea mediated by these toxins. This model is also used to study certain aspects of intestinal colonization by enteropathogens. According to this model animals are starved for 24 hours but given water ad libitum. Before surgery each rabbit is given Inovar (a muscle relaxant) intramuscularly. Surgery is done with a local anesthesia (2% lidocaine hydrochloride), which is given intradermally along the midline of the abdomen. The cecum is brought out through a midline incision and ligated with umbilical tape as close to the ileocecal junction as possible without compromising the blood supply to the area. The small intestine is then brought out, and a length of umbilical tape is tied in a slip knot around it to close it in the vicinity of the mesoappendix (Flow Chart 10.1). The challenge material is injected into the lumen of the anterior jejunum. Usually the desired dose is administered in an arbitrarily chosen volume of 10 mL of PBS. After the challenge is injected, the intestine and cecum are returned to the peritoneal cavity, and the incision is closed. The loose ends of the slip knot are brought through the incision in the muscle layers and held while the incision is sutured around them. The loose ends are left in the space between the muscle and skin layers and clipped to the skin by means of a surgical clip. The rest of the skin is sutured. The animal is kept in a holding box, and its temporary tie is removed 2 hours after the challenge. After the surgical clip is removed, the slip knot easily opened, and the umbilical tape could be pulled gently from the intestine of the animal. The unclosed portion of the skin incision is sutured. The animal is then returned to its cage and provided with food and water ad libitum.

Rabbits are monitored for the onset of diarrhea, signs of weakness, and death. Rectal swabs are taken



FLOW CHART 10.1 General workflow of rabbit ileal loop.

every day and plated onto Gelatin agar and MacConkey agar to identify shedding of the challenge organism. The dead animals are autopsied, and the appearances of the gut and the amount of fluid accumulation are documented. The key factor of the success of this procedure is the pressure that the tie exerted on the intestine. If it is too tight, it causes hemorrhage and leads to a stricture after its removal that inhibits the flow of luminal contents from the small intestine. Studies so far with the RITARD model have shown less susceptibility to the ETEC strains tested, although the strains in serogroup 078 appear to possess significant bioactivity in this model. The RITARD model identified a mechanism of ETEC pathogenesis, where it has been established that pilus adhesins also known as CF antigens permit attachment of ETEC strains to the intestinal mucosa, thereby facilitating colonization and delivery of enterotoxin to target epithelial cells.

The RITARD model procedure or certain derivative of it may be an efficient means of immunizing rabbits with potential live vaccine strains or virulent human pathogens so that the local immune response and the protection it engenders can be studied. One disadvantage of this approach is that it requires surgical intervention at the immunizing stage and at least once more, after an extended time, to evaluate protection.

Animal models for *Vibrio cholerae*

Vibrio cholerae is the causative agent of life-threatening diarrheal disease cholera that is thought to have distressed human populations for centuries. Today, cholera remains prevalent in several parts of the developing world and it is an ongoing threat to public health in regions where access to clean water and adequate sanitation is limited. Investigation of *V. cholerae* infections in numerous animal models and human volunteers have expedited identification of many factors that contribute to bacterial colonization and disease. A key element is *V. cholerae's* production of CT, an ADP-ribosylating toxin that accounts for cholera's hallmark secretory diarrhea. The pathogenesis is also dependent on *V. cholerae's* production of toxin-coregulated pilus (TCP), a type IV pilus whose expression is coregulated with CT. Additional genes and processes that are important for *V. cholerae* survival and growth in the intestine include LPS O-antigen, accessory CFs, a cell-associated mannose−fucose-resistant hemagglutinin, transport systems, such as RND efflux pumps, metabolic factors, such as iron and magnesium, as well as metabolic processes, including biosynthesis of certain amino acids. *V. cholerae* does not naturally colonize the intestines of adult mammals other than humans. Consequently to date infant and

suckling mice, infant rabbits, as well as mouse and rabbit ligated ileal loops are the most commonly used animal models to study the pathophysiology of diarrhea and to evaluate the potential anti-diarrheal therapies for cholera.

Infant mouse model

The infant mouse model discovers the distribution of infecting *V. cholerae* in the intestinal tract with the use of a technique, termed dosechase. ^{35}S-labeled dose of *V. cholerae* is administered orally to infant mice and followed by a chase to determine the location of radiolabel in the gut as well as distribution of viable organisms (Baselski and Parker, 1978). Essentially the infant mice are kept at fast for 6 hours, then ^{35}S-labeled *V. cholera* is inoculated orally with a blunt 21-gauge needle equipped with polyethylene tubing attached to a tuberculin syringe. Evans blue dye is included in the dose as a tracer to insure proper delivery of the inoculum. The inocula contain approximately 5×10^6 CFU in 0.05 mL. This model discovered differential colonization ability among strains in the infant mouse upper bowel and that the capacity to colonize the upper bowel is essential for the establishment of successful infection.

Suckling mouse model

The suckling mouse model is a modified version (Angelichio et al., 1999) of the method by Baselski and Parker (1978). The model is used to identify *V. cholerae* CFs. Prior to the recombinant DNA era, Baselski et al. determined that spontaneous lipopolysaccharide (LPS) rough *V. cholerae* strains are severely defective in colonization of the suckling mouse small intestine. Briefly, 4- to 5-day-old suckling mice are separated from their mothers 1 hour prior to inoculation with *V. cholerae*. The mice are then intragastrically inoculated with overnight grown cultures of *V. cholera*. The bacterial titers in each inoculum are determined by plating serial dilutions of the inocula on the appropriate plates. Infected mice are kept at 26°C in the absence of their mothers. Mice are sacrificed at the designated time points, and the small and large bowels as well as the ceca are removed. The small bowel is cut into three segments of equal length, designated the proximal, middle, and distal small intestines. The cecum and large bowel, extending all the way to the rectum, are also separated. Each of these five segments is then mechanically homogenized and serial dilutions are plated onto LB agar plate to enumerate *V. cholerae* CFU per segment. While colonization of the large bowel by *V. cholerae* is controversial among researchers, this model established that both El Tor and classical biotype strains can colonize this organ exceedingly well suggesting that the possibility of similar colonization may occur during human infections. This model also demonstrated that colonization by an El Tor biotype strain occur in the absence of TCP and lipopolysaccharide O-antigen is not essential for colonization in cecum and large bowel by either biotype.

V. cholerae readily colonizes the suckling mouse small intestine, and studies of newborn mice have been extremely useful in identifying *V. cholerae* gene products that promote intestinal colonization. However suckling mice do not develop overt diarrhea or other signs of cholera gravis; thus they have not been as useful for studying factors underlying cholera pathology, such as the bacterial and host factors important for the secretory response. Furthermore to date there has been very limited use of techniques (such as confocal microscopy) to investigate the fine localization of *V. cholerae* in the gastrointestinal tracts of suckling mice.

Infant rabbit model

More than a decade ago, Metchnikoff (1894) found that infant rabbits could develop profuse watery diarrhea after oral administration of *V. cholerae*, but the illness did not develop reliably. Recently Ritchie et al. (2010) developed a nonsurgical model of cholera gravis in infant rabbits. Accordingly 3-day-old infant rabbits are treated with cimetidine (50 mg/kg intraperitoneal injection) first followed by orogastric inoculation with *V. cholera* (1×10^9 CFU) with a 5-French catheter after 3 hours. The inocula are prepared from overnight cultures of *V. cholerae* grown at 30°C, harvested by centrifugation, and the cell pellets are resuspended in sodium bicarbonate solution. Subsequently the rabbits are monitored for clinical signs of illness and diarrhea is assessed by the presence of watery or fecal material on the hind legs, tails, or ventral surfaces of individual rabbits (Fig. 10.3). Using this model, it has been proved that *V. cholerae* mutants that do not produce CT and TCP are unable to induce cholera-like diseases in rabbits. Histological analysis of intestinal sections from infected rabbits revealed that CT induces extensive secretion of mucin from goblet cells in the small intestine. Furthermore confocal and scanning electron microscopy of small intestine of the infant mice showed the presence of mucin-rich aggregates of *V. cholerae* cells. This model established that CT-dependent mucin secretion plays key role in bacterial infectivity, perhaps by protecting the organism from host factors that limit infection. This model is also particularly useful for investigating the factors involved in

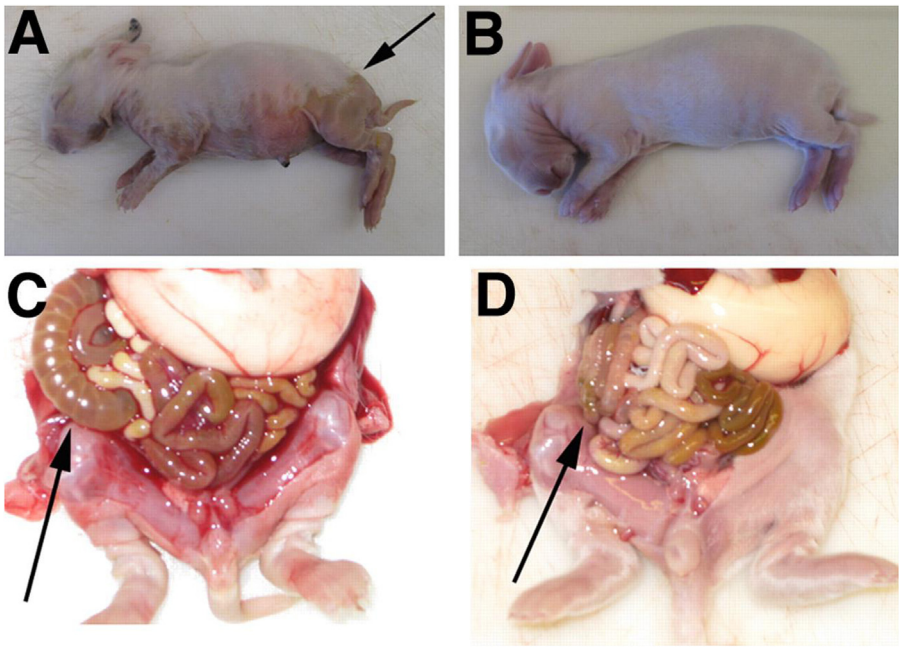


FIGURE 10.3 Gross findings in infant rabbits inoculated with *V. cholerae* (A and C) or buffer (B and D).

V. cholerae transmission, since it has been observed that infant rabbit littermates in the same cages as rabbits inoculated with *V. cholerae* frequently acquire cholera-like diseases.

Rabbit ligated ileal loop model

Rabbit ligated ileal loop model was introduced in the early 1900s but not extensively used until its revival by De and Chatterje (1953). The model has proven to mimic the effects of *V. cholerae* infection on the human gut rather well and it has the advantage that several samples or time periods could be examined with adjacent loops in the same animal. Ligated loops have also been used in classy scanning and transmission electron microscope studies of *V. cholerae* attachment to the small intestine. This study showed that the bacteria adhere directly via their surface coat to the microvilli tips. Most of the bacteria adhere horizontally with the surface, but some vibrios, especially at the early times of the infection attach in an end-on manner, with their flagella extending into the lumen. More recently this model together with elegant confocal microscopy have been employed to examine the pathogenic sequence of events that characterize the *V. cholerae* infectious process. This study revealed that late in the infection process *V. cholera* detaches from the epithelial surface and moves into the fluid filled lumen, a process named "mucosal escape response" and this phenomenon requires the stationary phase alternative sigma factor, RpoS. Expression profiling of bacteria isolated from ileal loop fluid and mucus showed a considerable RpoS-dependent upregulation of many chemotaxis and motility genes corresponding to detachment of bacteria from the mucosal surface and activate motility and chemotaxis functions, hence prepare the organism to leave the host, enter environmental reservoirs or be transmitted to another individual. However this model suffers from few disadvantages, the closed intestinal loop system requires abdominal surgery and bypasses the natural route of infection as well as several aspects of ordinary gastrointestinal tract physiology, such as peristalsis.

Ileal ligated mouse model of cholera

The use of mice over rabbits as animal models for cholera has several advantages, being small and with shorter life span, mice are relatively inexpensive, easy to handle, and require lesser quantities of compounds/vaccines for therapeutic/preventive evaluation. Transgenic mice are also available for studying cholera pathophysiology. Recently Sawasvirojwong et al. (2013) developed a ligated ileal loop model of *V. cholerae*-induced diarrhea in adult mice. Briefly 6-week-old mice are fasted for 24 hours and then anesthetized by intraperitoneal injection of nembutal (60 mg/kg). The body temperature of the mice is maintained at 37°C using a heating pad, a small abdominal incision is made and a loop of distal ileum is isolated by suture (2–3 cm in length). The closed ileal loop is instilled with *V. cholerae* (10^7 CFU/loop). After abdominal closure by suture, mice are allowed to recover from anesthesia. At different time points after bacterial inoculation the mice are reanesthetized and ileal loops are exteriorized for measurements of weight/length

ratio. Mice with intestinal weight/length ratio of at least 0.1 g/cm are considered as having positive diarrheal response.

This model established that cystic fibrosis transmembrane conductance regulator (CFTR)-mediated transepithelial Cl^- secretion plays central role in the pathogenesis of diarrhea and hence suggesting it as a valuable model for examining pathogenesis of diarrhea and evaluating potential anti-secretory therapies as well as vaccines for cholera.

Animal models for *Campylobacter jejuni*

C. jejuni is a leading cause of food-borne human gastroenteritis worldwide. Each year over 1.3 million people are infected with *C. jejuni* in the United States, resulting in 76 deaths annually. In developing countries, the incidence of *Campylobacter* enteritis is as high as 0.4 episodes per child per year. Compared with other common enteric bacterial pathogens, the molecular and cellular mechanisms of *C. jejuni*'s pathogenesis remain poorly defined, since it appears that the bacteria utilize unique pathogenic strategies, as it lacks many of the common toxins, effector proteins, and virulence factors found in other pathogenic bacteria. Indeed, the study of *C. jejuni* pathogenicity has largely been limited due to the lack of relevant and convenient animal models that mimic the various phases of acute human infection.

Nonhuman primate model

The closest model to the human infection is oral infection of nonhuman primates, especially *Macaca nemestrima*. Challenged infant *M. nemestrima*, develop vomiting and diarrhea, with blood in their stools. Most animals have a bacteremia. The onset and duration of disease is similar to that in human beings. The gross pathology and histology of these infections are also similar to that seen in human beings with acute campylobacter-associated colitis. Experimental infection induces acquired, and apparently protective, immunity. This model has been used to test the safety and immunogenicity of whole-cell, killed campylobacter vaccines (Baqar et al., 1995), providing evidence for human clinical trials.

Although this is apparently a good model of the naturally occurring human disease, there are sufficient problems to preclude the use of nonhuman primate models. In particular the availability of such animals for experimental purposes, the facilities and skills require to house these animals, the variability in their immune status and ethical considerations all prevent routine use.

Ferret model

One promising model has been developed in 3- to 6-week-old ferret kits challenged orally with *C. jejuni* (Bell and Manning, 1990). In a modified version of this model, at about 24 hours postchallenge, animals excrete greenish mucoid stools, frequently with occult blood, occasionally accompanied by anorexia, dehydration, and bacteraemia. These symptoms are self-limiting and the infection induces acquired immune responses, which appears to be partly protective. This model is used to assess the virulence potential of mutants of *pspA*, a gene associated with the expression of a *C. jejuni* pilus-like appendage and *cheY*. Recently the oral ferret model is used to compare the virulence of *C. jejuni* strains 81−176 and the genome sequence strain NCTC 11168. This study indicated that NCTC 11168 is poorly virulent, which may be due to the absence of certain genes. Thus this model appears to be an extremely useful in vivo tool in the study of virulence factors. However the relationship to human disease remains debatable. The use of tincture of opium to suppress peristalsis, the high doses (10^{10}−10^{11} CFU), and the short-lived symptoms of soft, but not diarrheic, stools raises a number issues. More importantly for most laboratories availability for routine testing, for example mutants, is restricted due to price, seasonal breeding, and a lack of campylobacter-free ferret breeding colonies. These plus the general scarcity of ferret immune reagents and the generally subjective analysis of the diarrhea means that the model requires considerable skill to establish and interpret.

Pig model

The similarity between the human and porcine gastrointestinal tracts allows the use of pigs as animal models of human enteric infections. Experimental infection of normal piglets is generally asymptomatic while colostrum-deprived neonatal animals develop diarrhea, which is occasionally bloody and mucoid and lasted for up to 6 day. Gross lesions and an abnormal histopathology are mainly confined to the large intestine. In experimentally infected gnotobiotic piglets clinical and histopathological effects are also observed. These effects include inflammation and edema of the cecum and colon with watery diarrhea from about day 2 to 12. These results suggest that neonatal piglets in the absence of maternally derived mucosal antibodies and possibly competing flora provide a useful model of campylobacteriosis. The symptoms and histopathology

appear to be consistent with human disease. However too few studies have been undertaken to assess the reproducibility of the model. Moreover a comparison of wild-type strains or mutants with this model has not yet been reported.

Mouse model

Many attempts have been made to induce disease in campylobacter-infected rodents. Although oral inoculation of *C. jejuni* induced reproducible, albeit short-term, colonization of the gastrointestinal tracts of both adult and infant normal mice, disease is rarely observed. However some studies reported diarrhea in about 10% of C.B-17 *scid beige* mice orally challenged with fresh clinical isolates of *C. jejuni*. This is accompanied by colonic inflammation. Nevertheless, some scientists were unable to detect lower intestinal lesions in similarly challenged SCID mice, although they did observe antral gastritis. This lack of reproducibility may be a reflection of the genetic background of the *scid* mutation but seems to indicate that the disease symptoms observed in the former report are not just a consequence of the lack of host immune competence. Thus such a model may be difficult to standardize. This oral mouse diarrhea model requires reproduction in other laboratories before it can be successfully used to test bacterial virulence properties.

Although the natural route of transmission for *C. jejuni* is oral challenge, alternative routes to enhance virulence potential have been considered. Intranasal inoculation of adult mice causes death within 6 days postchallenge. The degree of lethality varies with the dose, mouse strain, and campylobacter isolate used. At doses of 5×10^9 CFU, there was over 70% mortality of adult BALB/c mice with *C. jejuni* strain 81176. Organisms from mice challenged with this strain are recoverable from a variety of intestinal and extraintestinal sites, suggesting a systemic infection and splenic colonization persisted throughout the experiment. Thus this appears to be a model of acute infection involving extraintestinal colonization and invasion. It is also a model of immune protection and is used to assess vaccine efficacy. The advantages of being able to work with such immune-competent mice are considerable; however the relationship of this model to the human disease remains questionable. The abnormal route, high dose, and high mortality are a poor reflection of human disease symptoms. Moreover the reproducibility of this model in other laboratories has yet to be established.

Intraperitoneal challenge of adult mice with up to 1×10^9 CFU bacteria generally results in disseminated infection with recovery from liver and spleen but no obvious symptoms of disease even in different strains of mice. Supplementation of the challenge dose with iron dextran enhances virulence. Such treatment induces a dose–response observed in terms of diarrhea, weight loss, splenomegaly, and mortality. However the relevance of this model is debatable, especially as challenge with heat-killed organisms induces similar, albeit significantly weaker, symptoms.

Mice would normally provide a preferred infection model system; however they have repeatedly proven resistant to pathogenic infection by *C. jejuni*, and many strains are unable to even be reliably colonized. The basis for their resistance to *C. jejuni* colonization appears to at least partially reflect active competition from the resident intestinal microbiota, thereby preventing *C. jejuni* from establishing a niche within the murine gut. Secondly the murine immune system has proven very tolerant to the presence of *C. jejuni* and in wild-type (WT) mice, their presence only rarely elicits any overt intestinal inflammation. To overcome this tolerance several groups have tested genetically manipulated mice that develop exaggerated inflammatory responses to bacteria, such as interleukin-10 (IL-10) deficient (Il-$10^{-/-}$) mice. While Il-$10^{-/-}$ mice can be colonized by *C. jejuni* resulting in severe enterocolitis, the loss of IL-10 dramatically alters the murine immune system. As a result their immune system is unable to effectively clear *C. jejuni* from the GI tract, leading to chronic colonization rather than the acute infections seen in humans. Moreover the immune systems of Il-$10^{-/-}$ mice are so sensitive that the presence of any commensal microbe can potentially trigger spontaneous enterocolitis. The oral gavage and intraperitoneal injections of MyD88-deficient mice have also been employed for the study of *C. jejuni* colonization and dissemination in mice, but encounter the reverse limitation of Il-$10^{-/-}$ mice, where the immune response is attenuated, allowing for colonization of the intestine or systemic sites with limited host responses. Recently mice deficient in Single IgG Interleukin-1 related receptor (SIGIRR) are employed as an animal model for *C. jejuni* infection (Stahl et al., 2014). *C. jejuni* heavily colonizes the cecal and colonic crypts of *Sigirr*$^{-/-}$ mice, adhering to, as well as invading intestinal epithelial cells. This infectivity is dependent on established *C. jejuni* pathogenicity factors, capsular polysaccharides (*kpsM*) and motility/flagella (*flaA*). The inflammatory immune response elicited by *C. jejuni* in this model is principally dependent on TLR4. Furthermore it has been found that loss of the *C. jejuni* capsule leads to increased TLR4 activation and exaggerated inflammation and gastroenteritis. This study has given new insights into murine *Campylobacter* colonization, studying innate immune responses to *C. jejuni*, and

identification of pathogenic factors governing infection, but the relationship between the observations in this model and human disease is unclear.

Rabbit model

Rabbits are more closely related to primates than rodents in phylogeny and have far-reaching significance because they more closely resemble primates in anatomy and physiology. The oral administration of *C. jejuni* to rabbits results in transient colonization but no evidence of disease even in neonatal animals. However adult rabbits are an appropriate size for animal models in which the bacteria can be injected directly into an intestinal loop.

The RITARD model, originally developed to investigate the pathogenesis of *V. cholerae* and *E. coli*, involves the transient physical blockage of normal peristalsis by generating a temporarily sealed intestinal segment. *C. jejuni* detain in this segment for about 4 hours induces a mucoid, occasionally bloody, diarrhea within 3- to 5-day postchallenge. Acute inflammatory lesions and death are also frequently observed. The model detected qualitative differences between strains, wild types, and mutants. Because the intestinal tie is temporary, the infected animals can be utilized to investigate long-term pathological effects and may be allowed to induce detectable immune responses.

An alternative to the RITARD model is the rabbit ileal loop test (RILT). In this model sections of ileum (5 cm long) are ligated into loops. The loops (up to four per animal) are inoculated with bacterial suspension. The loop contents are harvested 18 hours postchallenge. The resulting pathology comprises an acute inflammatory reaction. The RILT model primarily detects fluid accumulation, which appears to be associated with host factors such as elevated levels of cyclic AMP, prostaglandin E2, and leukotriene B4. Thus this model may mimic some of the acute, host-related responses to infection.

Both rabbit models require considerable surgical skills and postoperative care and are consequently not widely available. The relationship between the observations in these models to human disease is unclear; the route of administration is artificial, the effects of surgical trauma are unknown and the severe pathological effects occasionally observed poorly reflect human disease. However they appear in particular to be models of host fluid secretion and acute inflammatory responses as well as translocation across the intestinal epithelial barrier via M cells. Because large numbers of organisms can be held in a confined space, the RILT model has also been useful for biomass production to investigate in vivo expression of antigens.

A novel infant rabbit model has been studied in the recent year (Shang et al., 2016). Newborn rabbits are inoculated orogastrically with 1×10^9 CFU of *C. jejuni*. It is capable of systemic invasion in the infant rabbit and develop a diarrhea symptom that imitate the symptoms observed in many human campylobacteriosis. The pathogen primarily colonizes the large intestine and induces intestinal inflammation with upregulated levels of interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α), IL-1β, IL-2, IL-6, IL-8, and IL-22 gene expression. Genes preferentially expressed during *C. jejuni* infection were screened, and acs, cj1385, and cj0259 seem to be responsible for *C. jejuni* invasion.

The infant rabbit shares many advantages with rodents, such as small size, short gestation period, large litter size, ease of breeding and colony maintenance, and a relatively low purchase and housing costs. The infant rabbit can be used as experimental model to study diarrheagenic *Campylobacter* species as well as to explore the survival mechanism of the bacterium in vivo and its molecular pathogenesis, however this model is not suitable for vaccine efficacy study because active immunization is not possible and immune system is not so much developed.

Chicken model

Given its optimal growth conditions, it is generally considered that *C. jejuni* has evolved to preferentially colonize the avian gut. As this colonization is both extensive and asymptomatic the organism appears to act as a commensal in this host. Consequently the chick has become an important model for the investigation of bacterial CFs.

One-day-old chicks, orally challenged with as few as 30 CFU of fresh isolates of *C. jejuni*, achieved maximal ceacal colonization within 3 day at levels of up to 1×10^{10} CFU per gram of ceacal contents. Older birds, up to 8 weeks of age, are equally susceptible to experimental colonization. This colonization is chronic and only start to decline after 6–7 weeks or so postchallenge. Although the main site of colonization is the cecum, organisms are recovered from throughout the gastrointestinal tract as well as the spleen and liver, indicating that some systemic infection occurred. The level and extensiveness of colonization in some chick models varied depending on the genetic lineage of the birds, the challenge strain, and the degree of laboratory adaptation of the strain.

Challenge chick models have also been developed for testing vaccine efficacy. The results indicated that vaccination with killed whole cell or subunit vaccines may induce partial protection. However this efficacy is

not reproducible. One problem with killed or subunit vaccine candidates is the inability, or inefficiency, of adjuvants to elicit effects at avian mucosal surfaces.

To date the oral chick model has proved remarkably useful for the assessment of the colonization potential of defined mutants and variants. One *C. jejuni* strain, 81116, produced a dose–response curve of colonization thus providing a quantitative model. This model has been particularly useful for detecting the compromised colonization potentials of deletion or insertion mutations. Mutants so far tested in this chick model have included *flaA* and *flab*, *sod*, *cat*, and *regX*.

The chicken provides a model of campylobacter colonization in the avian gut. The relationship between this and mammalian gut colonization is unknown. Experimental challenge of chickens of all ages appears to induce an identical outcome to that observed during natural infection, when *C. jejuni* acts like an avian gut commensal. In studies of over 30 strains tested from a variety of sources (human, chicken, ostrich, and water), even at levels of 1×10^{10} CFU per gram of ceacal contents, colonization is asymptomatic regardless of strain. The reason why such colonization levels do not usually induce disease in birds is unknown. Possible reasons include host-specific expression of bacterial virulence factors or lack of host-specific receptors for such factors.

Animal models for *Shigella*

Shigellosis or bacillary dysentery is endemic throughout the planet, although essentially a major health concern in its most impoverished areas, particularly in the pediatric population between 1 and 5 years old. It can be caused by any serotype: *Shigella dysenteriae* type 1, *Shigella flexneri*, *Shigella boydii*, and *Shigella sonnei*. Each year, over 164 million cases occur worldwide, with the majority occurring in children in developing countries, and 1.1 million cases result in death. The constant emergence of antibiotic resistance in *Shigella spp*., even to the newest antibiotics, underscores the need for an effective vaccine to help control *Shigella* disease. To screen vaccine candidates an animal infection model mimicking human shigellosis would be essential. However the four *Shigella* species; *S. flexneri*, *S. dysenteriae*, *S. sonnei*, and *S. boydii* do not produce acute rectocolitis in experimental animals. The lack of a relevant animal model reproducing human bacillary dysentery is one of the key barriers to developing a successful *Shigella* vaccine. To overcome this obstacle considerable effort over the years has been devoted to establish a reliable animal model of bacillary dysentery. Several established *Shigella* infection models (Marteyn, 2016) have proven to be useful and are discussed below.

Macaque monkey model

The most relevant model is the macaque monkey model. Indeed macaque monkeys infected intragastrically with *Shigella* develop typical bacillary dysentery, similar to humans, except that the needed inoculum is much higher than that required for human infection (10^9 and 100 CFU, respectively). It allows to follow *Shigella* adaptation to the gastric acidic environment, its survival to the small intestine-associated immune response and the invasion, colonization, and destruction of the colonic mucosa, associated with bloody stools, intestinal ischemia, and the rise of polymorphonuclear neutrophils. This model has been mainly used to assess the immunogenicity, and possibly protection induced by orally administered, live attenuated vaccine candidates. However high cost and ethical issues render its routine use almost impossible. In addition some monkeys are *Shigella* carriers, and this can interfere with the development of dysentery.

Pig model

Among other large animal models of shigellosis, a young (4-week-old) domestic pig (*Susscrofadomestica*) model is evaluated, although no colonic colonization by *Shigella* strains (*S. dysenteriae* and *S. flexneri*) is noticed upon oral administration of pathogens. On the contrary *S. dysenteriae* 1 oral administration in a piglet model lead to the gastrointestinal track epithelium invasion with a more profound destruction of the colonic mucosa and lamina propria, associated with high levels of IL-8 and IL-12 (Jeong et al., 2010). Elevated levels of TNF-α, IL-1β, IL-6, and IL-10 are also detected in feces and in gut segments of infected animals. Detached necrotic colonocytes are observed in the lumen, with inflammatory cells outpouring from damaged mucosa. These data conclude that piglets are highly susceptible to shigellosis providing a useful tool to compare vaccine candidates for immunogenicity, reactogenecity and response to challenge, investigate the role of virulence factors, and test the efficacy of microbial agents.

Rabbit model

The most informative model used to date involves injection of *Shigella* into ligated loops of rabbit intestine (Fig. 10.4). Infection of ligated ileal loops leads to bacterial invasion accompanied by induction of acute inflammation and subsequent destruction of epithelial

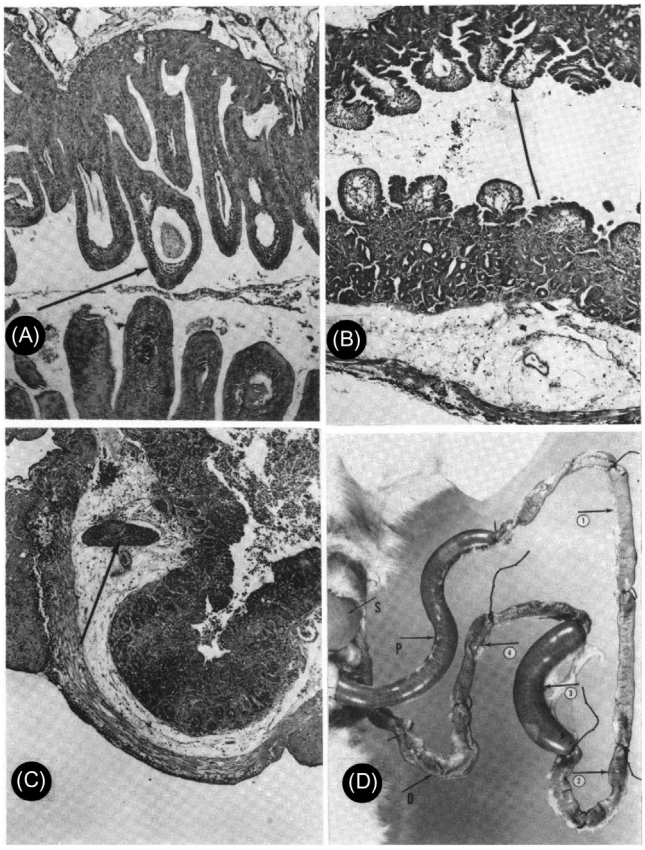


FIGURE 10.4 Section of rabbit small intestine 3 h after inoculation with *Shigella*. Lacteals are distended (arrow), causing prominent distortion of villi. Mild submucosal edema is present. Hematoxylin (H) and Eosin (E). Magnification, ×50.

cells about 18–24 hours after infection. This model allows the study of *Shigella* interaction with the intestinal barrier and subsequent induction of an innate response. In this model the targeted organ is the ileum, not the colon as observed in humans. More specifically it has been observed that M cells, located in the ileum Gut-associated lymphoid tissue (GALT) are preferentially invaded. Rabbit intestinal loops have been extensively used for phenotype characterization of *Shigella* mutants as well as to analyze the invasive phenotype of *Shigella* in Peyer's patches and the consequences of bacterial invasion. However this model is limited by the fact that genetic manipulation is not feasible and very few rabbit-specific reagents are available. Adult rabbits subjected to direct colonic intubation by cecal bypass also develop shigellosis. Although the model is sensitive to virulence attenuation, their suitability for protection studies has yet to be determined. More recently a nonsurgical rabbit model of enteric *Shigella* infection is characterized by oral administration of the pathogen. Consistently the ileum, not the colon, is the major site of tissue infection and necrosis observed in this model. However the use of this model of shigellosis is now also limited by ethical constraints (moderate animal suffering) since alternative small animal models become available.

Recently an infant rabbit intrarectal inoculation model was described in which animals develop disease and rectal pathology reminiscent of natural infections (Yum et al., 2019). Kuehl et al. (2019) also reported that an orogastric inoculation of infant rabbits with *S. flexneri* results in severe disease resembling human shigellosis. This model provides a powerful and accessible small animal model for further investigation of factors contributing to *Shigella* pathogenesis and for testing new therapeutics.

Guinea pig model

In guinea pigs, the keratoconjunctivitis assay, known as the Sereny test, is useful to assess invasiveness, intercellular spread and virulence of *Shigella* strains. Indeed, *Shigella* invades the corneal epithelium and spreads to contiguous cells, with the more virulent strains causing ulcerative keratoconjunctivitis. Despite its marked usefulness for immunogenicity studies and protective efficacy of *Shigella* vaccine candidates, a detailed analysis of host immune responses remains difficult in this model. Furthermore although this model is considered the gold standard assay for protective immunity, the other drawbacks include difficulty of quantitatively describing the inflammatory response and the irrelevance of the target organ. Recently a new guinea pig model has been described that represents typical bacillary dysentery under natural conditions. Guinea pigs are administered with virulent *S. flexneri* 2a (2457T or YSH6000) or *S. flexneri* 5a (M90T) by the intrarectal route develop severe and acute rectocolitis, which mimic human shigellosis. *Shigella* invasion and colonization of the distal colon are seen at 24 hours following intrarectal infection with significant damage and destruction of mucosal and submucosal layers, thickened intestinal wall, edema, erosion, infiltration of neutrophils, and depletion of goblet cells in the distal colon. Furthermore after *Shigella* infection robust expression of proinflammatory cytokines and inducible NO synthase mRNA are detected in the colon. The rectocolitis model also showed that guinea pigs vaccinated with attenuated *Shigella* elicit high levels of mucosal IgA antibody with milder symptoms of bacillary dysentery after *Shigella* infection. This guinea pig colitis model is useful for assessing the protective efficacy of *Shigella* vaccine candidates, suggesting that it can be used to reliably predict the protective immunity of vaccine candidates.

Mouse model

Murine model of shigellosis would be valuable for several reasons, including ease of handling, the possibility of genetic manipulation, and the availability of many murine-specific reagents. Moreover knock-out mice could potentially provide new insights into *Shigella* pathogenesis and the host immune response. Unfortunately *Shigella* does not develop a disease similar to shigellosis in adult mice. Why mice are refractory to *Shigella* infection is still unknown. Since induction of inflammation at early times postinfection is crucial for the establishment of the infectious process, the leading hypothesis to explain the absence of infection in mice is that mice are defective in some of the steps leading to acute inflammation, such as sensing of *Shigella* and/or expression of effectors required for polymorphonuclear leukocyte (PMN) recruitment.

Despite the lack of a relevant murine model strictly mimicking human infection, several murine models are available that allow one to address specific issues of the infection process. Mice can be used for pulmonary pneumonia studies after intranasal inoculation with *Shigella* to assess virulence attenuation, to run immunization experiments and to determine protection against infection. When administered intranasally, *Shigella* invades the tracheobronchial and alveolar epithelia, leading to the development of an acute tracheobronchitis and alveolitis characterized by a massive influx of PMNs. Even though this model provides an excellent tool to analyze the nature and dynamics of the innate and adaptive immune responses, it does not exactly replicate the natural intestinal disease, such as the epithelial disruption and invasion events seen at the intestinal barrier. Recently this murine model has been shown to reproduce the down regulation of INF-γ production induced by *Shigella* in infected patients at the acute phase of the disease. However the major drawback of this model is the lack of clinical relevance to the infection site.

Recently Fernandez et al. (2003) developed a model of intragastric infection in newborn mice using massive amounts of *Shigella* organisms (10^9 CFU). A 4-day-old mice infected with the invasive *Shigella* presented inflammatory destruction of the mucosa and significant infiltration of PMNs into the gut similar to those described in patients with shigellosis. Moreover cytokine and chemokine responses consistent with inflammation are observed. However this newborn mouse model cannot be used to evaluate protective immunity, as it must be employed within a narrow time window (i.e., 4–5 days after birth). This model is therefore useful to study the early interactions of *Shigella* with the intestinal mucosa and to decipher the molecular and cellular mechanisms leading to resistance/susceptibility to infection.

In a unique approach another group created the human intestine xenograft model in severe combined immunodeficient (SCID) mice. This model results in extremely high levels of IL-1β and IL-8 production as well as in marked infiltration of neutrophils following direct injection of bacteria into the lumen of the intestinal xenograft. Although this model holds enormous potential to characterize the phenotype of *Shigella* mutants at the molecular level (e.g., transcriptome analysis) in the context of a "reconstituted" human intestinal barrier, the model is too artificial and laborious for the purpose of assessing protective efficacy.

Another novel murine intraperitoneal model of infection has proven useful recently (Yang et al., 2014). Intraperitoneal challenge with virulent *Shigella* results in diarrhea and severe body weight loss in adult B6 mice. The bacteria invade and colonize in systemic tissues as well as in the serosa and lamina propria region of the large intestine. Epithelial shedding, barrier integrity, and goblet cell hyperplasia are also observed in the large intestine by 24 hours postintraperitoneal *Shigella* infection. Additionally following intraperitoneal challenge expression of proinflammatory cytokines (IL-1α/β, IFN-α, IFN-γ, IL-6, and TNF-α) and chemokines (CCL2, CCL3, CCL4, CCL5, CCL7, and CXCL10) are induced in the large intestine. Moreover mice prevaccinated with attenuated *Shigella* are protected against intraperitoneal challenge with virulent *Shigella*, suggesting that this model is useful for understanding the induction mechanism of bacillary dysentery and for evaluating *Shigella* vaccine candidates.

Animal models for *Salmonella typhi*

Salmonella enterica serovar Typhi (*S. typhi*) is a human-restricted facultative intracellular bacterium that causes a life-threatening systemic infection called typhoid fever. *S. typhi* causes significant morbidity and mortality across the globe, resulting in an estimated 21.5 million cases and 2,00,000 deaths each year, the majority of which occur in developing countries. The major sources of *S. typhi* infections in developed countries, including the United States and the United Kingdom, are from travelers returning from the Indian subcontinent or other endemic regions. *S. typhi* can cause lifelong infections in humans, most often by colonizing the gallbladder. The molecular bases for its host adaptation and ability to cause persistent infection are not known. There are currently no effective vaccines against typhoid fever and no vaccines that can be used in young children. The isolation of multidrug-resistant *S. typhi* has raised the worrisome possibility of the reemergence of untreatable typhoid fever. Since *S. typhi* is restricted to humans, which has hampered

the development of a conventional animal model to study *S. typhi* pathogenesis and test potential vaccines.

Mouse model

Currently there are no animal models that support productive infection with *S. typhi*. A recent study attempted to establish an in vivo *S. typhi* infection in BALB/c mice (Xu et al., 2009). Intraperitoneal infection of BALB/c with *S. typhi* induces neutrophil influx into the peritoneum and macrophages are the major cell type containing internalized bacteria at 0.5 and 4 hours postinfection, but the number of typhi in macrophages decreased substantially within 4 hours after infection.

Since *S. typhi* is in essence a pathogen of the reticuloendothelial it is possible that determinants of host specificity and restriction may reside within the reticuloendothelial system, as this is the most variable compartment across different animal species. Therefore attempts have been made to investigate the ability of a mouse with a humanized immune system to support infection by *S. typhi*. It has been found that immunodeficient $Rag2^{-/-}\gamma c^{-/-}$ mice engrafted with human fetal liver hematopoietic stem and progenitor cells support *S. typhi* replication and persistent infection when infected intraperitoneally. Infected animals mount a human innate and adaptive immune response to *S. typhi*, resulting in the production of cytokines and pathogen-specific antibodies, indicating that this animal model can be a useful resource for understanding *S. typhi* pathogenesis and to evaluate potential vaccine candidates against typhoid fever.

In another report the authors have shown that intravenous inoculation of *S. typhi* into humanized $Rag2^{-/-}\gamma c^{-/-}$ mice establishes *S. typhi* infections. High bacterial loads are found in the liver, spleen, blood, and bone marrow of mice reconstituted with human leukocytes. Importantly *S. typhi*-infected humanized mice lost significant body weight, and some of the infected mice display neurological symptoms, suggesting that the model can be utilized to study the pathogenesis of *S. typhi* to develop novel therapeutic strategies.

Recently Libby et al. (2010) has discovered that *S. typhi* are also able to replicate in vivo in nonobese diabetic (NOD)-scid $IL2r\gamma^{null}$ mice that lack the IL-2 receptor common γ-chain engrafted with human hematopoietic stem cells (hu-SRC-SCID mice) cause a lethal infection with pathological and inflammatory Th1 cytokines IFN-γ and TNF-α responses resembling human typhoid. The pathological changes in the infected mice including evidence of central lobular hepatocellular injury with vesiculation and cytoplasmic hyaline changes, Kupffer cell swelling, effacement of normal splenic architecture with lymphocyte depletion, and the presence of large splenic granulomas with palisading epithelioid macrophages and multinucleated giant cells. Screening of transposon pools in hu-SRC-SCID mice discovered that *Salmonella* Pathogenicity Islands 1, 2, 3, 4, and 6 are the virulence determinants. This finding indicates that the presence of human immune cells allows the in vivo replication of *S. typhi* in mice and that the hu-SRC-SCID mouse provides unique opportunity to gain insights into *S. typhi* pathogenesis and develop strategies for the prevention of typhoid fever.

Additionally it has been reported that mice lacking Toll-like receptor 11 (TLR11) could be lethally infected with *S. typhi* after oral or systemic inoculation and produce a febrile illness with features of human typhoid. It is postulated that TLR11-mediated recognition of *Salmonella* flagellin prevents *S. typhi* infection in wild-type (C57BL/6) mice and that the lack of functional TLR11 renders humans susceptible to the bacterial infection. This study also revealed that TLR11-deficient mice can be protected from infection by vaccination with heat-killed *S. typhi* or by passive transfer of serum from immunized mice. It is therefore proposed that TLR11-deficient mice could serve as a convenient animal model for typhoid fever. However the role of TLR11 and its binding partner flagellin in regulation of *S. typhi* infection and the use of TLR11-deficience mice as model system to study *S. typhi* pathogenesis are controversial (Song et al., 2016). Urgent efforts to develop a convenient animal model for typhoid fever must therefore persist.

Animal models for nontyphoidal *Salmonella*

Nontyphoidal *Salmonella* (NTS) are associated with gastroenteritis and bacteremia in immunocompetent individuals, in which the infection remains localized to the terminal ileum, colon, and mesenteric lymph node. NTS gastroenteritis is characterized by a short incubation period, averaging less than 1 day, followed by the development of diarrhea, fever, and intestinal inflammatory infiltrates that are dominated by neutrophils. The zoonotic pathogens *Salmonella enterica* serotype Typhimurium (*S. typhimurium*) is most frequently associated with this diarrheal disease. The increasing prevalence of multidrug-resistant *S. typhimurium* with resistance to ciprofloxacin and expanded-spectrum cephalosporins threaten to limit treatment options, emphasize the need to develop new intervention strategies, which will require the use of adequate animal models (Table 10.1).

TABLE 10.1 Animal models of human *Salmonella* disease.

Model	Advantages	Limitation
Mouse typhoid	Natural infection; availability of host genetics and immunological reagents; model for fecal–oral transmission; low cost	Not suited to study gastroenteritis; *S. typhimurium* does not cause typhoid fever in humans
Mouse colitis	Availability of host genetics and immunological reagents; suited to study intestinal inflammation; low cost	Requires disruption of the microbiota by pretreatment with antibiotics; colitis is accompanied by systemic infection
Humanized mouse	Provides a model to study *S. typhi*	Requires extensive manipulation
Guinea pig	Suited to study intestinal inflammation	Requires preconditioning through starvation and opium treatment; limited availability of host genetics and immunological reagents
Calf gastroenteritis	Natural infection that closely resembles human gastroenteritis	Limited availability of genetics and immunological reagents; requires specialized animal facilities
Rhesus macaque	Natural infection that closely resembles human gastroenteritis	Limited availability of genetics; requires specialized animal facilities; high cost

Opium-treated guinea pig model

In 1967 Akio Takeuchi performed electron microscopic studies of epithelial invasion by *S. typhimurium* in guinea pigs preconditioned by starvation and treatment with opium. In this model, he discovered that invasion of epithelial cells by *S. typhimurium* is accompanied by a local degeneration of microvilli and the formation of membrane extrusions. Later it was revealed that the epithelial invasion by *S. typhimurium* is mediated by a type III secretion system (T3SS-1) encoded by *Salmonella* pathogenicity island 1 (SPI-1). The main function of T3SS-1 is to translocate effector proteins into the host cell cytosol, where they trigger rearrangements in the actin cytoskeleton.

Calf gastroenteritis model

S. typhimurium is a natural cause of gastroenteritis in calves, and infection is associated with signs of disease that parallel the symptoms observed in humans. Upon oral infection with *S. typhimurium* calves develop clinical signs within 12–48 hours, which is similar to the short incubation period observed in human volunteer. Clinical signs of the disease during experimental infection include diarrhea, anorexia, fever, dehydration, and prostration. Usually oral inoculation with 10^4–10^7 CFU causes transient diarrhea, which persist for 2–8 days, whereas lethality may be caused at doses between 10^8 and 10^{11} CFU. Animals develop intestinal inflammation characterized by a severe diffuse infiltrate composed predominantly of neutrophils. Neutrophil recruitment is associated with necrosis of the upper mucosa and migration of neutrophils into the intestinal lumen. In severe cases necrosis of the upper mucosa leads to formation of a pseudomembrane, a gross pathological change observed in the terminal ileum and the cranial portions of the colon.

Analysis of *S. typhimurium* mutants in the calf model in the late 1990s provided important first insights into the role virulence factors play during gastroenteritis. Oral challenge studies revealed that T3SS-1 is essential for the ability of *S. typhimurium* to cause intestinal inflammation and diarrhea, while inactivation of T3SS-2 reduces the severity of intestinal lesions in calves.

Additionally a bovine ligated ileal loop model delivered the opportunity to examine the development of intestinal inflammation at early time points after *S. typhimurium* infection. Ultrastructural studies of infected ileal ligated loops explored that *S. typhimurium* induces ruffling of the plasma membrane at the apical side of intestinal epithelial cells, invading M cells or absorptive enterocytes. In ligated ileal loops, bacteria can be detected inside epithelial cells as early as 20 minutes postinfection, and in macrophages in the lamina propria containing bacteria were detected at 60 minutes after infection. The use of this model revealed that flagella contribute to neutrophil recruitment in the intestinal mucosa during *S. typhimurium* infection. The role of flagella in eliciting inflammatory responses is in part indirect by promoting bacterial invasion and in part direct, by serving as a pathogen-associated molecular pattern (PAMP) that stimulates innate pathways of inflammation. Limitations of the calf model include the scarcity of reagents available to manipulate the host and limited availability of animal facilities to perform the research. Another drawback of the ligated intestinal loop model is that it is only suited for studying early steps during infection.

Mouse model

Most laboratory studies are carried out using *S. typhimurium* in mice, where a disseminated infection with some similarities to human typhoid is observed. Mice infected with *S. typhimurium* do not develop gastroenteritis but instead contract a systemic disease characterized by bacterial multiplication in the liver and spleen, which results in hepatomegaly and splenomegaly. The small intestinal pathology is characterized by a predominantly mononuclear leukocyte infiltrate, with follicular hyperplasia, capillary thrombosis, hemorrhage, and ulcerations observed at areas of Peyer's patches in moribund animals. Mastroeni et al. (1993) used the mouse typhoid model to demonstrate that protective immunity to *S. typhimurium* infection requires both antibodies and $CD4^+$ T cells, a principle that still guides current efforts in vaccine development. Using the mouse typhoid model identified the second type III secretion system (T3SS-2) of *S. typhimurium* and latter it is found that T3SS-2 enables *S. typhimurium* to survive in host macrophages.

A unique mouse colitis model confirmed observations from the calf model that flagella, T3SS-1, and T3SS-2 contribute to intestinal inflammation during *S. typhimurium* infection. The mouse colitis model is used to demonstrate that inflammation promotes a luminal outgrowth of *S. typhimurium* (Fig. 10.5), because the respiratory burst of neutrophils, which transmigrate into the intestinal lumen (Barman et al., 2008). This model delivers a comprehensible mechanism of *S. typhimurium*-induced gastroenteritis; *S. typhimurium* employs its virulence factors to trigger inflammation by using flagella and T3SS-1 to invade the intestinal epithelium, followed by T3SS-2-mediated survival in tissue macrophages. The resulting inflammatory response generates a new respiratory electron acceptor that supports outgrowth of *S. typhimurium* in the gut lumen, thereby promoting its transmission by the fecal–oral route. One caveat of the mouse colitis model is that development of acute cecal inflammation requires the use of an antibiotic to disrupt the microbiota. Furthermore *S. typhimurium* disseminates systemically in both genetically resistant and genetically susceptible mouse lineages, thus making it difficult to study mucosal barrier functions in the mouse colitis model.

Coinfection model

The risk factors for *S. typhimurium* bacteremia include an underlying malaria infection or HIV disease. Initial studies suggest that animal models are well suited to interrogate the pathogenesis of these coinfections.

Toward examining the fundamental vulnerability of children with severe malaria to *S. typhimurium*

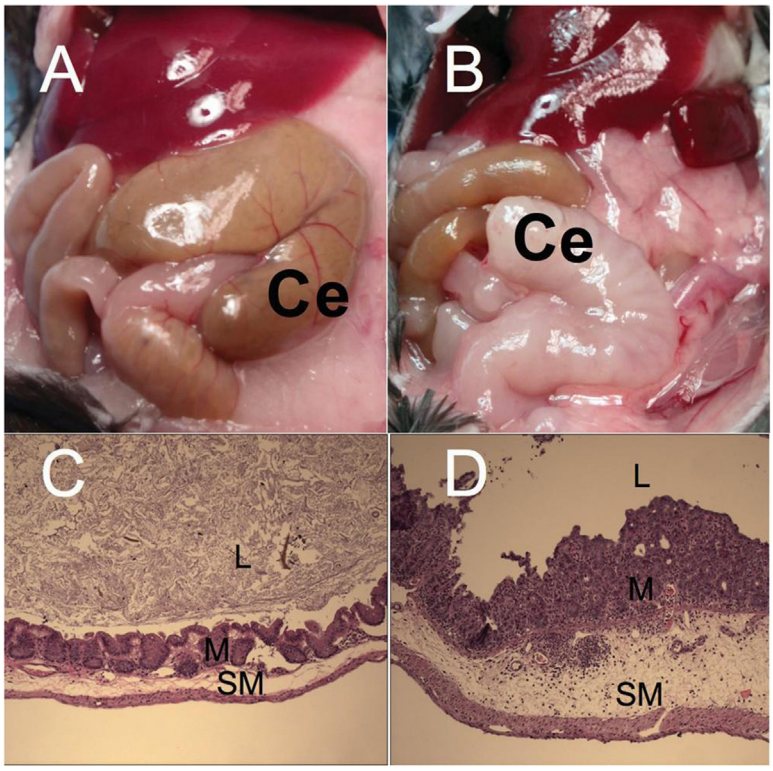


FIGURE 10.5 Inflammation of the cecum in the mouse colitis model. (A) Normal appearance of the cecum (Ce) in a mock-infected control animal. (B) Shrunken and edematous cecum (Ce) from a *S. typhimurium*-infected mouse. (C and D) Histopathological appearance of the murine cecum of a mock-infected mouse (C) or a mouse infected with the *S. typhimurium* wild type (D) 72 h after infection.

bacteremia, a mouse coinfection model was developed in resistant CBA mice, using the rodent malaria parasite *Plasmodium yoelii* subsp. *nigeriensis* and *S. typhimurium* (Roux et al., 2010). Using this model, the investigators demonstrated that increased systemic loads of *S. typhimurium* during coinfection are caused by both hemolytic anemia and malaria parasite-mediated immunosuppression. A reduction in circulating IL-12 is also observed in the coinfection model suggesting that impaired inflammatory responses in patients with severe malaria may be one factor that predisposes them to NTS bacteremia.

Ligated ileal loops in rhesus macaques infected with simian immunodeficiency virus (SIV) and *S. typhimurium* are currently employed to investigate NTS bacteremia in HIV-infected individuals (Raffatellu et al., 2008). Initial characterization of this model revealed that SIV-mediated depletion in the ileal mucosa of IL-17-producing $CD4^+$ T cells (Th17 cells) selectively blunted expression of IL-17, IL-22, IL-26, CCL-20, and lipocalin-2 in response to *S. typhimurium* infection and is associated with increased bacterial dissemination to the mesenteric lymph nodes. Furthermore IL-17 deficiency resulted in increased systemic dissemination of *S. typhimurium* in the mouse colitis model. These data suggest that Th17 depletion is one factor that predisposes HIV-infected individuals to NTS bacteremia.

Translational significance

The development of safe and efficacious vaccines and therapeutics relies on a proper understanding of the advantages and limitations of animal models available to researchers. In certain cases such as for new or emerging diseases for which human data are not available, the animal model is crucial for understanding the pathogenesis of the disease before the development of vaccines or therapeutics can even be considered. Beyond that a well-designed animal model provides a sound basis for supporting good science and ensuring the most beneficial use of both animal and human resources. A well-designed animal model requires a thorough understanding of similarities and differences in the physiology between humans and animals and incorporates that knowledge into the goals of the study.

Animals make good research subjects for a variety of reasons. Animals are biologically similar to humans. In fact chimpanzees share more that 99% of DNA with humans and mice share more than 98% DNA with humans, consequently, animals are susceptible to many of the same health problems as humans. Animals have a shorter life cycle than humans and as a result they can be studied throughout their whole life span or across several generations. In addition scientists can easily control the environment around animals (diet, temperature, and lighting), which would be difficult to do with humans.

Enteric bacterial pathogens present a significant risk to human health worldwide, and due to the severity of symptoms associated with infections, few opportunities have presented themselves to study host susceptibility to these microbes and disease pathogenesis in the human host. For this reason a variety of infection models have been developed to provide insight into our current understanding of the molecular, cellular, tissular, and genetic mechanisms leading to the development of the infectious process as well as to identify novel therapeutic strategies or development of successful vaccines. Furthermore in vivo infection models are crucial to identify and differentiate between virulence factors that may be common to all pathogens and those that are specific as well as to determine the basis for host resistance or susceptibility to these pathogens to properly control the public health burdens that they impose. Importantly animal models have been the basic translational model in the preclinical setting in elucidating key biochemical and physiologic processes of disease onset and propagation in a living organism. Experimental intestinal inflammation raised in animals, particularly in rodents, constitute the major preclinical tool of evaluating novel diagnostic and therapeutic drugs screening before clinical testing.

Animal models have been instrumental in establishing a number of conceptual advances in our understanding of human disease. These advances were made possible by approaches that exploited the unique strengths of each animal model. However each animal model also has shortcomings that limit its usefulness for studying certain disease manifestations. As a consequence researchers must proactively consider the advantages and disadvantages of each model and determine the most suitable models to address specific aspects of intestinal inflammation being investigated. Presently there is no “perfect” animal model that can address all the mechanisms involved in intestinal inflammation and immune response. However better design and conduct as well as further development of animal models are warranted.

Despite the advancement in biomedical research and the benefits derived by the society through animal models, which still remain a unique source of in vivo information, a wide range of alternatives to animal-based preclinical research has emerged. One of those potential alternatives is the use of experimental clinical trials, for example, in which disease is modeled in

healthy subjects, which allow the early clinical testing of vaccine or drug candidates without prior extensive animal model testing. Following several failures in clinical development of vaccine or drugs, experimental clinical protocols have been developed for providing early clinical proof of concept within the boundaries of a phase I clinical trial design (Olesen et al., 2012). While these human models have helped to bridge the translational gap, they constitute rather an addition in the translational research armamentarium than a substitute for well-designed animal models. A more recent approach is in silico modeling also called quantitative systems pharmacology (Schmidt et al., 2013). By modeling disease mechanisms, the efficacy of vaccine candidates and drugs addressing known or novel targets on clinical endpoints and biomarkers is predicted. Ideally quantitative systems pharmacology is combined with disease models in a way that certain specific hypothesis are generated that can subsequently be assessed in experimental animals and finally be fed back into the in silico model to potentially refine the hypothesis.

In summary animal models when adequately selected, designed, and conducted can provide precious information to our knowledge of biology and medicine, including the discovery and development of new vaccines and drugs. The translational value of animal models could be further improved and will fill the missing link between in vivo studies and clinical applications when combined with the emerging alternative translational approaches.

World Wide Web resources

The introduction of the World Wide Web provides an easy and cost-effective way of retrieving biomedical research information and a more flexible way of communicating with key opinion leader in the respective field. Searching for medical literature or references is extremely critical, especially in the era of evidence-based medicine.

Google (http://www.google.com) is helpful in finding articles from a wide variety of academic publishers, professional societies, preprint repositories and universities, as well as scholarly articles available across the web. In Google the key word "enteric diseases and animal models", "enteric pathogens and animal models", and Enteric bacteria and animal models revealed 66,30,000, 53,10,000, and 42,90,000 results, respectively.

Medline is produced by the United States National Library of Medicine, which is considered the most useful resource for searching the medical literature. A number of suppliers offer Medline access. Some Medline service providers offer free access to titles and abstracts but full-text articles can be purchased on-line. PubMed (http://www.ncbi.nlm.nih.gov/pubmed) located at the US National Institutes of Health is a very good Medline searching tool for accessing citations and links to full-text journals. In PubMed Advanced search one can employ the key words "Enteric Diseases" and "Animal models", 1483 studies will appear and 726 articles are freely downloadable.

In addition to Google and PubMed, PubTator (https://www.ncbi.nlm.nih.gov/research/pubtator/) is a Web-based tool for accelerating manual literature curation (e.g., annotating biological entities and their relationships) through the use of advanced text-mining techniques. PubTator has many unique features that distinguish it from existing annotation and literature search tools, as it is designed specifically for the needs of biocurators who have limited text-mining experience. PubTator is an all-in-one system that provides one-stop service for literature curation from searching and retrieving relevant articles to annotating selected articles.

The current trends in animal models for the development of novel therapeutics will also be available from the websites of different journals including *Nature Biotechnology*, *Journal of Experimental medicine*, *AMEM*, and others.

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Glossary

Diarrhea The condition of having at least three loose or liquid bowel movements each day.

Bacteremia The presence of bacteria in the bloodstream.

Reticuloendothelial cells A group of cells having the ability to take up and sequester inert particles and vital dyes, including macrophages and macrophage precursors, specialized endothelial cells lining the sinusoids of the liver, spleen, and bone marrow, and reticular cells of lymphatic tissue (macrophages) and bone marrow.

Gastroenteritis Also known as infectious diarrhea, is inflammation of the gastrointestinal tract that involves the stomach and small intestine.

Enterotoxin A toxin released by microorganism that specifically affects cells of the intestinal mucosa, causing vomiting and diarrhea.

Suckling An infant or young animal that is still taking milk from the mother.

Intragastric Administration of a drug or any substance within the stomach, for example, via gavage or gastrostomy.

Freund's Complete Adjuvant It is composed of inactivated and dried mycobacteria (usually *M. tuberculosis*) emulsified in mineral oil and used as an immunopotentiator (booster). It is named after Jules T. Freund.

Anorexia A psychological and possibly life-threatening eating disorder defined by an extremely low body weight relative to body mass index (BMI).

Colostrum A pre-milk fluid produced in the mammary glands of mammals that have recently given birth.

edema A build-up of fluid in the body that causes the affected tissue to become swollen.

Tracheobronchitis Also known as bronchitis, is a condition involving inflammation of the windpipe or bronchi, both of which carry air to the lungs.

Rag2$^{-/-}\gamma c^{-/-}$ mice Exhibit T cell, B cell and NK cell immunodeficiencies that make them effective transplant hosts for human immune cells.

Kupffer cells A specialized population of macrophages that reside in the liver.

Hyperplasia It is increased cell production in a normal tissue or organ. Hyperplasia may be a sign of abnormal or precancerous changes.

Long answer questions

1. Define mucosal escape response. How does RopS regulate the mucosal escape response?
2. What are the main advantages of mice compared to rabbit as animal model?
3. Explain the mechanism of action of cholera toxin.
4. What are the advantages and disadvantages of in vivo animal models as compared to in vitro models?
5. Define humanized mice and describe their role in studying *S. typhi* infection and immunity.
6. Why animals are necessary for biomedical research?
7. Why cannot alternative methods replace animals in research?

Short answer questions

1. What is toxin? Give few examples.
2. What are the functions of pili, flagella, and capsule?
3. What is the function of MacConkey Agar?
4. Which radiolabeled isotope is used for dosechase technique?
5. List the name of cytokines involved in *Shigella* pathogenesis and how they promote intestinal inflammation?
6. What is the role of TLR in the pathogenesis of *S. typhi?*

Yes/no types questions

1. Shigella is a gram-positive bacteria.
2. Heat-stable toxin (ST) is an endotoxin.
3. IL-10 is an anti-inflammatory cytokine.
4. CD4 + T cells drive innate immunity.
5. Heat-stable (ST) is secreted by *V. cholera*.
6. TLR11 serves as pattern recognition receptor for flagellin.
7. CS21 is essential for gut colonization of Vibrio.
8. Neutrophils belong to inflammatory cell type.
9. Actin cytoskeletal rearrangement plays role in bacterial colonization.
10. LPS is a major component of bacterial cell wall.

Answers to yes/no questions

1. No—Gram-negative bacteria are bacteria that do not retain the crystal violet stain used in the Gram staining method of bacterial differentiation.
2. No—STs are enterotoxins (STs), secretory peptides produced by some bacterial strains, such as enterotoxigenic *Escherichia coli*.
3. Yes—IL-10 is a cytokine with potent anti-inflammatory properties that plays a central role in limiting host immune response to pathogens, thereby preventing damage to the host and maintaining normal tissue homeostasis.
4. No—CD4 + T cells play important role in the adaptive immune system. The cells of the adaptive immune system are T and B lymphocytes. Innate immune cells include dendritic cells, monocytes, macrophages, neutrophils, and NK cells.
5. No—STs are secreted by enterotoxigenic *Escherichia coli*. *V. cholera* secrets cholera toxin (also known as choleragen and sometimes abbreviated to CTX, Ctx, or CT) is AB5 multimeric protein complex.
6. Yes—TLR11 belongs to the toll-like receptor (TLR) family and the interleukin-1 receptor/toll-like receptor superfamily. TLR11 plays a fundamental role in both the innate and adaptive immune responses, through the activation of Tumor necrosis factor-alpha (TNF-α), the interleukin 12 (IL-12) response, and interferon-gamma (IFN-γ) secretion.
7. No—Colonization surface antigen 21 represents key virulence-associated factors of enterotoxigenic *Escherichia coli* (ETEC). It is required for gut colonization, the first step of the diarrheal disease process induced by these bacteria.
8. Yes—During the beginning (acute) phase of inflammation, particularly as a result of bacterial infection, environmental exposure, and some cancers, neutrophils are one of the first-responders of inflammatory cells to migrate toward the site of inflammation.
9. Yes—The actin cytoskeleton is a key target of numerous microbial pathogens, including

protozoa, fungi, bacteria, and viruses. In particular, bacterial pathogens produce and deliver virulence effector proteins that hijack actin dynamics to enable bacterial invasion of host cells, allow movement within the host cytosol, and facilitate intercellular spread or block phagocytosis.

10. No—Lipopolysaccharide (LPS) is the major component of the outer membrane of gram-negative bacteria. Lipopolysaccharide is localized in the outer layer of the membrane and is, in noncapsulated strains, exposed on the cell surface.

CHAPTER

11

Chick chorioallantoic membrane assay: a 3D animal model for cancer invasion and metastasis

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Summary

The chick chorioallantoic membrane (CAM) assay is an attractive in vivo animal model widely used in cancer research. This chapter reviews the latest applications of the CAM assay for the assessment of tumor growth, invasion, metastasis, and patient-derived xenografts in a wide range of malignancies. Furthermore the utility of the CAM assay for investigating the efficacy of anticancer drugs is presented. Advantages and limitations of the CAM assay will be discussed. In summary the CAM assay represents a rapid, cost-efficient, reproducible, and excellent short-term in vivo animal model for preclinical cancer research.

What you can expect to know

The CAM assay is an in vivo animal model that involves the implantation of tumor cells or patient-derived tissues on the extraembryonic membrane or CAM of the developing chick embryo. Various methods for tumor cell inoculation and tumor tissue grafting have been developed to study tumor growth, invasion, metastasis, and PDXs. Tumor invasion and growth in the CAM assay can be observed over 3–9 days. Quantification methods for tumor progression in the CAM assay include immunohistochemistry (IHC), qPCR, fluorescence, bioluminescence, and magnetic resonance imaging (MRI). The CAM assay is an attractive in vivo animal model for cancer research with wide range of applications.

Introduction

The chick chorioallantoic membrane (CAM) assay is an established in vivo animal model that has been widely used in various applications to study tumor biology. It has many advantages compared to xenograft and transgenic mice models that will be discussed in this chapter. CAM assay protocols including quantification methods of tumor progression in the CAM assay and recent studies utilizing the CAM assay in cancer research will be presented.

History

The CAM assay is one of the earliest animal models utilized to grow tumor grafts. Murphy and Rous (1912) transplanted chicken sarcomas using the CAM assay. A study by Dagg et al. (1956) was one of the first studies that utilized the CAM assay to assess the growth of human cancer cell lines, and in 1980 the CAM assay was reported as a tool to study metastasis (Ossowski and Reich, 1980).

In recent years the CAM assay has become a popular in vivo 3D animal model for various malignancies.

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As the CAM contains a rich vascular network, the CAM assay has been extensively used to assess tumor angiogenesis and screen for novel antiangiogenic compounds (reviewed in the study by Tufan and Satiroglu-Tufan, 2005). Subsequently the CAM assay has also been utilized for evaluation of tumor growth, migration, invasion, and metastasis (Cimpean et al., 2008; Ribatti, 2016). Various studies have also employed CAM assays as patient-derived xenografts (PDXs) platform for preclinical research using surgical tumor specimens (recently reviewed in the study by DeBord et al., 2018). Moreover Kue et al. (2015) utilized the CAM assay as a preclinical model for screening and testing of novel anticancer drugs.

Principle

Structure and function of the CAM

During the chick embryonic development (ED), three extraembryonic membranes are formed; (1) the yolk sac membrane, (2) the amnion, and (3) the chorioallantoic membrane (CAM). The CAM is formed 3–4 days after incubation by the fusion of the chorion and allantois layers (Romanoff, 1960). The CAM has a rich vascular system and by ED16, the CAM covers most of the yolk sac and is pressed against the shell membrane. The CAM functions as a respiratory organ and acts as a gas-exchange organ receiving oxygen and eliminating carbon dioxide through the pores in the egg shell of the chick embryos (Romanoff, 1960). The development of the chick embryos as well as the morphological structure and physiological function of the CAM have been well described (reviewed in the study by Ribatti, 2016). The three major layers of the CAM are (1) the outer ectodermal layer attached to the shell membrane, (2) the mesodermal layer that includes the blood vessels and stromal components, and (3) the endodermal layer that faces the allantoic cavity (Valdes et al., 2002). The outer ectoderm is also known as the chorionic epithelium, the mesoderm is known as intermediate vascularized mesenchyme, and the endoderm is known as the deep allantoic epithelium (Ribatti, 2014). The mesoderm of the CAM consists of various extracellular matrix (ECM) proteins such as fibronectin, collagen, laminin, integrin alpha(v)beta3, and matrix metalloproteinase-2 (MMP-2) (Giannopoulou et al., 2001) as well as collagen type I, III, and IV (Rowe et al., 2009), which mimic the stroma in the tumor microenvironment. These key characteristics and features of the CAM layers make it an attractive model to study tumor growth and invasion.

Methodology

Various CAM assay protocols have been employed in cancer research (Table 11.1). The full ED for the

TABLE 11.1 Summary of CAM assay methods to assess tumor growth, invasion and metastasis.

Methods	Application	Advantages	Disadvantages	References
In ovo	Tumor graft inoculated into the CAM with anticancer drugs	Tumor metastasis to the lower CAM can be assessed	Only 1–2 grafts per chick embryo, can be difficult to visualize	Schomann et al. (2013); Herrmann et al. (2018)
Ex ovo	Tumor graft implanted with scaffold onto the CAM layer	More than 2 grafts can be placed on the chick embryo due to a larger exposed area, tumor grafts are easy to visualize	Lower embryo survival rate, chick embryo can become infected	Martowicz et al. (2015)
i.v. Injection	Tumor cells injected i.v. into chick embryo veins	Tumor metastasis can be assessed using *Alu* qPCR and fluorescent imaging	Tumor cells have to be labeled with GFP for fluorescence imaging	Palmer et al. (2011)
Inoculation of cells onto the CAM	To assess tumor metastasis to organs and lower CAM of the chick embryo	Tumor metastasis can be assessed using *Alu* qPCR	Tumor metastasis can only be assessed for a short-term (7 days)	Zijlstra et al. (2002)
Matrigel implants	To assess cancer cell invasion and tumor growth with anticancer drug treatment	Cell invasion into the CAM mesoderm can be assessed using IHC	Tumor cells have to invade into the CAM mesoderm	Lokman et al. (2012), Mélin et al. (2015)
Collagen plug	Tumor cell or spheroids invasion into the CAM	Assess tumor cells after knockdown of target genes	Tumor cells have to invade into the CAM mesoderm	Martowicz et al. (2015)
Thermanox discs	Cancer cells inoculated with the discs after gentle laceration of the CAM surface	Tumor growth can be visualized easily on the CAM	Only invasive tumor cell lines can be used in the CAM assay	Balke et al. (2010)

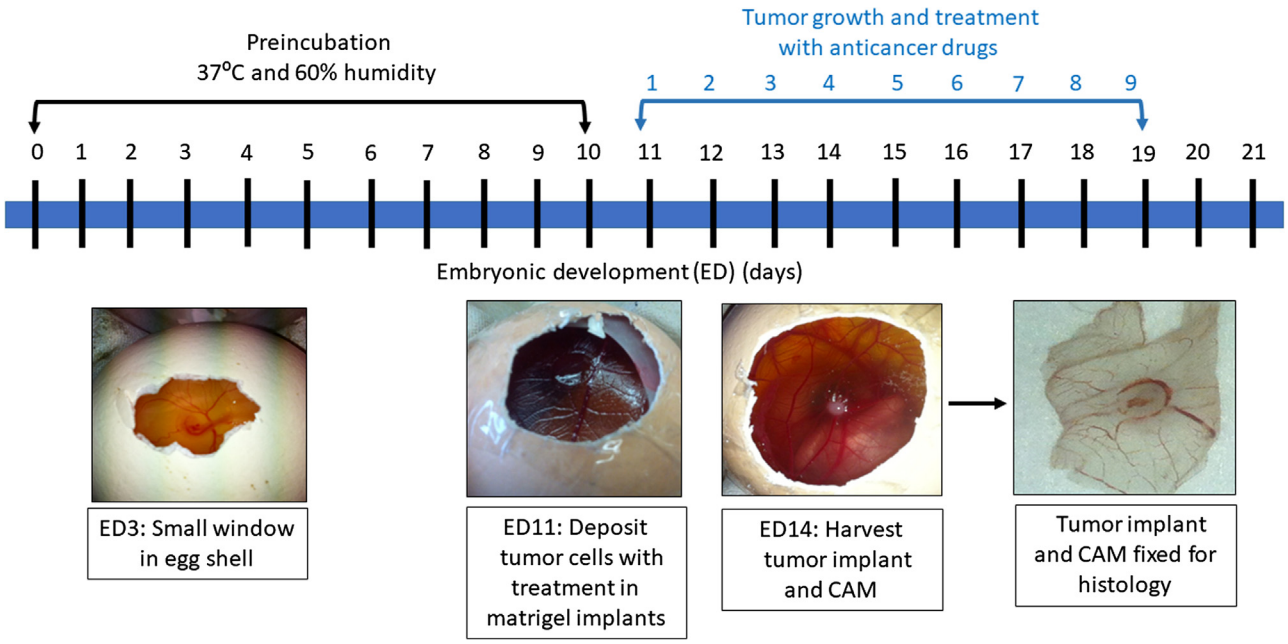


FIGURE 11.1 Chick embryo embryonic development (ED) (days) and CAM assay experimental time line. Preincubation of chick embryos on ED0 to ED10 and observation of tumor growth and treatment with anticancer drugs between ED11 to ED19. An example of a CAM assay protocol to assess tumor growth and invasion using matrigel implants. On ED3, a small window in the egg shell was made and on ED11, a matrigel implant including tumor cells and treatment was deposited on the CAM surface. On ED14 the tumor implant with CAM was harvested and fixed for histology analysis.

chick embryos takes 21 days. Most applications use the *in ovo* method in which a small window is made in the egg shell on ED3 and tumor cells are inoculated on ED11, followed by incubation for 3 to 9 days with tumor cells ± anticancer drugs (Fig. 11.1). The small window needs to be made on ED3 or ED4 when the extraembryonic vascular network and the heart of the chick embryo are already visible. On ED5, the CAM attaches to the inner egg shell, and opening of the egg without rupturing the CAM structure is very difficult (Romanoff, 1960).

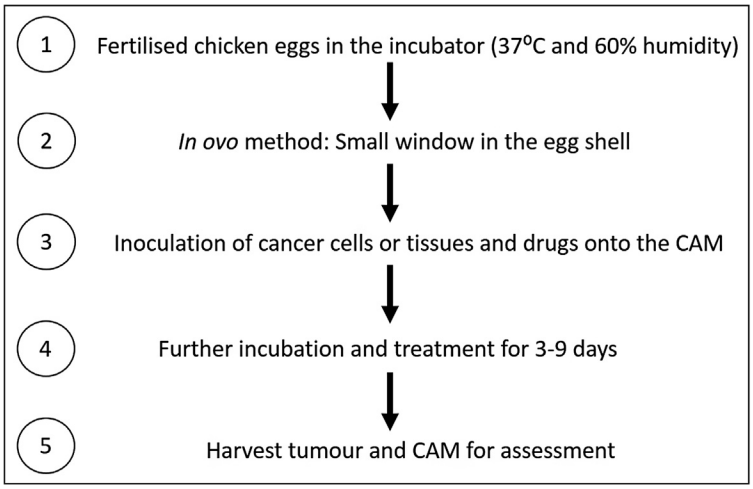
In the ex ovo CAM assay, which is also known as the shell-less method, the chick embryos are placed into a plastic weigh boat or a glass dish (Martowicz et al., 2015). Ex ovo chick embryos allow more replicates of cancer implants to be grafted onto the surface of the CAM as a larger area of CAM is accessible (Martowicz et al., 2015). Schomann et al. (2013) performed a comparison of the in ovo and ex ovo methods and reported no significant differences in size and weight of the chick embryos. However a lower embryo survival rate using the ex ovo compared to the in ovo method has been reported (Lokman et al., 2012).

Different techniques can be used for the inoculation of tumor cells on the CAM. For the study of invasion and growth, thermanox discs (Balke et al., 2010), Teflon O rings (Vu et al., 2018), matrigel implants (Lokman et al., 2012; Mélin et al., 2015), and collagen plugs (Martowicz et al., 2015) can be employed. For the study of metastasis, tumor cells can be injected: (1) into the yolk sac of ED3 chick embryos, (2) into the amniotic cavity of ED3 chick embryos, or (3) intravenously (i.v.) into the CAM veins of ED11 chick embryos (Taizi et al., 2006; Palmer et al., 2011) (Flow Chart 11.1).

Examples With Applications

Tumor Growth

The CAM assay has been successfully used for assessing tumor growth of various cancer cell lines and tissues (Table 11.2). It was employed to assess growth of breast (Jin et al., 2015), prostate (Das et al., 2017), ovarian (Lokman et al., 2012), pancreas (Rovithi et al., 2017), and head and neck carcinomas (Liu et al., 2013). Different methods have been developed to monitor and quantitatively assess tumor growth in the CAM assay. Assessment of tumor growth and cell proliferation in the CAM mesoderm was examined using Ki67 immunohistochemistry (Taizi et al., 2006; Sys et al., 2012; Martowicz et al., 2015; Mélin et al., 2015; Parkes et al., 2018; Swadi et al., 2018). Tumor growth in the CAM assay was also assessed using fluorescence imaging with green fluorescent protein (GFP)-labeled cancer cells in hematological malignancies (Taizi et al., 2006), cancers of the head and neck (Liu et al., 2013),



FLOW CHART 11.1 Flow chart of *in ovo* CAM assay.

TABLE 11.2 Summary of studies that have utilized cancer cell lines and patient derived xenografts (PDXs) in the CAM assay to assess tumor growth, invasion and metastasis (published articles from 2012 to 2018).

Cancer type (cell lines or tissues)	Applications	Quantification methods	References
Tumor of musculoskeletal system	PDXs tumor growth	IHC (Ki67, S100, CK7, CK20, ER, PR)	Sys et al. (2012)
Ovarian cancer cell lines (SKOV-3, OV-90 and OVCAR-3)	Tumor invasion, testing of anticancer drugs	IHC (pan-cytokeratin)	Lokman et al. (2012), Lokman et al. (2013)
Head and neck squamous cell carcinoma (HNSCC) (UM-SCC-29)	Tumor growth, angiogenesis, invasion, and metastasis	Fluorescence (GFP-labeled cells), *Alu* qPCR, IHC (vimentin and E-cadherin)	Liu et al. (2013)
Clear cell renal cell carcinomas (CCRCC) (RCC4, Caki-2 and 786-O)	PDXs tumor growth, invasion, and angiogenesis	IHC (CD31, CD34, and pan-cytokeratin)	Fergelot et al. (2013)
Colorectal cancer (WiDr and HCT116)	Tumor growth and angiogenesis	IHC (Ki67, cleaved caspase 3, CK20)	Mélin et al. (2015)
Breast cancer (MDA-MB-231)	Tumor growth	MRI, IHC (Ki67, desmin)	Zuo et al. (2015)
Multiple myeloma (OPM-2 and RPMI-8226)	Tumor spheroids, invasion, angiogenesis, and testing of anticancer drugs	Fluorescence (GFP-labeled cells), IHC (Ki67, CD138, vimentin)	Martowicz et al. (2015)
Nasopharyngeal carcinoma (NPC) cell lines (HONEI, 5−8 F,6−10B, C666-1) and tissues	PDXs tumor growth and invasion	Fluorescence (GFP-labeled cells) and IHC (CK34βE12)	Xiao et al. (2015)
Liver metastasis of ductal adenocarcinoma of the pancreas	PDXs tumor growth and testing of anticancer drugs	IHC (CD34, CK7, CK19 and CK8/18)	Ciolofan et al. (2017)
Pancreatic ductal adenocarcinoma	Tumor growth of primary cells and testing anticancer drugs	Bioluminescence, IHC (CD31, CK7, CK19, MUC1)	Rovithi et al. (2017)
Endometrial cancer cell lines (ISK, HECIA, RL85-2 and MFE280)	Tumor growth and testing anticancer drugs	IHC (Ki67, Bax, and Bcl-2)	Parkes et al. (2018)
Ovarian cancer (OVCAR-8) and ovarian cancer tissue	Tumor growth, PDXs tumor growth, and testing anticancer drugs	Fluorescence (GFP-labeled cells), IHC (vimentin)	Vu et al. (2018)
Neuroblastoma (SK-N-BE(2)C and IMR-32)	Tumor growth and testing anticancer drugs	Fluorescence (GFP-labeled cells), IHC (Ki67)	Swadi et al. (2018)

breast (Jin et al., 2015), ovary (Vu et al., 2018), nasopharynx (Xiao et al., 2015), and neuroblastoma (Swadi et al., 2018). Bioluminescence imaging has also been used in the CAM assay for prostate cancers, osteosarcomas (Jefferies et al., 2017), and pancreatic malignancies (Rovithi et al., 2017). Moreover, Zuo et al. (2015) also utilized a high-resolution MRI for the assessment of breast cancer growth. The analysis of breast cancer xenografts using the CAM assay identified different components of the tumor by immunohistochemistry such as cell density, proliferation, and angiogenesis, which correlated with different gray values in the MRI imaging (Zuo et al., 2015). Herrmann et al. also utilized MRI to examine primary neuroblastomas and their metastatic lesions in chick embryos. Tumor volumes were assessed with both microscopy imaging and MRI (Herrmann et al., 2018).

The CAM assay also enables the examination of 3D spheroid tumor growth in vivo. Liu et al. (2015) labeled spheroids of breast cancer cells (MCF-7) with monoclonal antibodies linked to nanoparticles and utilized the CAM assay to assess their growth. In addition Martowicz et al. (2015) utilized the CAM assay for growth of spheroids from multiple myeloma cells (OPM-2 and RPMI-8226) with primary human bone marrow mesenchymal cells and collagen type I as the ECM component. The tumor growth of the spheroids on the CAM was monitored using stereo immunofluorescence microscopy.

The CAM assay also allows the successful growth of tumors derived from primary cells. Primary cancer cells are a more ideal model when compared with commercial cell lines that have been cultured long term on two-dimensional (2D) culture surfaces. Farhat et al. (2018) used the CAM assay for growth and metastasis of primary leukemic stem cells (LSCs)-enriched patient cells via i.v. injection into the chick embryos and showed engraftment of primary human $CD34^{+}$ leukemic cells in the chick embryo liver. Rovithi et al. (2017) also utilized the CAM assay for growth of primary cells derived from primary pancreatic cancers.

Tumor Invasion

The CAM assay is an excellent 3D in vivo tumor model to study tumor invasion. Tumor growth and invasion occur within 2–6 days in the CAM assay in comparison with 3–12 weeks in the mouse model. The histological structure of the CAM includes the ectoderm, mesoderm, and endoderm (Fig. 11.2A). Cancer cells are seeded onto the CAM ectoderm and cell invasion results in the destruction of the ectoderm, followed by migration and invasion into the mesoderm (Fig. 11.2B). CAM layers have a type IV collagen-rich basement membrane and underlying stroma with type I and III collagen (Rowe et al., 2009). These key features of the CAM mesoderm mimic the stroma microenvironment of tumors and allow migration, invasion, and proliferation of tumor cells, making the CAM assay an attractive animal model to visualize tumor invasion.

CAM assays have been utilized to assess invasion of prostate cancers (Das et al., 2017), ovarian cancers (Lokman et al., 2012; Zhu et al., 2017), nasopharyngeal carcinomas (Xiao et al., 2015), and multiple myelomas (Martowicz et al., 2015). For quantification of cancer cell invasion after 2–6 days post inoculation, pan-cytokeratin immunostaining (Lokman et al., 2012; Das et al., 2017; Zhu et al., 2017) and immunofluorescence (Liu et al., 2013; Xiao et al., 2015) have been employed.

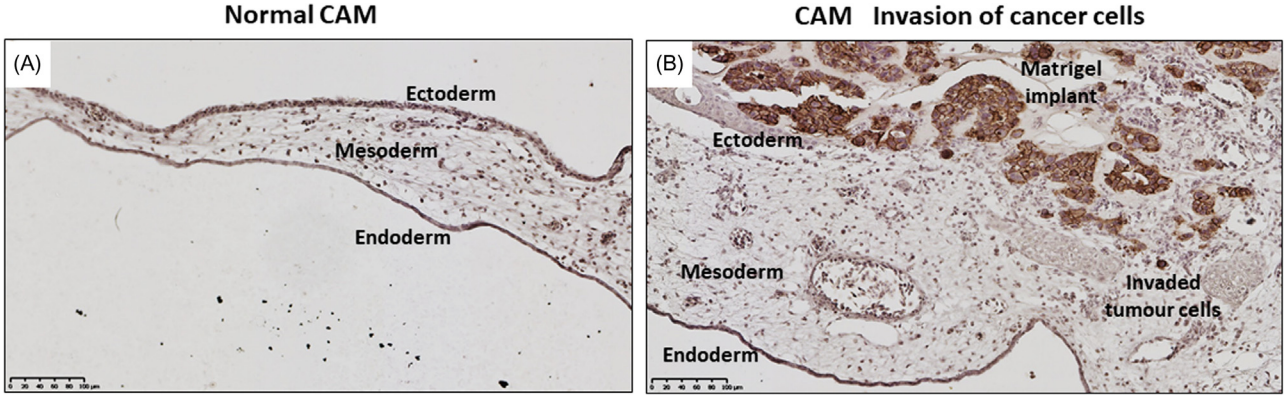


FIGURE 11.2 Cancer cell invasion into the CAM layer. (A) Structure of the CAM layer; ectoderm (outer layer), mesoderm (stroma) and endoderm. (B) Invasion of ovarian cancer cells (OV-90) from the matrigel implant resulted in destruction of the ectoderm and cancer cell invasion into the mesoderm. Tumor cells in the matrigel implant and the mesoderm were visualized by annexin A2 immunostaining (1/500, BD Biosciences; Lokman et al., 2013). Original scale ×200, scale bar 100 μm.

Tumor Metastasis

The CAM assay can be utilized to investigate the metastatic dissemination of tumor cells in vivo. Zijlstra et al. (2002) utilized two malignant human tumor cell lines [HEp3 (epidermoid carcinoma) and HT1080 (fibrosarcoma)] to assess spontaneous metastasis and secondary tumor growth in the CAM assay. Spontaneous metastasis occurred when tumor cells were inoculated after gentle laceration of the CAM of ED10 chick embryos and tumor formed after 7 days. Secondary growth was assessed 7 days after i.v. injection of cells by harvesting the lungs and lower CAM of the chick embryos (Zijlstra et al., 2002). The rapid tumor growth over a 5–7 days period post injection makes the CAM assay a unique experimental model to study intravasation, the entry of malignant cells into the vasculature, and to assess early colonization steps of the metastatic cascade (Deryugina and Kiosses, 2017). Conn et al. (2009) utilized the chick embryo spontaneous metastasis model together with mouse spontaneous metastasis model using prostate cancer cells (PC3) to examine intravasation. Liu et al. (2013) inoculated head and neck cancer cells into the upper CAM and assessed metastasis to the lower CAM and organs of the chick embryo such as heart, liver, and lung. Bobek et al. developed a chick embryo metastatic cancer model using Lewis lung carcinoma cells injected i.v. into chick embryos. Visualization of tumor cells metastasized to brain, heart, and sternum was performed using fluorescence imaging (Bobek et al., 2004).

Metastasis of tumor cells in the CAM assay can be quantified using human specific *Alu* quantitative PCR analysis, as the *Alu* sequence is specific for human DNA but not present in the chick embryo DNA (Zijlstra et al., 2002). Palmer et al. (2011) assessed spontaneous metastasis by i.v. injection of HEp3 epidermoid carcinoma cells onto the CAM and metastasis were assessed using *Alu* qPCR. The *Alu* qPCR was also used to assess metastasis of nasopharyngeal carcinoma cells to the heart and lung of the chick embryos (Xiao et al., 2015). Altogether these studies demonstrate that the CAM assay is a useful in vivo model to investigate the key steps and mechanisms involved in the metastatic cascade.

Patient-Derived Xenografts

The CAM assay can also be used for PDXs studies. It allows successful grafting of patient tissues that can then be easily visualized and monitored (Sys et al., 2012). PDXs in the CAM assay have been reported for many malignancies including sarcomas (Sys et al., 2012), renal cell carcinomas (Fergelot et al., 2013), pancreatic adenocarcinomas (Ciolofan et al., 2017), and ovarian carcinomas (Vu et al., 2018). PDXs in the CAM assay retain the 3D architecture of the tissues, accurately present the tumor pathology, and allow to predict treatment response to therapeutics (reviewed in the study by DeBord et al., 2018).

Sys et al. (2012) grafted bone and soft-tissue tumors onto the CAM and showed that the tumor grafts retained the original tumor characteristics. Moreover PDXs of laryngeal squamous cell carcinoma (LSCC) onto the CAM retained the characteristics of the original tumor and showed no signs of necrosis (Uloza et al., 2015). PDXs of nasopharyngeal carcinomas were successfully grafted onto the CAM and maintained good viability and growth (Xiao et al., 2015). PDXs and CAM assays were also reported for oral cell squamous carcinomas, and assessment of the grafts showed expression of epithelial mesenchymal transition (EMT) markers including E-cadherin and vimentin after the tissues were treated with TNFα and TNFβ (Kauffmann et al., 2018). The successful grafting of PDXs in the CAM assay in various tumor types makes it a promising and attractive preclinical cancer research model for personalized medicine.

Response to anticancer drugs

The CAM assay can be utilized to assess the activity, toxicity, biodistribution, and pharmacokinetics of cancer therapeutics and is an attractive alternative to mouse models (reviewed in the study by Vargas et al., 2007). Numerous FDA-approved anticancer drugs such as paclitaxel, carmustine, camptothecin, cyclophosphamide, vincristine, cisplatin, aloin, mitomycin C, antinomycin-D, and melphalan have been utilized in the CAM assay (Kue et al., 2015).

Various studies have investigated the effects of chemotherapeutic agents on tumor progression using the CAM assay. Martowicz et al. (2015) reported decreased invasion of multiple myeloma (OPM-2) cells after treatment with the drug bortezomib in the CAM assay. Malignant glioma tissues were successfully grafted in the CAM assay and could predict the efficacy of cytotoxic drugs injected into the CAM veins (Shoin et al., 1991). Tumor growth of leukemic cells (K562) was impaired after i.v. injection of doxorubicin in the CAM assay (Taizi et al., 2006). Bobek et al. (2004) reported on the combination of streptokinase and gemcitabine inhibiting Lewis lung carcinoma cell metastasis to the brain, heart, and sternum of chick embryos. Treatment of primary pancreatic ductal adenocarcinoma with a combination of gemcitabine and crizotinib resulted in reduced tumor growth by 63% in the CAM assay (Rovithi et al., 2017). Moreover Ciolofan et al. (2017) grafted metastatic pancreatic adenocarcinoma tissue fragments onto the CAM, and treatment with drugs including bevacizumab and rapamycin showed

reduced tumor growth. Vu et al. (2018) also reported that the CAM assay is a suitable model to assess the effects of nanoparticle-loaded doxorubicin on ovarian tumor growth. The utility of the CAM assay for the assessment of immunotherapeutics was shown by Lokman et al. (2013) who reported decreased ovarian cancer cell invasion after treatment with annexin A2 antibodies.

Advantages and limitations of the cam assay

The advantages of the CAM assay in comparison with other in vivo models are numerous and include cost-efficiency, reproducibility, short experimental time points, high embryonic survival rates, and simple technical setup, combined with well-characterized physiology and histology (reviewed in the study by Lokman et al., 2012). As the chick embryos are naturally immunodeficient, various cancer cell lines and patient tumor tissues can be successfully transplanted onto the CAM. Furthermore the CAM assay is an attractive metastasis model to assess key features such as intravasation, spontaneous metastasis, and assessment of both primary and secondary tumor growth. Finally the CAM assay allows large-scale, fast and cost-efficient screening of novel anticancer drugs to predict patient response.

Several studies have utilized both CAM assays and xenograft mouse models in parallel to investigate tumor growth and metastasis (Table 11.3). Both models are immunocompromised in vivo animal models that allow the formation and observation of tumor growth for several days (CAM assay) or weeks and months (mouse model). Jin et al. (2015) reported breast cancer cells (BICR-H1), overexpressing cancer and embryo expression protein 65 (CEP65), enhanced tumor growth and metastasis to the lungs of the chick embryos and in the severe combined immunodeficiency (SCID) mouse model. Both CAM assay and mouse model have also been utilized in cancers of ovary (Zhu et al., 2017), prostate (Das et al., 2017) and head and neck (Liu et al., 2013) as well as melanomas (Avram et al., 2017). Both animal models were useful for assessing tumor growth and overall similar results and observations were reported. The advantages of the CAM assay compared with the mouse model are its easier experimental setup, shorter time frame, reproducibility, and cost-effectiveness, allowing for large-scale experiments and high-throughput screening.

The limitations of the CAM assay are short observation periods (3–9 days) and the inability to study cancer and immune cell interactions. In addition chick embryos are highly vascularized organisms characterized by rapid morphological changes. Furthermore only a limited number of available antibodies for tissue characterization are specific to chick embryos. But

TABLE 11.3 Summary of studies that utilized both CAM assay and mouse model in cancer research.

Cancer type (cell lines)	CAM assay	Mouse model	References
Prostate cancer (PC3)	PC3 cell variant promotes intravasation in the chick embryo spontaneous metastasis model	PC3 cell variant promotes tumor growth and intravasation in the mouse spontaneous metastasis model	Conn et al. (2009)
Head and neck squamous cell carcinoma (UM-SCC-29)	Stable knockdown of enhancer of zeste homolog 2 (EZH2) in UM-SCC-29 cells reduced tumor size, angiogenesis, invasion, and metastasis in the CAM assay	Down-regulation of EZH2 in UM-SCC-29 cells inhibited tumor growth after injected subcutaneously in the athymic nude mice	Liu et al. (2013)
Ovarian cancer (OV-90 and SKOV-3)	Neutralizing annexin A2 antibodies decreased cancer cell invasion in the CAM assay	SKOV-3 tumor growth and peritoneal dissemination in nude mice was inhibited by neutralizing annexin A2 antibodies	Lokman et al. (2013)
Breast cancer (BICR-H1)	BICR-H1 overexpressing CEP65 enhanced tumor growth and metastasis to the lungs of the chick embryos	CEP65 promoted BICR-H1 tumor growth and metastasis to the lungs in the SCID mouse model	Jin et al. (2015)
Prostate cancer (LNCaP and PC3)	miR-194 inhibitor reduced prostate cancer cells (LNCaP and PC3) invasion in the CAM assay	Overexpression of miR-194 in PC3 cells promoted tumor growth and metastasis in the NOD/SCID mouse model	Das et al. (2017)
Ovarian cancer (SKOV-3)	Calcium and integrin binding proteins 2 (CIB2) inhibited SKOV-3 cancer cell invasion in the CAM assay	Overexpression of CIB2 in SKOV-3 cells promoted tumor growth in NOD/SCID mice	Zhu et al. (2017)
Melanoma (A375)	Primary and secondary tumors formed in the CAM assay	A375 melanoma cells formed tumors in Balb/c nude mice model	Avram et al. (2017)

overall the CAM assay has many advantages that outweigh the limitations, which makes it an attractive model for in vivo cancer research.

Conclusions

Numerous studies have shown the utility of the CAM assay in cancer research. The CAM assay is an established and well-characterized animal model to investigate tumor growth, invasion, metastasis, and PDXs. Various quantification methods have been developed and described for the analysis of tumor progression in the CAM assay. Preclinical in vivo testing of anticancer drugs in the experimental CAM assay allows to study drug activity, toxicity, biodistribution, and aids in the development of clinical trials. Many advantages of the CAM assay to study tumor behavior in parallel to the mouse studies have also been reported. In conclusion the CAM assay has a great potential as preclinical model for personalized cancer treatment.

Ethical Issues

The CAM assay complies with the 3R principles (replacement, reduction, and refinement). Experiments performed on the chick embryos in research are more acceptable than using rodents, as unhatched birds are not considered as living animals.

Ethical issues regarding the CAM assay:

1. Chick embryos should be euthanized by a humane method at the end of the experiment. Commonly used techniques include freezing at −20°C, decapitation or in ovo fixation with paraformaldehyde without prior anesthesia. E14 and younger embyros are not considered to feel pain, but analgesia protocols should be considered by researchers if conducting experiments after day 15 of chick embryo development.
2. The optimal dose of anticancer drugs to the chick embryos need to be assessed prior to experimental procedure to determine that the dose is tolerable and not toxic to the chick embryos.

Translational significance

Successful cancer treatment has to account for the heterogeneity of individual cancers, and novel individualized approaches are warranted. PDXs studies with the CAM assay as patient-derived preclinical tumor model have the potential to determine the optimum treatment in a fast and cost-effective way. Therefore the CAM assay has the potential to become a vital tool for personalized cancer medicine.

Clinical correlations

Various studies have successfully grafted fresh surgical patient tissues onto the CAM of chick embryos. Patient tissues have also been cryopreserved in liquid nitrogen and then grafted onto the CAM for assessing of tumor growth and testing of therapeutic targets. The assessment of the tumor drug response in the in vivo CAM model is a useful screening tool for personalized medicine with the potential to improve patient management and treatment outcome.

Turning point

Over the past 20 years there has been an increasing interest in utilizing the CAM assay to study tumor growth and progression in various malignancies. The CAM assay is an attractive and a well-established in vivo animal model that is simple, reproducible, and has various applications, including the study of tumor invasion, metastasis, angiogenesis, PDXs, and screening for novel drugs and therapeutic targets.

Box

In 1960, Sir Mac Farlane Burnet shared the Nobel Prize for Medicine with Peter Medawar for the discovery of acquired immunological tolerance. He developed techniques for growing influenza viruses in the chorioallantoic membrane of the chick embryos which then became a standard laboratory practice.

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Glossary

Chorioallantoic membrane (CAM) Fusion of the allantoic membrane and the chorion on day 3 or 4 of chick embryonic development.
Ectoderm A multilayer epithelium at the air interface of the chick embryo.
Endoderm A layer of thick inner epithelium at the interface of the allantoic sac.
Mesoderm A stroma layer of loose connective tissue.
In ovo The chick embryo remains within the egg shell and the inoculation of tumor cells occurs through a small window in the shell.
Ex ovo A shell-less method where the chick embryo is removed from the egg shell and placed into a petri dish
Chick embryonic development The process of chick embryo development from a fertilized chicken egg to a fully developed chick embryo on day 21.

Abbreviations

2D	2-Dimensional
3D	3-Dimensional
BCL-2	B-cell lymphoma 2
CAM	Chorioallantoic membrane
CD	Cluster of differentiation
CK	Cytokeratin
ECM	Extracellular matrix
ED	Embryonic development
EMT	Epithelial mesenchymal transition
ER	Estrogen receptor
GFP	Green fluorescent protein
i.v.	Intravenous
IHC	Immunohistochemistry
MUC1	Mucin 1
MRI	Magnetic resonance imaging
NOD/SCID	Nonobese diabetic/severe combined immunodeficiency
PR	Progesterone receptor
PDXs	Patient-derived xenografts
qPCR	Quantitative polymerase chain reaction
SCID	Severe combined immunodeficiency

Long answer questions

1. What are the applications of the CAM assay in cancer research?
2. What are the methods that can be used to inoculate tumor cells in the CAM assay?
3. What are the quantification methods that have been developed to assess tumor growth, invasion, and metastasis in the CAM assay?
4. Is the CAM assay an alternative animal model in cancer research?

5. What are the advantages of the CAM assay in cancer research?

Short answer questions

1. The CAM assay can be used to assess tumor growth, invasion, angiogenesis, PDXs, and metastasis in various malignancies and also for screening and testing of novel drugs and therapeutic targets.
2. The methods that can be used to inoculate tumor cells in the CAM assay including thermanox discs, matrigel implants, collagen plugs, and i.v. injection.
3. The quantification methods that are used to assess tumor growth, invasion, and metastasis in the CAM assay are immunohistochemistry, qPCR, MRI, fluorescence, and bioluminescence imaging.
4. The CAM assay using chick embryos is an alternative animal model for assessing tumor growth and metastasis. Comparable observations have been found when comparing the CAM assay with the mouse model.
5. The CAM assay is a high-throughput, rapid, cost effective, and reproducible model that makes it an attractive animal model in cancer research.

Answers to short answer questions

1. Can any cell line be used in the CAM assay to assess tumor growth?
2. Can patient tumor tissues from surgery be grafted onto the CAM?
3. Can the CAM assay be utilized for testing novel drugs and therapeutic targets?
4. Are high doses of drugs toxic for the chick embryos?
5. Does the chick embryo have an immune response?
6. Can the CAM assay be used to study cancer metastasis to secondary organs in the chick embryos?
7. Are studies using the CAM model and the mice model comparable?
8. Can fluorescence and bioluminescence imaging be used in the CAM assay?
9. Are there any limitations of the CAM assay?
10. Do we need to apply for animal ethics approval to conduct the CAM assay experiment?

Yes/no type questions

1. Yes—Any cell line can be used in the CAM assay, but the cell line has to be an invasive to be able to invade into the CAM mesoderm layer.
2. Yes—The CAM assay is an attractive PDX model as patient tumor tissues can be grafted onto the CAM for several days.
3. Yes—The CAM assay is an inexpensive and robust model for screening and testing of novel cancer therapeutics in vivo.
4. Yes—Optimal drug doses need to be determined as high doses can be fatal for chick embryos.
5. No—The chick embryo is a naturally immunodeficient animal model that accepts transplantations from different tissues and species. However nonspecific inflammatory response can develop after day 15 of chick embryo development.
6. Yes—Tumor cells inoculated into the CAM can metastasize to distant organs of the chick embryo such as the heart, lungs, and liver.
7. Yes—Various studies have reported similar observations using both the CAM model and the mice model.
8. Yes—Tumor cells can be visualized using fluorescence and bioluminescence in the CAM assay.
9. Yes—The limitations of the CAM assay are short observation periods and rapid morphological changes. Interactions of cancer and immune cells cannot be examined.
10. Yes—CAM assays using chick embryos require animal ethics approval before conducting experiments.

SECTION II

Animal biotechnology: tools and techniques

CHAPTER

12

Animal biotechnology as a tool to understand and fight aging

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Summary

Aging is a biological reality and has its own dynamics, which is beyond human control. It is accompanied by loss of normal function and, often, the onset of age-related diseases ultimately resulting in death. Human life expectancy is increasing due to the advancement in new technologies but aging-related health problems continue to seriously compromise the quality of life. The essential aim of aging research is not only to extend the life span but also to maintain good health and quality of life. Antiaging medicines is now a multimillion dollar industry. We are in the process of understanding the biochemical/molecular events that occur during the ageing process. Dietary flavonoids are nowadays emerging as antiaging compounds. Flavonoids have been demonstrated to have various health-associated properties. Thus, the development of preventive measures or interventions that slows down aging should be workout that will improve the quality of life for millions and reduce the costs associated with health care.

What you expect to know

This chapter provides an excellent introduction to an overview of aging, theories of aging, reactive oxygen species, and use of various animal experimental models for the study of human aging. Translational significance of aging and strategies that will help in delaying the aging process along with its ethical issues is also discussed.

Introduction

Aging is both an opportunity and a challenge. Increased life expectancy is a common feature of most countries today. At the same time, new technologies make even more costly procedures available to health-care systems. Aging is a biological reality and has its own dynamics, which is beyond human control. Aging is defined when two criteria are met. First, the probability of death at any point of time increases with the age of the organism. This statistical definition applies from yeast to mammals and reflects the progressive nature of organism. Second, characteristic changes in phenotype occur in all individuals over time due to the limiting processes. Biologically, aging is the accumulation process of diverse detrimental changes in the cells and tissues with advancing age, resulting in an increase in the risks of disease and death (Harman 2006). There are many theories, which attempt to explain the process of aging. The oxidative stress hypothesis offers the best mechanistic elucidation of the aging process and other age-related phenomenon (Fig. 12.1).

Theories of aging

There are more than 300 theories, none of which could qualify as being the theory of aging, and all of them could be, at best, labeled as "hypothesis" or "aspect theories" (Rattan 2006). Over the years, gerontologists have become resigned to the futility of formulating a unified theory of aging which can encompass its evolutionary, biological, and sociological aspects. Most significantly, it has been clearly shown that the phenotype and the rate of progression of aging are

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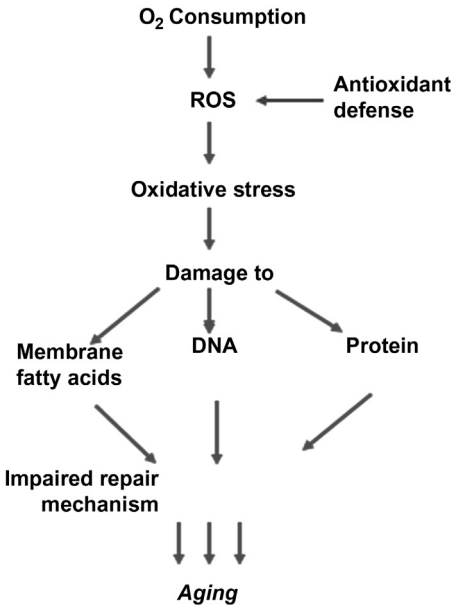


FIGURE 12.1 Role of reactive oxygen species in the process of aging.

highly variable in different species, in organisms within a species, in organs and tissues within an organism, in cell types within a tissue, in sub-cellular compartments within a cell type, and in macromolecules within a cell. These observations necessarily lead to the conclusion that aging has no universal cause, phenotype, and consequence, except death.

Evolutionary theories

The evolutionary theory describes aging as an emergent phenomenon that take place primarily in protected environments which allow survival beyond the natural life span in the wild. The natural life spans of a species, termed essential life span (ELS) or the warranty period. Species that undergo fast maturation and have the early onset of reproduction with large reproductive potential generally have a short ELS, whereas slow maturation, late onset of reproduction, and small reproduction potential of a species are concurrent with its long ELS.

Evolutionary theories argue that aging results from a decline in the force of natural selection. Because evolution acts primarily to maximize reproductive fitness in an individual, longevity is a trait to be selected only if it is beneficial for fitness. Life span is, therefore, the result of selective pressures and may have a large degree of plasticity within an individual species, as well as among species. The evolutionary theory was first formulated in the 1940s based on the observation that Huntington's disease, a dominant lethal mutation, remained in the population even though it should be strongly selected against. The late age of onset for Huntington's disease (30–40 years) allows a carrier to reproduce before dying, thereby allowing the disease to avoid the force of natural selection. This observation inspired the mutation accumulation theory of aging, which suggests that detrimental, late-acting mutations may accumulate in the population and ultimately lead to pathology and senescence. Currently, there is scant experimental evidence for this theory of aging.

However, the basic concept that aging results from a lack of selection enjoys a wealth of experimental support. Long-lived *Drosophila* strains can be bred by selecting the offspring of older adults, demonstrating that life span can be altered directly by selective pressure. Life span is species-specific because it is largely a function of survivability and reproductive strategy in a competitive environment. Consequently, organisms that die primarily from predation and environmental hazards will evolve a life span optimized for their own particular environment. This idea was tested in a natural environment by comparing mainland opossums that are subject to predation to a population of opossums living on an island free of predators. The evolutionary theory predicts that the protected island opossums would have the opportunity to evolve a longer life span, if it were beneficial to fitness. Indeed, island opossums do live longer and age more slowly than their mainland counterparts. The observation that organisms can age in a natural environment indicates that although extending life span can be beneficial to fitness, other considerations might necessitate sacrificing longevity for reproductive fitness. This basic idea of the disposable soma theory of aging argues that the somatic organism is effectively maintained only for reproductive success; afterward, it is disposable. Inherent in this theory is the idea that somatic maintenance, in other words, longevity, has a cost; the balance of resources invested in longevity versus reproductive fitness determines the life span.

Molecular theories

The gene regulation theory of aging proposes that senescence results from changes in gene expression. Although it is clear that many genes show changes in expression with age, it is unlikely that the selection could act on genes that promote senescence directly. Rather, life span is influenced by the selection of genes that promote longevity. Recently, DNA microarrays have been used to assay genome-wide transcriptional changes with age in several model organisms.

Despite vigorous research, there is yet no agreement on the biochemical mechanism responsible for the loss of replicative potential of diploid culture cells. The aging process is not programmed, but, rather, the absence of selection for maintenance. The life span of

an organism is the sum of deleterious changes and counteracting repair and maintenance mechanism that respond to damage. Aging is defined when the longevity and embryonic development are met. The probability of death at any point in time increases with the age of the organism.

The importance of specific kind of genome instability in aging is becoming increasingly apparent. The accumulation of genomic changes, i.e., point mutations, loss of repeated DNA sequence such as ribosomal DNA rearrangements, and changes in chromosome number have been proposed as the cause of aging. Age-dependent changes have also been observed in the hypoxanthine phosphoribosyltransferase (HPRT) and human leukocyte antigens (HLA-A) genes of peripheral blood lymphocytes in humans. Hypoxanthine-guanine phosphoribosyltransferase (HGPRT) is an enzyme encoded by HPRT gene. HGPRT catalyzes the conversion of guanine to guanosine monophosphate (GMP) and hypoxanthin to ionosine ionophosphate (IMP). HGPRT has an important role in purine metabolism. HLA-A is a components of certain major histocompatibility complex I (MHC I) cell surface receptor isoforms which are present on the surface of all nucleated cells and platelets. These play an important role in immune system. The low frequency of these genomic changes, even in old individuals, casts doubt on their importance in aging. Aging is manifested by both the limited number of cell divisions and the change in phenotype undergone by mother cells prior to senescence. The pace of aging in mother cells is dictated by changes occurring in the ribosomal DNA. One of the most exciting developments in aging research is the identification of an insulin-like signaling pathway that regulates the life span in worms, flies, and mice. Life span extension results from the activation of a conserved transcription factor in response to a reduction in insulin-like signaling, indicating that gene expression can regulate the life span.

Studies of human centenarians and their relatives have identified a significant genetic aspect of the ability to survive to exceptional ages. A recent study supports the idea that exceptional longevity has a genetic component by identifying a locus on chromosome 4 that may contain gene(s) that promote longevity (Puca et al., 2001). Genetic analysis of human longevity is especially important given that genetic aspects of aging are studied primarily in short-lived model organisms. The main molecular theories of aging are as follows:

1. Gene regulation theory
2. Codon restriction theory
3. Error catastrophe theory
4. Somatic mutation theory
5. Dysdifferentiation theory.

Cellular theories

Cell senescence/telomere theory

Telomeres, the repeated DNA sequences at the ends of linear chromosomes, are unable to be fully replicated by DNA polymerases. Telomeres consist of the six-base repeating sequence TTAGGG. With each cell division, some of the telomeres are lost. But the number of times that most dividing cells can divide is limited. Thus, they will shorten with cell division unless maintained via telomerases, a ribonucleopreotein enzyme that can add telomeric repeat sequences to chromosome ends. For humans, the length of the remaining telomere is usually an indicator of how many divisions a dividing cell has left. Higher levels of oxidative stress increase the rate of telomere shortening. It is proposed that telomere shortening could be a molecular clock that signals the eventual growth arrest. Strong support for this has been recently provided by demonstration that the reactivation of telomerases in certain cultured human cells can extend their life span beyond their normal limits. There are several findings that relate telomere shortening to aging in vivo. There appears to be a rough correlation between telomere length and age in human soma. The cellular senescence theory of aging was formulated in 1965 when cell senescence was described as the process that limits the number of cell divisions normal human cells can undergo in culture.

Free radical theory of aging

The free radical theory of aging was first proposed in 1956 (Harman 1956). It is one of the best-known theories and remains controversial to this day. All organisms live in an environment that contains reactive oxygen species (ROS); mitochondrial respiration, the basis of energy production in all eukaryotes, generates ROS by leaking intermediates from the electron transport chain. The free radical theory is further divided into several hypotheses focusing on the exclusive role of particular organelles and types of damaged molecules in the aging process. One such hypothesis argues that mutations in mitochondrial DNA accelerate free radical damage by introducing altered enzyme components into the electron transport chain. Faulty electron transport results in elevated free radical leakage and ultimately more mitochondrial DNA mutation and exacerbated oxidant production. This "vicious cycle" of mutation and oxidant production eventually leads to cellular catastrophe, organ failure, and senescence. Another hypothesis argues that free radicals cause aging when oxidized proteins accumulate in cells. An age-dependent reduction in the ability to degrade oxidative modified proteins may contribute to the build-up of damaged, dysfunctional molecules in the cell.

System-based theories

In system-based theories, the aging process is related to the decline of the organ systems essential for

1. The control and maintenance of other systems within an organism, and
2. The ability of organisms to communicate and adapt to the environment in which they live.

In humans, all systems may be considered indispensable for survival. However, the nervous, endocrine, and immune systems play a key role by their ubiquitous actions in coordinating all other systems and in their interactive and defensive responsiveness to external and internal stimuli.

Neuroendocrine theory

This theory proposes that aging is due to changes in neural and endocrine functions that are crucial for coordinating communication and responsiveness of all body systems with the external environment, programming physiological responses to environmental stimuli, and maintaining an optimal functional state for reproduction and survival while responding to environmental demands. These changes, often detrimental in nature, not only selectively affect the neurons and hormones that regulate evolutionarily significant functions such as reproduction, growth, and development, but also affect those that regulate survival through adaptation to stress. Thus, the life span, as one of the cyclic body functions regulated by biological clocks, would undergo a continuum of sequential stages driven by nervous and endocrine signals. Alterations in the biological clock, for example, decreased responsiveness to the stimuli driving the clock or excessive or insufficient coordination of responses, would disrupt the clock and the corresponding adjustments. An important component of this theory is the perception of the hypothalamo-pituitary-adrenal (HPA) axis as the master regulator, the pacemaker that signals the onset and termination of each life stage. One of the major functions of the HPA axis is to muster the physiological adjustments necessary for preservation and maintenance of the internal homeostasis despite the continuing changes in the environment.

With aging, a reduction in sympathetic responsiveness is characterized by a decreased number of catecholamine receptors in peripheral target tissues; a decline of heat shock proteins that increase stress resistance in many animal species, including humans, and a decreased capability of catecholamine to induce these heat shock proteins. The hormones of the adrenal cortex are glucocorticoids, for the regulation of lipid, protein, and carbohydrate metabolism; mineralocorticoids, for that of water and electrolytes; and sex hormones. Among the latter is dehydroepiandrosterone, which decreases with aging; dehydroepiandrosterone replacement therapy has been advocated in humans, despite unconvincing results. Glucocorticoids, as well as other steroid hormones, are regulated by positive and negative feedback, between the target hormones and their central control by the pituitary and hypothalamus. With aging and in response to continuing and severe stress, not only feedback mechanisms may be impaired, but also glucocorticoids themselves may become toxic to neural cells, thus disrupting feedback control and hormonal cyclicity.

Caloric restriction theory

Evidence that calorie restriction (CR) retards aging and extends median and maximal life span was first presented in the 1930s (McCay et al., 1935). Since then, similar observations have been made in a variety of species including rats, mice, fish, flies, worms, and yeast. Although not yet definitive, results from the ongoing calorie-restriction studies in monkeys also suggest that the mortality rate in calorie-restricted animals will be lower than that in control subjects. Furthermore, studies show that calorie-restricted monkeys have lower body temperatures and insulin concentrations than do control monkeys and both of those variables are biomarkers for longevity in rodents (Heilbronn and Ravussin, 2003). Calorie-restricted monkeys also have higher concentrations of dehydroepiandrosterone sulfate. The importance of dehydroepiandrosterone sulfate is not yet known, but it is suspected to be a marker of longevity in humans, although this is not observed consistently. In humans, a major goal of research into aging has been the discovery of ways to reduce morbidity and delay mortality in the elderly. The absence of adequate information on the effects of CR in humans reflects the difficulties involved in conducting long-term calorie-restriction studies, including ethical and methodological considerations. There is also evidence that DNA damage is reduced by CR, possibly as a result of increased DNA repair capacity. If normal feeding is resumed, these animals remain fertile far longer than other animals. CR diverts energy from growth and reproduction toward somatic maintenance and thus may explain the life-prolonging effect of CR.

Principle

The basic principle that governs aging is the generation of reactive oxygen species (ROS). Although the fundamental mechanisms of aging are still poorly understood, a growing body of evidence points toward the oxidative damage caused by ROS as one of the

primary determinant of aging. Aerobic cells produce ROS as a byproduct of their metabolic processes. The defense system of body tried to neutralize the ROS but when it increases beyond the defense system ROS cause oxidative stress that will damage to proteins, lipids and nucleic acid under conditions when the antioxidant defense of the body is overwhelmed. A certain amount of oxidative damage takes place even under normal conditions; however, the rate of this damage increases during the aging process as the efficiency of antioxidative and repair mechanisms decreases.

Reactive oxygen species—causative agent of aging

A free radical exists with one or more unpaired electron in atomic or molecular orbital. Free radicals are generally unstable, highly reactive, and energized molecules. ROS can be classified into oxygen-centered radicals and oxygen-centered nonradicals (Table 12.1)

Oxygen-centered radicals are superoxide anion (O_2^-), hydroxyl radical ($\cdot OH$), alkoxyl radical ($RO\cdot$), and peroxyl radical ($ROO\cdot$). Oxygen-centered nonradicals are hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$). Other reactive species are nitrogen species such as nitric oxide ($NO\cdot$), nitric dioxide ($NOO\cdot$), and peroxynitrite ($OONO^-$). ROS or free radicals in biological systems can be formed by prooxidative enzyme systems, lipid oxidation, irradiation, inflammation, smoking, air pollutants, and glycoxidation. Clinical studies reported that ROS are associated with many age-related degenerative diseases, including atherosclerosis, vasospasms, cancers, trauma, stroke, asthma, hyperoxia, arthritis, heart attack, age pigments, dermatitis, cataractogenesis, retinal damage, hepatitis, liver injury, and hypertension (Kumar et al., 2012). Free radicals have been implicated in the activation of nuclear transcription factors, gene expression, and a defense mechanism to target tumor cells and microbial infections. Superoxide anion may serve as a cell growth regulator. Singlet oxygen can attack various pathogens and induce physiological inflammatory response. Nitric oxide is one of the most widespread signaling molecules that participate in every cellular and organ function in the body. Nitric oxide acts as a neurotransmitter and an important mediator of the immune response which is also altered as a function of age (Maurya and Rizvi, 2009).

TABLE 12.1 Reactive oxygen species.

Oxygen centered radicals	Oxygen centered nonradicals
$O_2^{\bullet}$	H_2O_2
$^{\bullet}OH$	$^1O_2$
$HOO^{\bullet}$	
$ROO^{\bullet}$	

Free radicals (oxidants) come from two major sources: (1) endogenous and (2) exogenous. Endogenous free radicals are produced in the body by four different mechanisms.

1. From the normal metabolism of oxygen-requiring nutrients. Mitochondria—the intracellular powerhouses which produce the universal energy molecule, adenosine triphosphate (ATP)—normally consumes oxygen in this process and convert it to water. However, unwanted by-products—such as the superoxide anion, hydrogen peroxide and the hydroxyl radical—are inevitably produced, due to incomplete reduction of the oxygen molecule. It has been estimated that more than 20 billion molecules of oxidants per day are produced by each cell during normal metabolism. Imagine what happens with inefficient cell metabolism.
2. White blood cells destroy parasites, bacteria, and viruses by using oxidants such as nitric oxide, superoxide, and hydrogen peroxide. Consequently, chronic infections result in prolonged phagocytic activity and increased exposure of body tissues to the oxidants.
3. Other cellular components called peroxisomes produce hydrogen peroxide as a byproduct of the degradation of fatty acids and other molecules. In contrast to the mitochondria which oxidize fatty acids to produce ATP and water, peroxisomes oxidize fatty acids to produce heat and hydrogen peroxide. Hydrogen peroxide, which can then be degraded by the catalase. Under certain conditions, some of the hydrogen peroxide escapes to wreak havoc into other compartments in the cell.
4. An enzyme in the cells called cytochrome P450 is one of the body's primary defenses against toxic chemicals ingested with food. However, the induction of these enzymes to prevent damage by toxic foreign chemicals like drugs and pesticides also results in the production of oxidant by-products.

Superoxide anion (O_2^-)

Superoxide anion is a reduced form of molecular oxygen created by receiving one electron. $\cdot O_2^-$ is an initial free radical formed from mitochondrial electron transport system. The $\cdot O_2^-$ plays an important role in the formation of other ROS in living systems. The superoxide anion can react with nitric oxide ($NO^{\cdot}$) and form peroxynitrite ($ONOO^-$), which can generate toxic compounds such as hydroxyl radical and nitric dioxide.

Hydroxyl radical (OH)

Hydroxyl radical is the most reactive free radical and can be formed from $\cdot O_2^-$ and H_2O_2 in the presence of metal ions such as copper or iron. Hydroxyl radicals have the highest one-electron reduction potential and are primarily responsible for the cytotoxic effect in aerobic organism. Hydroxyl radicals react with lipids, polypeptides, proteins, and nucleic acids, especially with thiamine and guanosine. Hydroxyl radicals also add readily to unsaturated compounds. When a hydroxyl radical reacts with aromatic compounds, it can add on across a double bond, resulting in hydroxycyclohexadienyl radical. The resulting radical can undergo further reactions, such as reaction with oxygen, to give peroxyl radical or decompose to phenoxyl-type radicals by water elimination.

Hydrogen peroxide (H_2O_2)

H_2O_2 can be generated through a dismutation reaction from superoxide anion by superoxide dismutase (SOD).

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

Enzymes such as amino acid oxidase and xanthine oxidase also produce H_2O_2 from superoxide anion. H_2O_2 is highly diffusible and crosses the plasma membrane easily and is the least reactive molecule among. It is a weak oxidizing and reducing agent and is thus regarded as being poorly reactive. It can generate the hydroxyl radical in the presence of metal ions and superoxide anion, and produce singlet oxygen through reaction with superoxide anion or with HOCl or chloroamines in living systems. It can also degrade certain heme proteins, such as hemoglobin, to release iron ions.

Singlet oxygen

Singlet oxygen is a non-radical which is found in excited status. Singlet oxygen has been known to be involved in cholesterol oxidation. Oxidation and degradation of cholesterol by singlet oxygen was observed to be accelerated by the co-presence of fatty acid methyl ester.

Peroxyl and alkoxyl radicals

Peroxyl radicals ($ROO\cdot$) are formed by a direct reaction of oxygen with alkyl radicals ($R^\cdot$). Decomposition of alkyl peroxides (ROOH) also results in peroxyl ($ROO^\cdot$) and alkoxyl ($RO^\cdot$) radicals. Irradiation of UV light or the presence of transition metal ions can cause hemolysis of peroxides to produce peroxyl and alkoxyl radicals. Peroxyl and alkoxyl radicals are good oxidizing agents. They can abstract hydrogen from other molecules with lower standard reduction potential. This reaction is frequently observed in the propagation stage of lipid peroxidation.

Nitric oxide and nitric dioxide

Nitric oxide ($NO^\cdot$) is a free radical with a single unpaired electron. NO itself is not a very reactive free radical, but the overproduction of NO is involved in ischemia reperfusion, and neurodegenerative and chronic inflammatory diseases such as rheumatoid arthritis and inflammatory bowel disease. When NO is exposed in human blood plasma, can deplete the concentration of ascorbic acid and uric acid, and initiate lipid peroxidation. Nitric dioxide adds to double bonds and abstract labile hydrogen atoms initiating lipid peroxidation and production of free radicals.

Peroxynitrite

Reaction of NO and superoxide anion can generate peroxynitrite. Peroxynitrite is a cytotoxic species and causes tissue injury and oxidizes low-density lipoprotein (LDL). Peroxynitrite appears to be an important tissue-damaging species generated at the sites of inflammation and has been shown to be involved in various neurodegenerative disorders and several kidney diseases. Peroxynitrite ($OONO^-$) can cause direct protein oxidation and DNA base oxidation and modification acting as a "hydroxyl radical-like" oxidant. The significance of peroxynitrite as a biological oxidant comes from its high diffusibility across cell membranes. Nitrotyrosine, which can be formed from peroxynitrite-mediated reactions with amino acids, has been found in age-associated tissues.

Enzymatic formation

Pro-oxidative enzymes, including NADPH−oxidase, NO−synthase, or the cytochrome P−450 chain, can generate reactive oxygen species. Lipoxygenase generates free radicals. Lipoxygenase needs free polyunsaturated fatty acids (PUFA), which are not present in healthy tissue. Membrane-bound phospholipase produces PUFA and lysolecithins. Lysolecithins change the cell membrane structures, and free PUFA are oxidized to form lipid hydroperoxides. Once Fe (II) oxidized to Fe (III) lipoxygenase can convert polyunsaturated fatty acids (PUFA) into hydroperoxides. These enzymes can oxidize arachidonic acid, a PUFA rich in the central nervous system, into hydroperoxyeicosatetraenoic acid (HPETE). HPETE is then converted into leukotrienes, which regulate the immune responses. The production of Leukotrienes is accompanied by prostaglandins and histamine which act as inflammatory mediators. 15−Lipoxygenase has been identified within atherosclerotic lesions, which suggests that this enzyme may be involved in the in vivo formation of oxidized lipids.

Methodology: measurement of free radicals and methods to monitor aging

During the life span, the organism is confronted with oxidative stress on one side from intrinsic origins like the mitochondrial power generation is leaking ROS/reactive nitrogen species (RNS) and on the other side from extrinsic origins such as UV light, smoking and so on. Various methods on diverse systems have been documented to study the oxidative stress and aging. However, most of the methods get influenced by several factors such as life style, nutrition, and types of models. A commonly used alternate approach measures markers of free radicals rather than the actual radical. Markers of oxidative stress are measured using a variety of different assays.

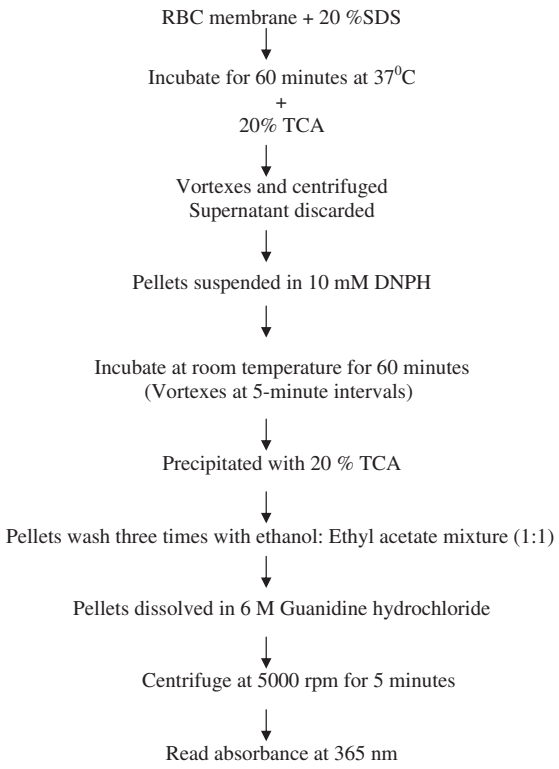
Protein oxidation/protein carbonyl content

Proteins are the building blocks of the body and likely to be major targets, as a result of their abundance in cells, plasma, and most tissues, and their rapid rates of reaction with ROS/RNS. ROS can lead to oxidation of amino acid residue side chains, formation of protein-protein cross-linkages, and oxidation of the protein backbone resulting in protein fragmentation. Oxidative stress leads to damage of proteins which results in loss in specific protein function; since proteins have unique biological functions, there are often unique functional consequences resulting from their modification. Most of the enzymes are made up of proteins. Any damage in the enzyme will lead to disturbance in all metabolic activities. It is estimated that almost every third protein in a cell of older animals is dysfunctional as enzyme or structural protein due to oxidative damage (Pandey and Rizvi 2010). The measurement of the protein oxidation is an important factor for the prediction of the aging process.

Carbonyl group content was determined following the method as described by Renzick and Packer (1994), using the 2′4 di-nitro phenyl hydrazine (DNPH) assay with a slight modification.

Approximately 1 mg of protein was precipitated with 20% trichloroacetic acid (TCA) (1:1vol/vol) in Eppendorff tube and vortexed for 30 seconds. After centrifugation, the clear supernatant was discarded and the pellet was resuspended with 0.5 ml of 10 mM DNPH in 2 M HCl and allowed to stand at room temperature for 60 minutes vortexing at every 5-minute intervals, to facilitate the reaction of DNPH with pellet proteins. The protein was precipitated again with 20% TCA and then the precipitated protein (pellet) was washed three times with 1.0 ml of 1:1(vol/vol) ethanol: ethyl acetate mixture. Finally, the pellets were dissolved in 0.7 ml of 6 M guanidine hydrochloride at 37°C. After centrifugation for 5 minutes at 6000 × g to precipitate the insoluble material, the clear supernatant was read against a complimentary blank at their maximum absorbance of 365 nm. Parallel blank was also run with the same procedure using 2 M HCl alone instead of 2,4 DNPH reagent. Carbonyl group content is expressed in nanomoles per milligram of protein using a molar absorbance coefficient of 22000 mol/L/cm (Flow Chart 12.1).

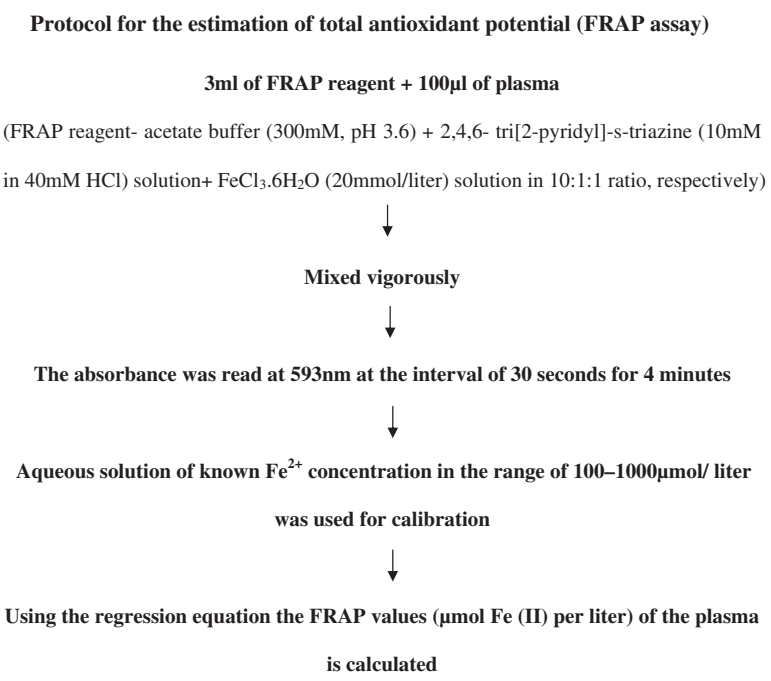
Protocol for the estimation of protein carbonyl content



FLOW CHART 12.1 Protocol for the estimation of protein carbonyl content.

Antioxidant capacity

Antioxidant capacity is the primary measurement to evaluate the state and potential of oxidative stress in aging and other age-related diseases. Since the imbalance between antioxidant and oxidants generates the condition of oxidative stress, the estimation of the reducing power/antioxidant capacity is the first step in the prediction of oxidative stress in the aging process. There are several methods to measure total antioxidant capacity in vitro. These methods are based on quenching of free radicals such as 1,1-diphenyl-2-picrylhydrazyl free radical ($DPPH^{\cdot}$), 2,2-azobis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS) by antioxidants; inhibition of lipid peroxidation,



FLOW CHART 12.2 Protocol for the estimation of total antioxidant potential (FRAP assay).

etc. The ferric reducing ability of plasma (FRAP) assay gets superiority because it is not dependent on the enzymatic/nonenzymatic method to generate free radical prior to the evaluation of the antiradical activity of plasma. The FRAP assay offers a putative index of antioxidants, or reducing potential of biological fluids and is simple, convenient, less time-consuming and reproducible. The total antioxidant capacity (FRAP value) decreases as a function of human age (Rizvi et al., 2006).

The Ferric Reducing Ability of Plasma (FRAP) values were determined by the following method (Benzie and Strain, 1996) (Flow Chart 12.2).

Common laboratory animal experimental models for aging research

Animal species phylogenetically close to humans may be used as animal models for the study of human aging. Many species with close genetic homology may serve as translational models to study aging and even age-related diseases. Mice and fish are effective models for the study of mechanism of aging and helps in better understanding of genetic and physiological bases of longevity.

Mice

It has a relatively short life span and share 99% genes of humans. Various genetic engineering technologies are available that can easily manipulate the genes of mice which helps in the understanding genetics of human aging. Mouse is used to test diets and compounds/drugs for their ability to delay aging and extend longevity in a mammalian model. Caloric restriction studies demonstrate to extend the life span of mice.

Fish

Fishes have been used as a gerontological model because of their many characteristics features. Investigators have cited a number of advantages for studying aging including the availability of large cohorts of offspring from single matings, the ectothermic nature of fish, and their reasonably short life span relative to many mammalian species. Other features include the low costs for breeding and maintenance, the ability to manipulate life span by both temperature reduction and food restriction.

In particular, guppies have proved to be an invaluable model for evolutionary analyses of aging, killifish are short-lived and may be exploitable for life span manipulation studies, and zebra fish come with a formidable armament of associated biological tools from their widespread use as a model of vertebrate development. These fish are well suited for the investigation of basic processes implicated in aging, such as insulin signaling, oxidative stress, and comparative studies of species with widely divergent longevities (Gerhard, 2007).

Many other model systems are used to study human aging that includes the following:

1. Human cells
2. Unicellular organisms such as the yeast
3. Roundworm
4. Fruit fly.

Polyphenols as an agent to fight aging

Polyphenols in food plants are a versatile group of phytochemicals. It has been reported that phenolic compounds have antioxidant, antimutagenic, and free radical scavenging activities. Epidemiological studies showed that increased consumption of phenolic compounds reduced the risk of cardiovascular disease and certain types of cancer and help in delaying the aging process (Maurya and Prakash, 2011). Moderate consumption of red wine, which contains high content of polyphenols, is associated with the low risk of coronary heart disease. Fruits and vegetables contain diverse phytochemicals of which large fractions are polyphenols. Polyphenols which have antioxidant property react with the ROS and form the product with much lower reactivity.

Flavonoids

The flavonoids are a large and complex group of compounds that occurs throughout the plant kingdom, providing flavors, color, antifungal and antibacterial activity, and contributing to many aspects of plant physiology. Most plant tissue can synthesize flavonoids. The flavanoids are diphenypropane derivatives that include flavonols, flavonones, antocynidines, flavones, and flavonols. More than 4000 flavonoids have been found in plants, fruits, and vegetables. There is a great interest in these phenolic compounds because of their potential role as an antiaging and cancer chemopreventive agents. This beneficial effect is considered to be mainly due to their antioxidant and chelating activities. However, some flavonoids such as quercetin have also been reported to be mutagenic and cocarcinogenic. The ability of flavonoids to scavenge free radicals and block lipid peroxidation raises the possibility that they may act as protective factors against cardiovascular disease and hypertension (Kumar et al., 2010), and there is epidemiological evidence consistent with this hypothesis. Any physiological significance of dietary flavonoids depends upon their availability for absorption and their subsequent interaction with target tissues, but little is known about their transport across the intestine. In general, flavanoid glycosides are resistance to processing, cooking, and digestion.

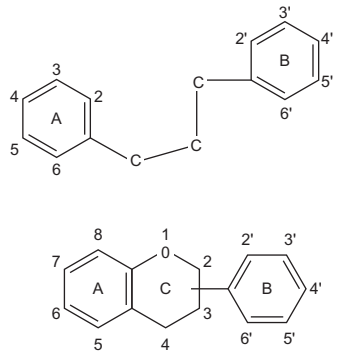


FIGURE 12.2 Basic structure of polyphenols.

The flavonoids are polyphenolic compounds possessing a basic structure of 15 carbon atoms; two benzene rings joined by a linear three-carbon chain. The chemical structure of flavonoids is based on this C_{15} skeleton, which forms a chromane ring bearing a second aromatic ring B at positions 2, 3, or 4 (Fig. 12.2).

Various sub-groups of flavonoids are classified according to the substitution patterns of ring C. Both the oxidation state of the heterocyclic ring C and the position of ring B are important in classification. Multiple combinations of hydroxyl groups, sugars, and oxygen and methyl groups attached to these structures create various types of flavonoids. The A and C rings collectively are often termed as the flavonoid nucleus (Fig. 12.3).

Flavanoids has many biological effects countering inflammatory, bacterial, viral, microbial, hormonal, carcinogenic, neoplastic, and allergic disorders and have been reported for in both *in vitro* and *in vivo* systems. Flavonoids exert antioxidant effects by neutralizing all types of oxidizing radicals including the superoxide and hydroxyl radicals and by chelation. A chelator binds to metal ions in our bodies to prevent them being available for oxidation. Flavonoids can also act as powerful chain-breaking antioxidants due to the electron-donating capacity of their phenolic groups.

Tea as antiaging compound

Tea is a natural beverage brewed from the leaves of an evergreen plant called *Camellia sinensis*. The *C. sinensis* is a very versatile plant that can grow under almost any conditions. Thus, tea is grown around the world from the Indian Sub-continent in India, Nepal, and Sri Lanka, to China, Japan, Indonesia, Vietnam, to the African subcontinent in Kenya, to Latin America in Argentina. As can be imagined, the quality of tea varies dramatically from region to region, with most of the variations originating in the variation in the climactic

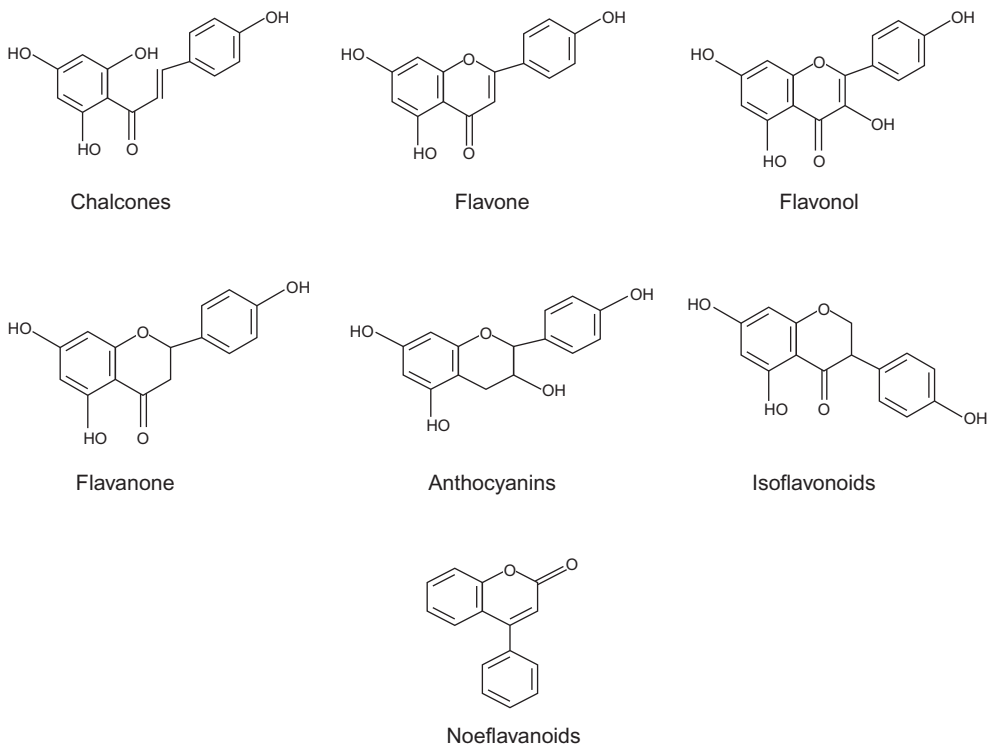


FIGURE 12.3 Structure of different subgroups of flavonoid.

conditions of the regions where the tea is grown and not from the differences in the tea bush itself.

Types of teas

Green Teas

Green tea is high in an important beneficial substance: polyphenols. Polyphenols, acting as antioxidants, reduce the formation of many types of cancer. It seems that flavonoids slow or even halt the oxidation process that allows cholesterol to harden and build on artery walls. In this way, flavonoids have the ability to lower cholesterol and reduce the incidence of heart disease. Green tea is also high in fluoride. That's right, the same stuff in toothpaste.

Black Teas

Tea in general contains fluoride, and all tea aids in digestion and bolsters the immune system. Black tea has both flavonoids and polyphenols, just to a lighter degree. Recent studies from the country of Denmark and from the Harvard Medical School both state that drinking several cups of tea a day (either green or black) can reduce heart attacks and heart disease by as much as 60%.

Herbal Teas

Camomile contains a mild and gentle sedative. It is recommended when people want a tea that will calm their nerves or when they want a tea that will help them sleep, but camomile also soothes the stomach and eases gas pains. After an illness, chamomile will even re-induce an appetite. In women, camomile acts as an antispasmodic. For generations, women have used it to ease menstrual cramps. It's gentle and natural.

Cinnamon is a strong spice whose chief health benefits have to do with soothing or correcting the stomach and intestines. It helps in releasing gas. This not only helps in digestion and in settling the stomach, but it can also help in treating a mild case of diarrhea.

Cloves have been used by herbalists for many centuries. By inhaling the strong odor of cloves, nausea is curbed and the stomach is settled. Drinking the clove tea aids in digestion and curbs flatulence.

Echinacea is one of the most recognized and accepted of herbal remedies. It increases resistance to illness and helps in fighting off infections. Echinacea is a catalyst in producing white blood cells. They are the cells responsible for fighting off illness and infection.

Ginger was used in ancient times by Chinese healers. Ancient (and present day) Chinese medicine has to do with achieving balance. If something appears out of balance, add or subtract to re-balance. If, for example, something seems cold, warm it up—this is exactly what ginger does. It warms the insides. It has been used for stomach cramps and to ward off cold and flu.

It is used for dieting as ginger speeds up the metabolism. Ginger is used to ward off nausea, especially nausea due to motion sickness. Drinking a cup of ginger tea before a nausea-inducing motion experience will prevent sickness.

Ginkgo helps to alleviate hypertension. Perhaps, this in itself is enough to slow aging. Ginkgo helps with circulation. It increases circulation in all parts of the body.

Ginseng is another one of those ancient Chinese medicines. Long touted as a "restorer of vitality," taken regularly, ginseng increases energy while releasing stress. The real trick is to use ginseng consistently over a period of time. Doing so really increases the energy.

Licorice teas are cooling, soothing, and coating. They are especially recommended for coughs or a sore throat.

Mate has caffeine in it. Grown in the rain forests of South America, it is a highly caffeinated/energizing drink. Passed around and drunk out of a gourd, in its native lands, it is used in communal ceremonies and celebrations. Yerba Mate is also high in vitamin C, which in turn is an antioxidant.

Peppermint is among our most popular herbal tea. Nearly everyone is familiar with it, and it is an easy choice. A lot of people already know something about the health benefits of peppermint. It aids in digestion, relieves nausea, even reduces flatulence, but there are a few benefits most do not know about. Peppermint teas can help to alleviate the pain of headaches associated with menstrual cramps. It can also help those with breathing troubles, like asthma. The smell of peppermint opens blocked breathing passages.

Rosehip is used in the blends of many teas. It is high in many vitamins, slightly acidic, and, containing pectins, is fruit-like. It acts as a mild laxative and diuretic.

Tea catechins

Green tea has attracted a significant attention recently, both in the scientific and in consumer communities for its health benefits for a variety of disorders, ranging from cancer to weight loss. Historically, green tea has been consumed by the Japanese and Chinese populations for centuries and is probably the most consumed beverage besides water, in Asian society.

The beneficial effects of green tea are attributed to the polyphenolic compounds present in green tea, particularly the catechins, which make up 30% of the dry weight of green tea leaves. The main characteristics catechins present in green tea are (-) epicatechin (EC), epicatechin-3-gallate (ECG), (-) epigallocatechin (EGC), and (epigallocatechin-3-gallate (EGCG) (Mukhtar and Ahmad 2000). EGCG, the most abundant catechin in green tea, accounts for 65% of the total catechin content. A cup of green tea may contain 100−200 mg of EGCG. Catechin and gallocatechin are present in trace amounts (Fig. 12.4).

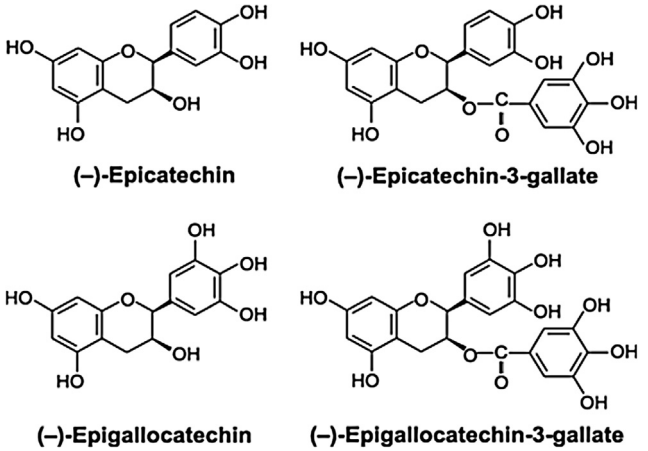


FIGURE 12.4 Structure of tea catechins.

Health benefits of tea

Green tea and its constituent catechins are best known for their antioxidant properties, which has led to their evaluation in a number of diseases associated with (ROS), such as cancer, cardiovascular, and neurodegenerative diseases. Several epidemiological studies as well as studies in animal models have shown that green tea can afford protection against various cancers such as those of the skin, breast, prostate, and lung. In addition to the cancer chemopreventive properties, green tea and EGCG have been shown to be antiangiogenic and antimutagenic. Green tea has also shown to be hypocholesterolemic and to prevent the development of atherosclerotic plaques. Among age-associated pathologies and neurodegenerative diseases, green tea has been shown to afford significant protection against Parkinson's disease, Alzheimer's disease, and ischemic damage. The tea has also shown antidiabetic effects in animal models of insulin resistance and has been shown to promote energy expenditure. Other health benefits attributed to green tea include antibacterial, antiHIV, antiaging, and antiinflammatory activities.

Molecular mechanisms of green tea effects

The health benefits of green tea are mainly attributed to its antioxidant properties and the ability of its polyphenolic catechins to scavenge ROS. These properties are due to the presence of the phenolic hydroxy groups on the B-ring in ungalloylated catechins (EC and EGC) and in the B- and D-rings of the galloylated catechins

(ECG and EGCG). The presence of the 3, 4, 5-trihydroxy B-ring has been shown to be important for antioxidant and radical scavenging activities. The green tea catechins have been shown to be more effective antioxidants than Vitamins C and E. The metal-chelating properties of green tea catechins are also important contributors to their antioxidative activity. Recent studies have shown that misregulated iron metabolism may be a central pathological feature in Parkinson's disease and that the iron-chelating properties of EGCG are important for its protective effects in neurodegenerative diseases (Mandel et al., 2004). In addition to antioxidant effects, green tea catechins have effects on several cellular and molecular targets in signal transduction pathways associated with cell death and cell survival. These effects have been demonstrated in both neuronal cells and in tumor epithelial/endothelial cells. Green tea also inhibits angiogenesis and tumor invasion by inhibiting metalloproteinases and the vascular endothelial growth factor receptor expression and signaling in tumor and endothelial cells. In neuronal cells, however, green tea catechins serve as a neuroprotective pro-survival function. Moreover, these effects have been observed at doses far lower than those at which antitumor activities have been demonstrated.

Green tea in aging and neurodegenerative diseases

Oxidative stress is believed to be a major contributor to the pathogenesis of Parkinson's disease, especially the death of dopaminergic neurons. Recently, misregulated iron metabolism in the brain has been shown to be involved in the generation of the pathological Lewy bodies in Parkinson's disease through iron-induced aggregation of alphasynuclein. Various studies have shown that green tea and EGCG significantly prevent these pathologies in animal models. Although there is no epidemiological evidence in human studies of the benefit of green tea for Alzheimer's disease, several studies in animal and cell culture models suggest that EGCG from green tea may affect several potential targets associated with Alzheimer's disease progression. Choi et al. (2001) showed that EGCG protects against beta-amyloid-induced neurotoxicity in cultured hippocampal neurons, an effect attributed to its antioxidant properties.

Animal biotechnology as a tool to understand aging and fight aging

The aging process is an inevitable part of life for humans, and animal biotechnology play an important role in understanding the process of aging and age-related diseases. It also provides various methods to fight aging. We are at the beginning of the biological revolution. Two centuries ago, the industrial revolution changed the way inanimate objects were manufactured. Today, the biological revolution is providing the means to create novel living organisms and combat aging and age-related diseases. The increase in the human life span and the decrease in disability at older ages are a testament to the economic and social progress.

Biotechnology is a set of techniques which helps to modify the living things. Biotechnology continues to deliver an impressive supply of new treatment options and interventions that will further extend healthy lives, policy–makers, and economists worry about the social implications of the future demand for health care. It holds out the promise of significantly improving elderly health and quality of life by alleviating the disabling conditions which plague our later years. According to both biologists and epidemiologists, the human life span continues to increase and the estimates of maximal life span may be greater than initially believed.

Biotechnologies are revolutionizing the aging experience by offering earlier diagnoses, new treatments such as regenerative and genetic interventions and ultimately disease prevention. Genomic studies make it possible to estimate the risk of age-related diseases. Biotechnologies are revolutionizing the ageing experience by offering earlier diagnoses, new treatments such as regenerative and genetic interventions, and ultimately disease prevention. Genomics make it possible to estimate the risk of age-related diseases. Techniques to prevent or replace lost functions are borrowing from the body's own development processes.

Aging and age-related diseases like Alzheimer's disease, Parkinson's disease, and Huntington's diseases may increase in the coming years. However, successful treatment strategies for age-related diseases are so far limited. Plants have always played a major role in the treatment of human and animal diseases. World-wide interest in the use of medicinal plants and its products is increasing, as they contain the recipe for chemical compounds of potential value in pharmaceutical products. Several Indian medicinal plants have also been proved to have antidiabetic, antihypertensive, and antiaging activities. In the last decade, approaches to search for biologically active compounds have changed dramatically for a number of reasons including advances in technology, new molecules of significant interest, changing ethical principles for organism collection, and increasing awareness of the chemical and biological potential of tropical rain forest. The pharmaceutical industry worldwide plays a major role in

developing new approaches to drug discovery aiming at faster and more efficient way to bring new medicines to the market. Several drugs and pant products are tested in animal models to evaluate the efficacy of compounds as antiaging. Thus, we can say that animal biotechnology play an important role in fighting aging and age-related diseases.

Human aging: a translational perspective and significance

The application of findings derived in basic science to the development of new understanding of disease mechanisms, diagnoses, and therapeutics in humans is known as translational research. It is the flow of ideas from basic science to clinical application. With the development of diverse new technologies, remarkable advances have occurred in the understanding of the molecular and genetic bases of aging and age-related diseases. Translational research in aging are generally involved nutrition, exercise, and metabolism of humans.

The involvement of posttranslational modifications in aging has been more clearly demonstrated in recent years. Carbonylation, a hallmark of protein oxidation in general, is paradoxically decreased in histone with aging and increased by CR, acetylation of lysine 9, and phosphorylation of serine 10 in histone H3 are decreased and increased, respectively, with aging, and the acetylation level of multiple extranuclear proteins decreases significantly with aging, and the change was not only retarded but increased remarkably by CR in rat liver. Based on above findings, Nakamura et al. discuss possible implications of the posttranslational protein modifications in biochemical processes underlying aging and CR-induced extension of the life span (Nakamura et al., 2010).

Ethical issues related with aging research

1. *Aging*: Aging is a biological reality and has its own dynamics, which is beyond human control. Aging is the accumulation process of diverse detrimental changes in the cells and tissues with advancing age, resulting in an increase in the risks of disease and death.
2. *Production of new animal models*: Biogerontologist employs short-lived laboratory models like yeast, free living nematodes, fruit flies, fish, and laboratory mice. For in vitro studies, fibroblasts are commonly studied animal cell type, originating from humans or mice. Animals that are used for aging research are typically maintained in a nonreproductive state, physically inactive, socially isolated, and exposed to minimal natural stressors as possible. There are well developed molecular tool kits and transgenic technologies that are used for the production of new animal models for aging research.
3. *Ethical implications for clinical and experimental research*: Ethics refer to the moral aspects of human conduct and personal character. Often ethical dilemmas arise in relation to personal freedoms, responsibilities, and rights or obligations (http://www.aging.pitt.edu/seniors/ethical-issues.asp). These are some common situations in which ethical issues may arise in older adults:
4. Advance care planning: Advance care planning involves advance preparation for life's unexpected emergencies. Regardless of age, advance care planning provides a greater control over decisions that affect a person's future and takes into consideration the person's beliefs and preferences in the event they are unable to make decisions on their own.
5. Right to privacy: Medical treatment often involves sensitive subjects that we would rather not share with other people—even those who are very close to us. If you are receiving treatment, and you do not want information shared with family, friends, or anyone else, you should know that knowledge is kept private between you and your health-care team.
6. Religion and health care: Religion may be a very important part of your life. If so, your religious beliefs may influence the types of medical treatment you desire. It is crucial to know your religion's true beliefs about certain medical procedures. Many times, even the most devout people are unclear about some of their religion's rules. They may refuse medical treatment that is allowed by their faith or consent to medical treatment that is not permitted.

World wide web resources

Studying human aging is an enormous challenge. The complexity of the aging phenotype and the near impossibility of studying aging directly in humans oblige researchers to resort to models and extrapolations. Bioinformatics offer various powerful set of tools to study aging and age-related disorders. There are data-mining methods and comparative genomics to DNA microarrays, to retrieve information in large amounts of data. There are several web-based resources that provide information to study aging. National Center for Biotechnology Information (NCBI)

web page www.ncbi.gov is an important resource for data mining and microarray studies. Aging-related information can be obtained from HAGR (http://genomics.senescence.info/), AGEID http://uwaging.org/genesdb/index.php), the meta-analysis of age-related gene expression Profiles, and aging-related yeast2hybrid experiments. The above web-based resources will help in downloading promoter sequences and other genome-related information. The Digital Ageing Atlas (http://human.ageing-map.org/) integrate molecular/physiological and pathological age-related data. Some other useful web-based resources for aging are below:

http://www.uwaging.org

http://www.antioxidants-for-health-and-longevity.com/causes-of-aging.html

http://www.biotecharticles.com/Biology-Article/Aging

Clinical correlation

Aging is characterized by morphological and biochemical changes that take place in a single cell and even in the whole organism. Oxidative stress, which decreases the antioxidant capacity, irreversibly damages cells. Clinical correlation between oxidative stress biomarkers and aging has been well established. It is reported that the level of lipid peroxidation in terms of malondialdehyde (MDA) increases as a function of human age while the level of enzymatic antioxidant activity and glutathione decreases during human aging. Telomere length shortening also takes place during normal human aging.

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Glossary

Aging Aging is the accumulation of changes in a person over time. E.g. Human aging.

Biotechnology Biotechnology is a field of applied biology that involves the use of living organisms and bioprocesses in engineering, technology, medicine and other fields requiring bioproducts. E.g. Many biotechnology companies are developing antiaging products such as antiaging creams. Juvista, is based on

a recombinant form of human transforming growth factor-β3 (TGF-β3), which is normally present at high levels in developing embryonic skin and in embryonic wounds that heal without a scar.

Oxidative stress Oxidative stress represents an imbalance between the production and manifestation of reactive oxygen species and a biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage. E.g. Lipid peroxidation, protein oxidation and DNA damage is higher in diabetic, hypertensive patients as compared to healthy normal.

Polyphenols Polyphenols are a structural class of natural, synthetic, and semisynthetic organic chemicals characterized by the presence of large multiples of phenol structural units. E.g. Tea catechins in green tea, resveratrol in grapes.

Abbreviations

CR	calorie restriction
EC	(−) epicatechin
ECG	(−) epicatechin gallate
EGC	(−) epigallactocatechin
EGCG	(−) epigallactocatechin gallate
FRAP	ferric reducing ability of plasma
H_2O_2	hydrogen peroxide
NO	nitric oxide
$O_2^{\cdot}$	superoxide radical
$^1O_2$	singlet oxygen
OH	hydroxyl radical
ROS	reactive oxygen species

Long-answer questions

1. What is aging? Why is there a decline in regenerative potential of an organism over time?
2. When does aging begin in humans? Discuss various theories of aging giving emphasis on free radical theory of aging.
3. What is polyphenols. How will you classify them based on their chemical structures?
4. How biotechnology helps in understanding aging and age-related phenomenon?
5. What is green tea? Discuss its health benefits.

Short-answer questions

1. What is aging?
2. Why do caloric restriction, delay the onset of a number of age-related physiological and pathological changes and increase the average and maximal life span in animals?
3. What are flavonoids?
4. How biotechnology helps to delay aging?
5. What are tea catechins?

Answers to short-answer questions

1. Aging is the accumulation process of diverse detrimental changes in the cells and tissues with advancing age, resulting in an increase in the risks of disease and death. Aerobic cells produce reactive oxygen species (ROS) as a byproduct of their metabolic processes. ROS cause oxidative damage to macromolecules (proteins, lipids and nucleic acids) under conditions when the antioxidant defense of the body is overwhelmed. A certain amount of oxidative damage takes place even under normal conditions, however the rate of this damage increases during the aging process as the efficiency of antioxidative and repair mechanisms decrease.
2. Evidence that calorie restriction (CR) retards aging and extends median and maximal life span was first presented in the 1930s. Since then, similar observations have been made in a variety of species including rats, mice, fish, flies, worms, and yeast. Although not yet definitive, results from the ongoing calorie-restriction studies in monkeys also suggest that the mortality rate in calorie-restricted animals will be lower than that in control subjects. Furthermore, calorie-restricted monkeys have lower body temperatures and insulin concentrations than do control monkeys and both of those variables are biomarkers for longevity in rodents. Calorie-restricted monkeys also have higher concentrations of dehydroepiandrosterone sulfate. The importance of dehydroepiandrosterone sulfate is not yet known, but it is suspected to be a marker of longevity in humans, although this is not observed consistently. In humans, a major goal of research into aging has been the discovery of ways to reduce morbidity and delay mortality in the elderly. The absence of adequate information on the effects of CR in humans reflects the difficulties involved in conducting long-term calorie-restriction studies, including ethical and methodological considerations. There is also evidence that DNA damage is reduced by CR, possibly as a result of increased DNA repair capacity. If normal feeding is resumed, these animals remain fertile far longer than other animals. CR diverts energy from growth and reproduction towards somatic maintenance and thus may explain the life-prolonging effect of CR.
3. The flavonoids are a large and complex group of compounds that occurs throughout the plant kingdom, providing flavors, color, antifungal and antibacterial activity, and contributing to many aspects of plant physiology. Most plant tissue can synthesize flavonoids. The flavonoid are diphenypropane derivatives that include flavonols,

flavonones, antocynidines, flavones and flavonols. More than 4000 flavonoids have been found in plants, fruits, and vegetables. There is great interest in these phenolic compounds because of their potential role as antiaging and cancer chemopreventive agents. This beneficial effect is considered to be mainly due to their antioxidant and chelating activities. However, some flavonoids such as quercetin have also been reported to be mutagenic and co-carcinogenic. The ability of flavonoids to scavenge free radicals and block lipid peroxidation raises the possibility that they may act as protective factors against cardiovascular disease and hypertension and there is epidemiological evidence consistent with this hypothesis. Any physiological significance of dietary flavonoids depends upon their availability for absorption and their subsequent interaction with target tissues, but little is known about their transport across the intestine. In general, flavonoid glycosides are resistance to processing, cooking and digestion.

4. Biotechnologies are revolutionizing the ageing experience by offering earlier diagnoses, new treatments such as regenerative and genetic interventions and ultimately disease prevention. Genomic studies make it possible to estimate the risk of age-related diseases. Biotechnologies are revolutionizing the ageing experience by offering earlier diagnoses, new treatments such as regenerative and genetic interventions and ultimately disease prevention. Genomics make it possible to estimate the risk of age-related diseases. Techniques to prevent or replace lost functions are borrowing from the body's own development processes.
5. Most of the polyphenols in green tea are flavonols commonly known as catechins. The main characteristics catechins present in green tea are the following:

1. Epicatechin (EC);
2. Epicatechin-3-gallate (ECG);
3. Epigallocatechin (EGC); and
4. epigallocatechin-3-gallate (EGCG).

Yes/no-type questions

1. Is oxidative stress is one of the causes of aging?
2. Is free radical theory of aging explains human aging?
3. Caloric restriction theory retards aging?
4. Can oxidative stress be measured?
5. Total antioxidant capacity of a cell can be measured?
6. Can mice, fish be used as a model to study aging?
7. Polyphenols acts as an agent to fight aging?
8. Is Green tea good for health?
9. Are posttranslational modifications involved in aging process?
10. Ethical issues are associated with aging research?

Answers to yes/no-type questions

1. Yes—Justification: Oxidative stress damages biomolecules which causes aging.
2. Yes—Justification: The free radical theory of aging was first proposed in 1956. It is one of the best-known theories to explain aging.
3. Yes—Justification: Evidence that calorie restriction (CR) retards aging and extends median and maximal life span.
4. Yes—Justification: Markers of oxidative stress are measured using a variety of different assays.
5. Yes—Justification: There are several methods to measure total antioxidant capacity in vitro. These methods are based on quenching of free radicals such as 1,1-diphenyl-2-picrylhydrazyl free radical ($DPPH^{\cdot}$), 2,2-azobis-3-ethylbenzthiazoline- 6-sulfonic acid (ABTS) by antioxidants
6. Yes—Justification: Animal species phylogenetically close to humans such as mice and fish may be used as animal models for the study of human aging.
7. Yes—Justification: Polyphenols in food plants are a versatile group of phytochemicals. It has been reported that phenolic compounds have antioxidant, antimutagenic, and free radical scavenging activities. Epidemiological studies indicate that they helps to fight aging.
8. Yes—Justification: Green tea is high in an important beneficial substance: polyphenols, which are good for health.
9. Yes—Justification: The involvement of posttranslational modifications in aging has been more clearly demonstrated. For example, carbonylation, a hallmark of protein oxidation in general, is paradoxically decreased in histone with aging.
10. Yes—Justification: Ethics refers to the moral aspects of human conduct and personal character. Often, ethical dilemmas arise in relation to personal freedoms, responsibilities, and rights or obligations.

CHAPTER

13

Multicellular tumor spheroids as in vitro models for studying tumor responses to anticancer therapies

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Summary

This chapter highlights the rationale, potential, and implementation of multicellular tumor spheroids (MCTSs) in cancer research. MCTS is a three-dimensional in vitro model system that bridges the gap between two-dimensional monolayer cell cultures and an in vivo tumor tissue model system. Compared to monolayer cultures, MCTS resembles tumor tissue in terms of structural and functional properties and is suitable for studying metastasis, invasion, and therapeutic screening of drugs. Spheroid models are helpful in accelerating translational research in cancer biology and tissue engineering.

What you can expect to know

Over the past few decades, three-dimensional (3D) cultures or spheroid emerged as a promising cancer model. Spheroids are the best mimics of solid tumors in vivo as they exhibit the characteristics of solid tumors such as cellular heterogeneity, diffusion-limited distribution of oxygen and nutrients, cell–cell signaling, growth kinetics, cell–cell interactions, and therapeutic resistance to treatment modalities. Based on the cancer cell sources and preparation protocols, four major types of spherical cancer models have been proposed/exists, namely, the multicellular tumor spheroid (MCTS) model, tumorospheres, tissue-derived tumor spheres, and organotypic multicellular spheroid (MCS). MCSs are generated from single-cell suspension culture of permanent cancer cell lines in a medium supplemented with fetal bovine serum and lacking any form of exogenous extracellular matrix (ECM). Tumorosphere is a model of cancer stem cell expansion in which spheroid develops from a single cancer stem/progenitor cell. The tumorosphere formation assay allows researchers to assess that whether a single-cell possesses the potential to both initiate and maintain tumors in the absence of cellular interaction and adhesion. Tissue-derived tumorospheres are generated from tissue remodeling and compaction after partial dissociation. Organotypic MCSs can also be termed as biopsy spheroid as it involves culturing of cut and minced tumor fragments without dissociation. This model has very close resemblance to tumor tissue (Weiswald et al., 2015).

The major focus of this chapter is on MCTSs, the best described 3D tumor model that offers an excellent in vitro screening system that mimics to a great extent the microenvironment prevailing in the tumor tissue, supporting studies on tumor-specific processes like angiogenesis, invasion, and metastasis, as well as assessment of responses to various therapies and underlying mechanisms.

Developed nearly 30 years ago as an alternative and advanced in vitro model to monolayer cultures, their role as a part of a high-throughput, cell-based assay system in drug discovery is gaining considerable

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importance. Spheroids bridge the gap between monolayers and animal tumors, facilitating mechanistic studies and evaluation of anticancer therapies, particularly relevant to solid tumors. The distinct possibility of establishing MCTS from primary tumor cells and also coculturing with different normal cells enhance the value of MCTS in cancer research and drug development. Recent developments in the generation of novel MCTS systems that allow analysis of a vast number of parameters can be conveniently employed in the high-throughput screening system with cell-based assays, thereby significantly shortening the time required for translational research, bridging the gap between discovery and clinical application. Thus, spheroids have the potential to enhance predictability of clinical efficacy and may minimize, if not replace, animal studies to a large extent in the near future. Together with novel and emerging tools of biotechnology, this 3D in vitro model is expected to substantially reduce the cost of new drug discovery.

History and methods

Introduction

Model systems play an important role in biomedicine, as they form the backbone of translational research by bridging the gap between basic concepts and discovery, with applications in the management of diseases involving diagnosis, prognosis, and therapy. In the field of oncology, they are useful in therapeutic screening, in preclinical evaluation, and to even study the basic biology of tumors. It is generally observed that the degree of complexity of various models available and deployed in experimental oncology bear an inverse relationship with the level of predictability for either diagnostic purposes or therapeutic evaluation (Khaitan et al., 2006b). In vitro cell cultures are important experimental tools in understanding the biology of neoplastic cells, as well as for the evaluation of potential therapeutic agents, and understanding the mechanisms underlying their actions. They can be easily created and manipulated and hence help in the systematic studies of multicellular systems. Among the in vitro models of tumors, two-dimensional (2D) models such as monolayers and suspension cultures have been used widely to study various aspects of tumor biology. However, in 2D cell culture, cells adhere, spread, and grow on the plastic surface and form unnatural cell attachments to proteins that are deposited and denatured on this synthetic surface. The abnormal cell morphology in 2D culture influences many cellular processes including cell proliferation, differentiation, apoptosis, and gene and protein expression (Edmondson et al., 2014). Therefore, extrapolation of findings from these models has limited value in clinics, as only a fraction of the tumor cells generally develop fully into a cell line and do not necessarily reflect the primary tumors from which they were derived. Most importantly, they lack 3D architecture and host tissue microenvironment, which are critical features of tumors (Khaitan et al., 2006b; Vinci et al., 2012). Therefore, there has been considerable amount of effort to develop and deploy various 3D in vitro culture systems (organ culture, spheroid culture) that are associated with enhanced reliability and predictability for clinical efficacy and to minimize studies with animal models (Khaitan et al., 2006b). It is very well realized that cell-based models used in experimental studies need to recapitulate both the 3D organization and multicellular complexity of the tumor as well as the organs to translate findings from these studies into clinical applications. In the 3D spheroid cell culture, cells develop contacts with each other and form natural cell-to-cell attachments. The new cells and the extracellular matrix that they synthesize and secrete in three dimensions are the natural materials to which cells are attached. In this 3D cell culture environment, cells can exert forces on one another and can move and migrate like in vivo. These cell-to-cell interactions in 3D cell cultures also include gap junctions, which directly couple one cell to another. Therefore, 3D cultures have been utilized in biomedical research since the first half of the 20th century to gain deeper insight into the mechanisms of organogenesis and expression of malignancy. However, only a small number of 3D model systems are sufficiently well characterized to simulate the pathophysiological cellular microenvironment in a tumor or to reconstitute a tissue-like cytoarchitecture, with cell-to-cell and cell-to-matrix interactions, growth, differentiation, and therapeutic responses similar to tumors in vivo. Organ culture is one of the 3D cultures, where small pieces of tumor explants are cultured in a moist gas or air phase on the surface of a relatively large volume of stationary nutrient medium (to retain the original structural relationships and differentiation of the tissue organization) and can be used to study the interactive function and the effect of drugs and other agents (Dwarakanath et al., 1985, 1987; Lasnitzki et al., 1992). However, lack of characterized reference stock, limited availability, and high variability, as well as ethical restrictions (at least with respect to human tissue), have limited the use of organ cultures.

Multicellular tumor spheroids

Spheroid models are sphere-shaped cell colonies that permit growth and functional studies of diverse normal and malignant tissues. The growth of

spheroids from tumor cells mimics the growth of naturally occurring human tumors, as their extracellular matrix and network of cell to-cell and cell-to-matrix interactions are similar to in vivo conditions and differ from the corresponding monolayer cultures (Khaitan et al., 2006b). Unlike traditional two-dimensional cell culture, 3D cell culture/spheroid does not grow in monolayer. In 3D culture cells rather grow into aggregates to form a high cell-density and closely packed, 3D tumor like structure. This densely packed cell-spheroid creates severe diffusion limitation for glucose and oxygen, mimicking a solid tumor perfectly (Khaitan et al., 2006a). Moreover, spheroids are also heterogenous in their composition, with external layer of highly proliferating cells. The high rate of proliferation is attributed to the constant exposure of cells to oxygen and nutrients in this layer. Once, the nutrients and oxygen diffusion starts to become a limiting factor, the rate of growth starts to decrease and cells begin attaining senescence, making a middle region of senescent cells. Furthermore, depletion of oxygen and glucose creates hypoxia, which ultimately leads to the formation of necrotic core. A pH gradient is also a remarkable feature of spheroid just as in solid tumors. The necrotic core of spheroid is the zone of lowest pH (6.5−7.2) owing to the conversion of pyruvate to lactate by cells in the hypoxic environment. Formation of spheroid has a growth pattern, which is the characteristic of solid tumors, beginning with an exponential volume increase until a diameter of 200−500 μm is achieved. After attaining a diameter of the definite size, the volume growth starts to decrease until a growth plateau is reached. This growth profile is essential for the establishment of multilayered organization of spheroids (Costa et al., 2016).

Various possibilities of manipulating the spheroid environment using different techniques for their generation has not only provided insights into the complexity of tumor physiology but also facilitated research efforts in developing novel therapeutic agents (Khaitan et al., 2006b). This model was adopted to cancer research several decades ago by Sutherland and his coworkers and has since then considerably contributed to our knowledge regarding various biological mechanisms of tumors, as well as in studies related to cellular response to diverse therapeutic interventions (Hirschhaeuser et al., 2010). Over the years, a wide variety of techniques have been developed for the cultivation of spheroids that span from simple culture systems using Petri dishes to novel methods like hanging drop, spinner culture, roller bottle culture, scaffold culture, microstructure-based cultures, etc., that generate spheroids from a wide variety of tumor and normal cells. More recently, microfluidics-based microtechnologies have been developed for the production of uniform tumor spheroids, which are capable of generating spheroids from diverse tissue origins that can be used to model various types and stages of cancers (Hirschhaeuser et al., 2010). Schematic diagrams illustrating some of the widely used approaches and methods for establishing homologous and heterologous spheroids are shown in Fig. 13.1.

Several well-established methods can be used to assess the effects of various therapeutic agents on spheroids, including the measurement of spheroid volume and growth, which follows a Gompertz function, similar to the growth of experimental tumors in animals (Khaitan et al., 2006a, 2006b). A combination of many analytical imaging techniques, autoradiography, the tunnel assay, bioluminescence imaging, and microelectrode-based oxygen analysis, has revealed concentric arrangement of cell proliferation, viability, and the micromilieu in large spheroids (Hirschhaeuser et al., 2010). Frozen or chemically fixed spheroid sections can be investigated for antigen expression by using various microscopy and immuno enzymatic techniques, while cells from dissociated spheroids can be used to analyze the information at the single-cell level (Khaitan et al., 2006b). Spheroids produced from various tumor cell types have been used to study responses to various treatment modalities like radiation, chemotherapy, hyperthermia, immunotherapy, and a combination of therapeutic interventions. Spheroids, as in naturally occurring tumors, develop cell-to-cell communication, numerous communication channels including gap junctions, desmosomes, and electrical coupling, which have provided insight into the molecular mechanisms regulating cell proliferation and differentiation in tumors (Mueller-Klieser, 1997). Fig. 13.2 illustrates various approaches currently employed using MCTS in evaluating the influence of various tumor-associated parameters on the in vivo response of tumors. Coculturing normal cells with malignant cells within a spheroid can provide information on tumor invasion and angiogenesis. Growing spheroid tumor aggregates with matrigels also provide valuable information about the metastatic property of tumor cells. Although MCTS offers many advantages over the monolayer cell cultures by mimicking in vivo conditions of the tumor to a very great extent, it does not eliminate the use of animal models as it has certain limitations. For example, pharmacokinetics, pharmacodynamics, and bioavailability of drugs cannot be studied using MCTS. Furthermore, the influence of the immune system on the systemic response of the organism can be evaluated only using appropriate animal models. Therefore, MCTS models cannot totally replace the testing of complete biological mechanisms relevant to the drug development. The concentric geometry of spheroids is widely used as a model of

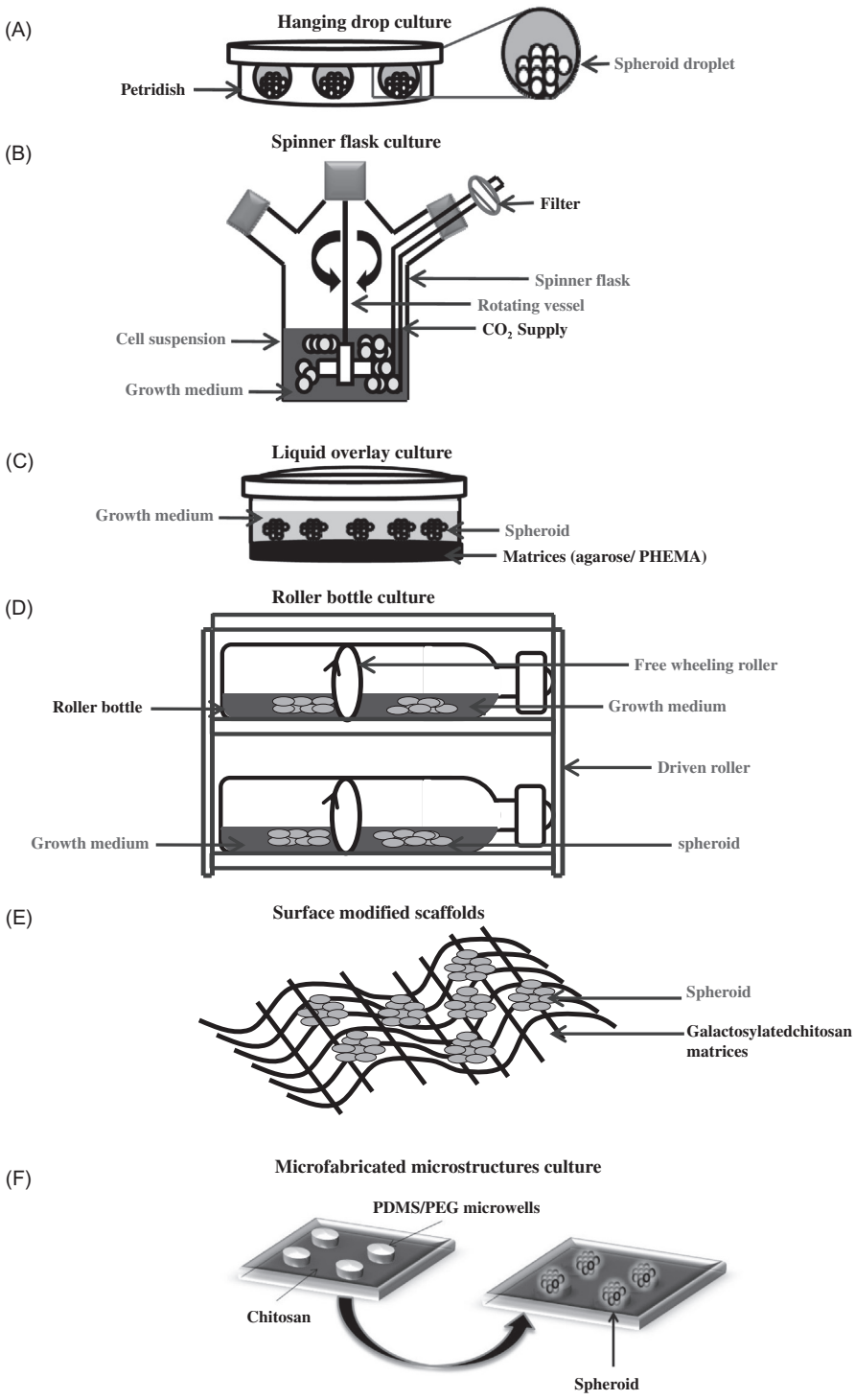


FIGURE 13.1 Different methods for the generation of multicellular tumor spheroids (MCTS).

diffusion-limited tissue with central necrosis, which develops when diffusion of oxygen becomes limited. The modes and mechanisms of cell death in tumor spheroids appear to be complex and involve multiple mechanisms. The three vital parameters for quantification of cellular viability in spheroids (the diameter of the spheroid, the diameter of the necrotic core, and the viable rim thickness) are different in different types of spheroids and depend on culturing conditions. The viable rim thickness may gradually increase or decrease with the expanding spheroid diameter, thereby indicating that different mechanisms are involved in the onset and expansion of the central necrosis in different spheroid types (Khaitan et al., 2006a).

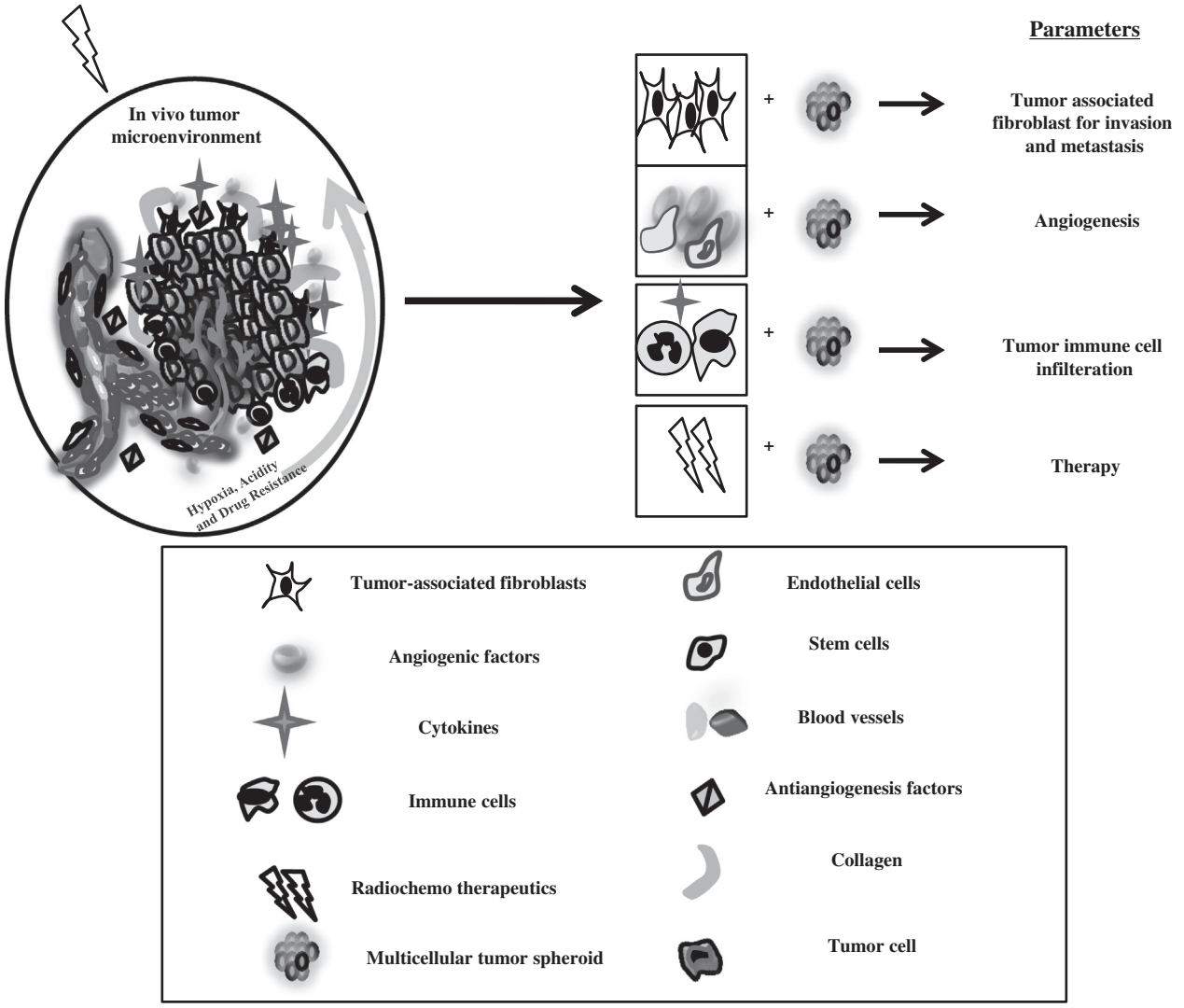


FIGURE 13.2 Approaches for studying the effects of tumor associated parameters on the in vivo response of tumors using multicellular tumor spheroid (MCTS)

Both multiple foci of necrotic (dead) cells confined not only to the central region and the presence of apoptotic cells have been found in spheroids similar to in vivo tumors (Khaitan et al., Khaitan et al., 2006a, 2006b). Since the mechanisms of apoptosis differ in several ways from that of necrosis, these could be independent predictors of cellular sensitivity to a particular therapeutic agent, and monitoring the level and mode of cell death may be useful in modulating treatment or in predicting the response of tumors to treatment (Khaitan et al., 2006b; Hirshhaeuser et al., 2010). Various parameters associated with the spheroids (such as growth, viability and cell survival, cell death, metabolic and mitochondrial status, gene expression status, levels of proteins, oxidative stress, and glutathione levels) influence the end results of in vitro studies aimed at understanding tumor response to various cytotoxic agents and metabolic inhibitors (Khaitan et al., 2006a). In culture, these parameters vary as a function of age and spheroid size, similar to the status in growing tumors. An exhaustive characterization in spheroids generated from a human glioma cell line clearly showed significant differences between monolayer cultures and spheroids at different ages with respect to many of the parameters (Khaitan et al., 2006a). Therefore, results obtained with any given tumor model must be interpreted carefully, and extensive characterization of the model is necessary before extrapolating responses from spheroids to therapeutic responses. Furthermore, since spheroids can also be cultured from primary cells of tumors, which retain to a very great extent the

biological as well as the metabolic behavior of the tumor, detailed characterization coupled with drug responses using appropriate parameters will facilitate the optimization and interpretation of predictive therapy. Tumor spheroid cultures have been widely used in experimental radiotherapy, photodynamic therapy (PDT), hyperthermia, chemotherapy, and target-specific approaches, as well as other contemporary and emerging therapies, such as antiangiogenesis therapy, gene therapy, cell- or antibody-based immunotherapy, etc. Evidence in the literature suggest that many treatments are expected to be less effective in the 3D pathophysiological environment. For example, 5-fluorouracil (5-FU) has higher antiproliferative effects on 2D cultures but not in spheroids. On the other hand, the hypoxia-activated drug tirapazamine is more effective against 3D cultures (Tung et al., 2011). Therefore, spheroids are most frequently considered as appropriate tools for evaluating drug candidates with enhanced tissue distribution and efficacy and can also be used for negative selection to reduce animal testing. Indeed, most therapeutic approaches were found to be less effective in 3D cultures than in 2D cultures. However, this cannot be generalized because some potential targets, especially signaling pathways, or even exclusively, play a role in the 3D environment or milieu (Barbone et al., 2008). Consequently, the spheroid model has also been increasingly recognized as a primary tool for positive selection in innovative drug development initiatives.

Genome-wide gene expression analysis in spheroids formed by human malignant gliomas and primary porcine hepatocytes has indicated that several genes express differently during spheroid formation and resemble the tissue of origin more closely than the monolayer. Moreover, comparative transcriptomic studies in cells from epithelial ovarian cancer, hepatocellular carcinoma, and colon cancer show that numerous genes associated with cell survival, proliferation, differentiation, and resistance to therapy are differentially expressed in cells grown as MCSs (compared to monolayer cultures, which mainly regulate the drug response in tumor cells). The expression profiles more closely resemble the profiles of the respective tumor tissue in vivo and are thus highly relevant for the establishment and testing of novel therapeutic interventions (Lin and Chang, 2008).

Historical facts toward the development of tissue culture technology from 2D and 3D cultures

In vitro 2D cultures of animal cells were first grown in the 20th century. In 1912, Alexis Carrel maintained chick heart cells in drops of horse plasma for the first time. After a few days, death of the explants was observed due to exhaustion of nutrients. Cells from a given explant could be maintained indefinitely if they were periodically subdivided and fed with a sterile aqueous extract of whole chick embryos. In the early 1950s, Earle used trypsin to dissociate the cells of a whole chick embryo. When this suspension of single cells was mixed with plasma and embryo extract and placed in a sterile glass container, the cells adhered to the glass and divided to form a primary culture. The primary culture contained a variety of cell types including macrophages, muscle fibers, etc. In 1952, Gey established a continuous cell line from a human cervical carcinoma known as HeLa (Henrietta Lacks) cells. In 1961, Hayflick and Moorhead isolated human fibroblasts (WI-38) and showed that they have a finite lifespan in culture. In 1965, Harris and Watkins were able to fuse human and mouse fibroblast cells using viruses. Simultaneously, Holtfreder and Moscona pioneered the field of biomedical research by their observations on morphogenesis in spherical reaggregated cultures of embroyonic or malignant tissues. However, it was not until the early 1970s that Sutherland and coworkers systematically investigated the response of tumor cell aggregates to antineoplastic therapy. Because the cell lines formed nearly perfect sphere-shaped aggregates, they were called "spheroids." As a consequence, a number of investigations were simulated on the studies of basic biological mechanisms [such as the regulation of proliferation, differentiation, cell death, invasion, cell−cell interaction, cell−matrix interaction, angiogenesis, or immune response (Mueller-Klieser, 1997)] and structural similarity to human tumors on a large scale. Thereafter, advancement in tissue culture technologies for the development and maintenance of 3D cultures has always been helpful in therapeutic applications.

Examples where 3D culture is more beneficial over 2D culture

Some of the advantages and limitations of 3D culture over 2D cultures are summarized in Table 13.1. For example, studies with thyrocytes (thyroid epithelium cells) by Manuchamp demonstrate that the loss of follicular organization in conditions of the monolayer culture results in the loss of polarization of thyrocytes (Mauchamp et al., 1998). This leads to considerable decrease in the ability to capture and transport iodine because there are decreased intercellular contacts and connections to the basal membrane. Similar modifications were observed for the cells of other organs and tissues, including mammary gland, kidneys, etc. Therefore, cultivation in three-dimensional conditions

TABLE 13.1 Advantages and limitations of 2D and 3D culture models.

Cell culture	Merits	Limitations
2D culture (monolayer and suspension culture)	• Absolute control on cell environment. • Cell observation, measurement, and manipulation are easier	• Cells lose their histological organization, polarity, and differentiation • Lack of host–tissue microenvironment • Altered gene expression and growth characteristics due to a deficiency in cell–cell and cell–matrix interactions. • Increased drug sensitivity. • Use of suspension culture is also limited by the sensitivity of some cell lines to shear stress
3D culture (organ and spheroid culture)	• Sufficiently well characterized to simulate the pathophysiological cellular microenvironment • Show enhanced reliability and predictability of clinical efficacy and minimize studies with animal models • Reconstitute a tissue-like cyto architecture with cell–cell and cell–matrix interactions, growth, differentiation, and therapeutic responses similar to tissue *in vivo*. • Gene expression profiles of 3D model reflect clinical expression profiles of tumors	• Lack of vasculature, host–immune interactions • Diffusional transport limitations: O2 and other essential nutrients may not reach all of the cells; accumulation of toxic waste products within scaffold space

TABLE 13.2 Advantages, limitations and applications of different methods of spheroid generation

Method	Applications	Advantages	Limitations	References
Hanging drop	Useful for studying tumor physiology, metabolism, toxicology, cellular organization, and development of bioartificial tissue	Affordable Spheroids uniform in cell number, size, and compositions Coculture of different cells	Labor intensive Difficulty in high-scale production	Vinci et al., 2012 Kelm et al., 2003
Soft agar liquid overlay	To manufacture 3D aggregates for studying tumor and fibroblast interactions and their role in tumor development	Affordable No shear stress Easy to set up	Spheroids formed are nonuniform in cell number, size, and shape Limited mass transfer Limited cell survival	Vinci et al., 2012, Yuhas 1977
Rotating wall vessel (NASA bioreactor)	Useful for the production of large number of spheroids	Maintain cells in under low shear stress Provide constant culture conditions Efficient mass transfer Efficient for long term maintenance of tissue-like functions and cell viability	Not useful for drug testing Expensive and require special setup Nonuniform cell size and number	Vinci et al., 2012 Ingram et al., 1997
Spinner flask/ roller bottle/ gyratory shaker	To generate large number of porcine hepatocyte spheroids with a concentration higher than 500 MCS/mL	Simple to perform Efficient mass transfer Increase cell viability and allow long-term culturing Coculture of different cell types	Intermediate to high shear stress Difficult to use at large scale	Vinci et al., 2012
Microfabricated microstructures	Useful in generating 3D liver or stem cell spheroidsHigh-throughput drug screening, real time imaging of cellsCoculture of different cells	Uniform and well-controlled spheroid size, cell number, and size Spheroids on chip	Requires specialized facilities	Hirschhaeuser et al., 2010 Dean et al., 2007
3D scaffolds	Tissue engineering and bioartificial liver Serve a better *in vitro* 3D system for screening cancer therapeutics	Provides 3D support Easy to set up	Expensive	Fischbach et al., 2007 Glicklis et al., 2000

was suggested to solve these issues. Cancer cells grown in 2D can be killed easily by low doses of chemotherapeutic drugs or low doses of radiation. However, same cells grown in 3D show resistance to the same doses of chemotherapeutic drugs or radiation, just like tumors in the body (Khaitan et al., 2006b). Therefore, 3D models are more valid targets for testing and discovering new drugs for cancer. Another benefit of 3D model over 2D is that cells in 2D form a monolayer of cells surface, where the drug needs to diffuse very short distance. However, 3D form multilayers of cells and drug molecule need to diffuse across multilayers of cells to reach to every cell in a spheroid. Moreover, cells grown in 3D will form natural barriers to drugs such as tight junctions that bind cells tightly together and block or slow the diffusion of drugs, making this model relatively more realistic for testing drugs. Therefore, more accurate results can save the resources and time involved in drug development and move drugs from the bench to bedside much faster.

Techniques for the generation of spheroids

Success in the generation of spheroids has been possible due to availability of simple and reproducible techniques on spheroid-based applications. While generating a multicellular spheroid, it is essential to prevent cells from being attached to the culture ware substratum. Number of techniques have been developed to culture and grow MCS from cell suspension, and Table 13.2 summarizes the advantages and limitations of some popular techniques that have been described for the generation of spheroids and that widely differ on the basis of spheroid size, cell specificity, efficiency in production, influence on cellular physiology, convenience, and suitability for subsequent applications. Some of the methods for spheroid generation are briefly discussed in this chapter.

Hanging-drop method

The hanging-drop method was primarily developed for culturing the in vitro aggregation of embryonic cells for studying morphogenesis and tissue formation. The technique does not require the coating of plates for spheroid formation, but it is useful only for short-term culturing. This is a very simple method in which roughly 4000 cells in volume of 40–50 μL are placed as a hanging drop onto the underside of the lid of a tissue culture dish. When the lid is inverted, the drops are held in place by surface tension and the microgravity environment in each drop concentrates the cells, and thereafter, these drops are incubated under physiological conditions until they form true 3D spheroids, in which cells are in direct contact with each other and with extra cellular matrix components at the free liquid–air interface (Kelm et al., 2003). The method requires no specialized equipment and is useful for generating MCS of defined sizes and cell numbers. In addition, spheroids generated by this method can be either embedded in matrigel to study angiogenesis or various viability assays like 3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyltertazolium bromide (MTT) (Khaitan et al., 2006b). This method has also been applied to the cocultivation of mixed cell populations, including the cocultivation of endothelial cells and tumor cells as a model of early tumor angiogenesis. Efficiency of MCS formation by this method can be enhanced using cell–cell cross-linking agents such as cell matrix proteins, for example, collagen, fibronectin, the synthetic polymer Eudragit, anti-B1 integrin monoclonal antibody, and poly (lactic-co-glycolic acid).

Liquid overlay method

In this method, bacteriological or ELISA 96-well plates are used to generate MCSs. Since bacteriological plates may not always promote MCS formation due to the lack of an appropriate surface for cell attachment, agarose–Dulbecco's modified eagle medium (DMEM)-coated plates have been used to generate MCS (Yuhas et al., 1977). This improvisation not only provides attachment of cells to the surface but also offers a nutritional requirement from the agar–Eagle's Basal Medium (EBME) combination for the growth of spheroids. Interestingly, other hydrophobic polymers, including poly (2-hydroxyethl methacrylate) or poly-*N*-*p*-vinylbenzyl-D-lactonamide, can also be used in place of agar (Tong et al., 1992). The advantage of this method is that it is inexpensive and simple to perform and provides heterogeneous size, cell number, and shape of spheroids.

Microfabricated microstructures method

Advancement in the semiconductor industry has given rise to microfabrication techniques that have been adopted for applications in life sciences. The techniques involve the use of soft lithography in the construction of polydimethylsiloxane (PDMS)- and polyethylene glycol (PEG)-based microstructures. These structures can be used for biomaterial microprinting and microfluidics. One of the greater applications of microfabricated techniques is in generating MCS with uniform size, cell composition, and geometry. The apparatus consists of a microfabricated device, namely, microwells that are loaded with suspended cells, redistributed by gravity and hydrodynamic forces, and finally assembled into aggregates based on

microwell geometry. With varying diameter and geometry of microwells, different shapes and types of spheroids can be generated, such as rods, tori, or honeycombs (Dean et al., 2007). This technique has potential for the mass production of tumor spheroids to generate 3D liver or stem cell spheroid arrays for high-throughput drug screening and in 3D coculture to investigate the effect of carcinoma-associated fibroblasts on cancer cell invasion.

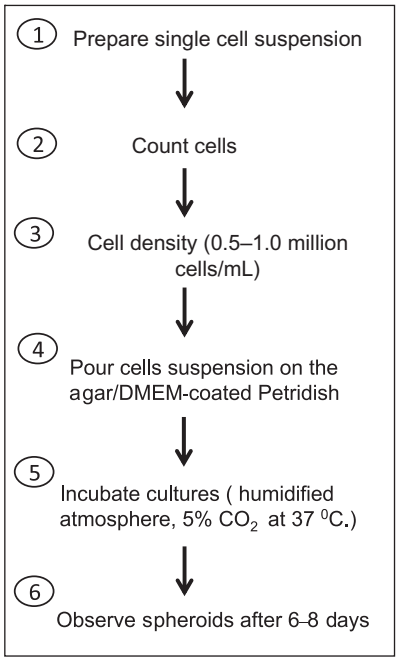
Rotatory flask methods

The spinner flask method is also one of the most attractive and robust approaches for the generation of large numbers of spheroids of defined size ranges under optimum growth conditions. Here, tumor cells aggregate to form spheroids in the flask without attachment to any other substrate. Spheroids of different diameter can be generated by optimizing cell seeding density, composition of medium, culture time, and spinning rate. For example, Sakai et al. (1996) used the spinner flask method for the production of porcine hepatocyte spheroids, which has great potential in the generation of bioartificial liver (BAL). In addition, the presence of Eudragit as an artificial matrix promotes hepatocyte cell aggregation, which further enhances liver function by preventing damage to the cells due to agitation. The spinner flask method generally provides high shear stress, which has an effect on cellular physiology. To overcome this problem, the National Aeronautics and Space Administration has developed a rotating wall vessel that provides a constant supply of medium and a constant nutrient flow rate, thus resulting in better differentiation and formation of multicellular aggregation (Ingram et al., 1997). Similarly, roller bottles and gyratory shakers are also used for the generation of large amount of spheroids to avoid effects caused by stagnant medium. This system provides low shear stress due to high surface-to-volume ratio, which allows gas exchange at an increased rate, and thus better constant and gentle agitation.

The merits of rotatory culture methods are as follows: they are simple to perform, they can be scaled to massive production and set up as long-term cultures, there is dynamic control on culture conditions, and coculture of different cell types is possible. The demerits are the variation in cell size and number and that special equipment is required.

Surface modification-based methods

A variety of modified substrates are also available for the generation of spheroids. For example, polyvinyldifluoride surfaces coated with galactose-tethered pluronic polymer are useful for hepatocyte attachment; their spheroids generate with high cell viability and enhanced functional maintenance (Du et al., 2007). Similarly, the thermoresponsive polymer, poly-*N*-isopropyl acrylamide (PNI-PAAm), in conjugation with collagen as modified substratum, can be used for the culture of human dermal fibroblasts. As PNI-PAAm solubility is dependent on the low critical solution temperature, changes in temperature can result in the formation of spheroids from monolayer cultures. Spheroids generated via these techniques are highly suitable for tissue reconstruction (Takezawa et al., 1990). Likewise, highly porous (sponge-like) scaffolds are useful in generating immobilized 3D arrangements of hepatocyte spheroids by providing a favorable environment and enhancing their aggregation. The scaffolds are generally made up of biomaterials such as alginate, gelatin, hyaluronan, or an alginate/galactosylated chitosan hybrid polymer. The use of scaffolds facilitates performance of implanted hepatocytes by enabling their aggregation and reexpression of differentiated function before implantation (Fischbach et al., 2007). These porous 3D scaffolds are useful in liver tissue engineering and BAL. Seeding primary epithelial cells and certain immortal epithelial cell lines (e.g., MCF-10A, DU 4475) on matrigel promotes the generation of monoclonal spheroids, which are useful for understanding the mechanism of epithelial morphogenesis.



FLOW CHART 13.1 Flow chart for the preparation of spheroids by liquid overlay culture method.

Chip-based spheroid generation

This is a more efficient method where numerous spherical multicellular aggregates (spheroids) are generated with nearly the same diameter on a microfabricated chip. These spheroids mimic real tissue morphology. Generally, the chips are made up of silicon and elastomeric microchannels with various cavities of 100−500 μm in diameter. Cells are loaded by a microfluidic channel into a silicon microchip or microwell and thus form a localized, single spheroid within each microstructure. This method is most suitable for various biomedical applications such as cell-based biosensors for toxicological and pharmacological examinations and in BALs (Kunz-Schughart et al., 2004).

Emerging technologies for the generation of spheroids

Currently, many new technologies are emerging that promises high throughput, better reliability, and simplicity to facilitate a wide variety of applications of multicellular spheroids (MCS) in the area of biomedical sciences. A new high-throughput and simple fabrication methods has been recently described that uses 3D acoustic tweezers platform based on the surface acoustic wave (SAW) cell manipulation methods (Chen et al., 2016). This technique uses a combination of drag force for microstreaming cells in the vertical direction, while a radiation force from Gor'kov potential aggregates cells in the horizontal plane. It has a remarkable high-throughput capacity by which more than 300 size-controllable spheroids can be created and transferred to the target container (like Petri dishes) every hour. This high-throughput system is likely to make a big impact on the drug development and discovery besides finding use in biomedical research and tissue engineering. A microfluidic technique that combines T-junction droplet generation and external gelation has also been recently developed that uses cell-laden alginate droplets in PDMS microfluidic devices (Sabhachandani et al., 2016). A continuous growth model of heterogeneous MCTSs has been recently generated that takes into account a diffusing nutrient from the surrounding facilitating both the proliferation rate and the mobility of tumor cells (Giverso and Ciarletta, 2016). A microplate technology coupled with the CellTiter-Glo 3D Cell Viability Assay has been recently developed that generates uniform and reproducible single spheroids in every well of a multiwell plate, thereby allowing rapid generation and assessment of 3D MCTSs in the same well (Vinci et al., 2012).

The issue of reproducibility in observations made from 3D (spheroid) models has been recently addressed using a novel open-source software capable of performing an automatic image analysis of 3D tumor colonies (Zanoni et al., 2016). These studies have shown that a number of parameters related to the shape and volume of the spheroids affect the response of large spheroids to different treatments. This emphasizes the importance of the preselection of spheroids of homogeneous volume and shape to enhance the reproducibility and reliability of results obtained from studies employing MCTSs.

Protocol for tumor spheroid generation

1. Thaw tumor cells from frozen stocks and subculture for >1 and <20 passages. Use standard medium for routine culturing. Keep cultures in a humidified atmosphere, with 5% CO_2 in air at 37°C.
2. Prepare single-cell suspensions by mild enzymatic dissociations using trypsin/EDTA solution. Transfer stock cultures every third to fourth day by seeding an appropriate number of cells in tissue culture flask, depending on the cell type and doubling time of cells.
3. To generate spheroids of 600 μm diameter, inoculate 0.01 × 106 viable cells of any cancer cell line in a 96-well plate, noncoated or coated with agarose in 0.2 mL DMEM-low glucose dextrose media supplemented with 5% fetal calf serum, antibiotics, and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid. Clusters of cells should be observable after 24 hours of initiation, but it will take nearly 4 days for these clusters to form spheroids. Individual spheroids can be used to monitor and analyze spheroid integrity, diameter, and volume under phase contrast microscope. The pH of the medium should be monitored daily to prevent acidosis.
4. These spheroids can be then further used to monitor the efficacy of drugs.

Application of the protocol: MCTSs of human glioma cell line (BMG-1) were used to understand their behavior, particularly related to the metabolism and radiation response in their application to anticancer therapy (Flow Chart 13.1) (Khaitan et al., 2006a, Khaitan et al., 2006a, 2006c).

Drug treatment protocol

1. For drug treatment, dilute the drugs in standard medium (e.g., dimethylsulfoxide, ethanol) at 2 × final concentration just before use.
2. Treat growing spheroid cultures by replacing 50% (100 μL) of the supernatant with drug-supplemented

standard medium. Simultaneously, replace medium of untreated reference spheroid cultures with solvent-containing or solvent-free standard medium.

3. Incubate cultures for a definite treatment time in a humidified atmosphere with 5% CO_2 in air at 37°C.

Parameters to monitor drug efficacy in 3D cultures

Different parameters can be used to monitor the effect of drugs on these 3D cultures. For example, cell viability can be determined by the use of an 3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyltertazolium bromide assay, analysis of cell cycle kinetics can be done by bromo-deoxy-uridine (Brdu) labeling assay, and the percentage of apoptotic cells can be measured by a Annexin V labeling assay.

Radiation response of tumor cells and its modifications

Ionizing radiation plays an important role in the management of a majority of malignancies. Although many tumors like gliomas and several carcinomas are known to be refractory to radiotherapy with marginal benefits in survival, approaches that help in individualizing therapeutic designs based on appropriate predictive assays can improve the efficacy of these therapies. MCTSs provide a useful predictive platform for the evaluation of tumor response to radiotherapy and/or chemotherapy, as well as adjuvant like radiosensitizers and chemosensitizers including metabolic modifiers and other modifiers of biological response (Khaitan et al., 2006a, 2006b). Radiation-induced cytotoxicity is associated with two mechanistically and morphologically distinct forms of cell death that contribute to the loss of clonogenicity. Programmed cell death (apoptosis), characterized by distinct membrane alterations and DNA degradation, is induced by both extrinsic and intrinsic pathways. On the other hand, mitotic death, linked to the cytogenetic damage expressed as chromosomal aberrations in the metaphase and manifested as micronuclei formation in the postmitotic daughter cells, arise from residual DNA damage following induction and repair of DNA lesions. Mitotically dead cells also undergo apoptosis as a secondary response referred to as delayed apoptosis, which may be generally observed 1 to 2 days after irradiation. The in vivo response of cancer cells to treatment may, in fact, be simulated in spheroids more closely than in conventional monolayer cultures, as many microenvironmental factors that contribute to the tumor cell response are better simulated in the spheroids. Since hypoxic areas in naturally occurring tumors may limit curability, one promising approach utilizes agents that label the hypoxic fraction, which has been well demonstrated using spheroids (Franko, 1985); many sensitizing agents have been developed using spheroids for screening and testing (Hall, 1988). However, the clinical efficacy remains to be determined. A more recent study provides a mathematical analysis of the radiation response of tumor cells, taking in to account oxygen status with the help of spheroids (Bertuzzia et al., 2010). Radiation produces a variety of lesions in the cell. These lesions induce lethal damage in a fraction of cells (clonogenically dead cells), which lose the capability of unlimited proliferation and die, after some cell cycle delay, at a subsequent time. Thus, after irradiation, the living tumor cell population will be composed by a subpopulation of intact, viable cells and a subpopulation of live but lethally damaged, clonogenically dead cells. The death of lethally damaged cells may occur by premitotic apoptosis or after one or more cell divisions (postmitotic apoptosis). The response of spheroids to irradiation can be performed on single cells obtained after disaggregation of these spheroids, or on intact spheroids, using elimination (disintegration) of spheroidal structure and growth delay as end points. Clonogenic cell survival is difficult to perform on spheroids of human tumor cells, but the cells show tumor type dependent radiation responses and offer an approach for comparison of radiosensitivity of tumor cell lines of different histologic origin (Bertuzzia et al., 2010). Because of the oxygen concentration gradient, the potential lethal damage to sublethal damage ratio changes with the radial distance from the spheroid boundary, leading to radioresistance in spheroids, similar to the scenario in tumors. Therefore, radiation response using spheroids has played an important role in generating new ideas for combining radiation with drugs that can amplify the effects of radiation (radiosensitizers, biological response modifiers, etc.) to achieve the enhanced therapeutic gain in radioresistant tumors. Since tumors with high glucose usage are generally resistant to radiotherapy and chemotherapy, it has suggested that glycolytic inhibitors can selectively enhance the effect of radiation and chemotherapeutic drugs (Jain, 1996). Indeed, studies using monolayer cultures of several human and murine tumor cells have shown that the glycolytic inhibitor, 2-deoxy-D-glucose (2-DG), enhances radiation and chemotherapeutic drug-induced toxicity that involve multiple mechanisms (Dwarakanath, 2009); however, heterogeneous responses are observed in local tumor control, tumor-bearing mice, suggesting contributions of

tumor physiology and variations in host factors (Gupta et al., 2009). Interestingly, spheroids (which are relatively more radioresistant) have been found to be sensitized to a greater extent by 2-DG: a nearly threefold (~300%) increase was observed in the radiation-induced cell death, compared to an increase of only 40% in monolayers under similar conditions (Khaitan et al., 2006c). Furthermore, the radiosensitization by 2-DG in spheroids was primarily due to enhancement of apoptosis, while enhanced cytogenetic damage was the major contributing factor responsible for cell death in monolayers. The differential mechanisms underlying radiosensitizing effects of 2-DG in spheroids and monolayers are consistent with the idea that these processes are regulated by different proteins and depend on different signaling pathways, which may be different in these two models. Many studies have also shown enhanced radiosensitization by chemotherapeutics (namely, tumor necrosis factor, doxorubicin, misonidazole) in multicellular spheroids compared to monolayer cultures (Wen-Hong et al., 2012). Thus, it is apparent that predictions and/or evaluation of newer approaches and biological response modifiers (particularly the metabolic inhibitors) are better accomplished using MCSs rather than monolayer cultures. The MCS model has also been found to be particularly effective in evaluating combinations of various drugs and/or radiosensitizers with fractionated irradiation regimens. Transient G2 delay, induction of apoptosis, and late onset of DNA strand breaks following radiosensitization have been found to be more pronounced in the spheroids, similar to those used clinically. Therefore, MTS may prove to be a valuable tool in studying the response of human tumors to clinical exposure protocols, including hyper fractionation (Yuhas et al., 1984).

Response to anticancer drugs

Inherent as well as acquired resistance to chemotherapeutic drugs is a major obstacle that limits the success of cancer chemotherapy. Tumor response to anticancer therapeutics is influenced by many factors, including drug penetration to different parts of the tumor, the physiological status of the tumor and tumor cells (e.g., hypoxia), proliferation level (growth fraction), cell signaling status, intercellular interactions and tumor cell–extracellular matrix interaction, etc. Limited success of many chemotherapeutic drugs in clinics that have otherwise been found effective against tumor cells has been partly due to the fact that many studies on the mechanisms of drug resistance have been carried out using monolayer cultures. Due to their three-dimensional architecture, MCSs have been found to be very useful in drug sensitivity testing (Tofilon et al., 1984) as they overcome many of the limitations posed by monolayer cultures. Several studies have demonstrated that tumor cells cultured as three-dimensional spheroids respond to drugs similarly to the in vivo scenario and justify the use of this in vitro system to develop clinically useful testing for individual human cancer patients. Since spheroids also represent the heterogeneous nature of individual tumors, they can also measure the drug sensitivities of specific cell types of cultured tumors. Disruptions of intercellular interaction and inhibition of cell adhesion show significant reduction in the resistance against many drugs in tumor spheroid (Shane et al., 2004). The usefulness of spheroids established from genetically manipulated breast cancer cell lines less sensitive to apoptosis have been recently demonstrated as a high-throughput in vitro system for screening drugs (Bartholoma et al., 2005). MCTS coupled with the analysis of metabolic status has been extensively used for investigating the treatment response of tumors to chemotherapeutic drugs. More recent studies using 2-Deoxy-2-[^{18}F]fluoroglucose (FDG) measurement of glucose utilization in spheroids of breast cancer cell lines strongly suggest that the combination of positron emission tomography radiotracers and image analysis in MCTS provides a good model to evaluate the relationship between tumor volume and the uptake of metabolic tracer before and after chemotherapy (Azita et al., 2006). This feature could be used for screening and selecting PET tracers for the early assessment of treatment response of tumors.

Response to photodynamic therapy

PDT is a treatment that uses photosensitizing compounds along with light to kill cancer cells. While pioneering contributions of Finsen, Raab, and Von Tappeiner—using a combination of light and drug administration—led to the emergence of photochemotherapy as a therapeutic tool, the isolation of porphyrins and their phototoxic effects on tumor tissue led to the development of modern photodetection and PDT. MCSs represent the ideally suited model for studying the effects of PDT and to better understand tumor response to PDT without interacting with vascular systems. The use of potent photosensitizers [such as polyvinylpyrrolidone-hypericin and photosan-3] has shown high therapeutic effects on spheroids of human urothelial cell carcinoma cell line as well as U-251 Mg (Terzis et al., 1997). MCSs have also proven useful for the study of complex PDT treatment regimens and combined therapies involving PDT and ionizing

radiation or hyperthermia (Khaitan et al., 2006b). Computer simulations have been found to be very useful in modeling the growth of MCS and predict the effects of investigational therapies, including PDT.

Response to antiangiogenesis therapeutics

Development of tumors and their response to therapy depend on the ability of tumor cells to adopt to different environments and compete with normal cells for space and nutrients. This process of tumorigenesis involves the interaction of tumor cells with the microenvironment, where host endothelial cells (or various types of other cells) are recruited to contribute to the formation of tumor vasculature to provide an adequate amount of oxygen and nutrition for the growing tumor mass (termed "tumor angiogenesis"). Angiogenesis drugs act to inhibit survival of newly formed blood vessels required for tumor growth and progression.

These drugs have recently shown good activity for the treatment of breast, lung, colon, and kidney cancers. However, these drugs can be toxic and even cause death. Hypoxia is a potent inducer of vascular endothelial growth factor (VEGF). Since MCTS develop hypoxic regions similar to in vivo conditions, they are good model systems to better understand mechanisms governing induction of VEGF, angiogenesis, and tumor progression and thus provide significant therapeutic potential for antiangiogenesis and anticancer drugs. Differential steps of tumor-induced angiogenesis have indeed been studied by a novel in vitro confrontation culture of a vascular multicellular prostate tumor spheroids and embryoid bodies grown from pluripotent embryonic stem cells. These show that tumor-induced angiogenesis results in growth stimulation of tumor spheroids, disappearance of central necrosis, and a reduction of the pericellular oxygen pressure, coupled with an increase in the levels of hypoxia inducing factor-1α, VEGF, heat shock protein 27, and P-glycoprotein (Khaitan et al., 2006b). Unequivocal induction of VEGF by hypoxia has also been confirmed using spheroids of C6 rat gliomas grown under different conditions, which was reversible following re-oxygenation (Khaitan et al., 2006b). Furthermore, under stressed conditions like glucodeprivation and low oxygen, VEGF mRNA shows more than an eight-fold extended half-life compared to nonstressed conditions. To study the induction of VEGF and GLUT-1, MCSs of glioma cells have been used in which each layer is differentially stressed by in situ hybridization. These findings show that two different consequences of tissue ischemia, namely, hypoxia and glucose deprivation, induce VEGF and GLUT-1 expression by similar mechanisms. By using prelabeled tumor cell spheroids prepared from Lewis lung carcinoma, quantitative analysis of spatiotemporal development of vasculature and its influence on the tumor growth has been carried out using microscopic examination exploiting the double labeling of tumor and vasculature (Khaitan et al., 2006b). These studies clearly demonstrate that development of tumor-induced vascular network precedes the rapid growth of tumors. Tumor mass-related stress-induced studies strongly suggest that stress related to tumor mass controls tumor growth at both the macroscopic and cellular levels and may influence tumor progression and delivery of therapeutic agents. Stress-induced VEGF activity has also been demonstrated under in vivo conditions using implanted neovascularizing spheroids in nude mice. Recently, a mini tumor model in the form of a 3D spheroid system consisting of endothelial, fibroblasts, and breast tumor cell line MDA-MB-231 has been developed to study the effects of antiangiogenesis drugs on tumor growth (Sampaio et al., 2012). It was demonstrated that this model is useful; for example, Galardin (GM6001), an inhibitor of metalloproteases, showed inhibition of spheroid sprouting. Endostatin was found to inhibit angiogenesis through binding to integrin a5b1. Furthermore, this model has also been used to unravel new roles for mesenchymal MT1-MMP in regulating endothelial sprout formation (Reilly et al., 1997). Recent studies with matrigel-based mammalian carcinoma spheroids have clearly demonstrated that tumor growth may not be critically dependent on angiogenesis as long as a minimal intratumoral microvessel density is maintained (Jelena et al., 2006). Since there is a considerable interest in targeting tumor angiogenesis for therapy, MCTS can facilitate the identification of various tumor-specific factors responsible for constitutional and stress-induced neovascularization, thereby aiding the development of more effective antiangiogenesis therapies.

Evaluation of response to immunotherapy

Tumor cells interact with various host cells like fibroblasts, endothelial cells, and infiltrating immune cells (macrophages, lymphocytes, etc.) for their development. Tumor-associated immune cells play an important role in promoting or inhibiting tumor growth and progression. Since MCTS mimics the in vivo environment to a large extent, coculturing spheroids with several immune cells like monocytes, macrophages, dendritic cells, T cells, or natural killer cells is helpful in studying heterologous interactions between tumor and immune cells, as well as their cellular toxicity and therapeutic effects. Tumor microenvironment influences the differentiation of monocytes

into tumor-associated macrophages (TAM). Studies using coculture of 3D MCS of urothelial–bladder–carcinoma cell lines J82 and RT4 with human monocytes or macrophages have been performed to determine the release of cytokines levels. The tumor spheroid of poorly differentiated 82 cells stimulates the secretion of interleukin (IL)-1 beta and IL-6 and also promotes the differentiation of monocytes to TAM (Konur et al., 1998). Cocultures of urothelial bladder cell spheroids with either Bacillus-activated killer cells or IL-2 (lymphokine)-activated killer cells have shown that Bacillus-Calmette-Guérin (BCG) is an effective immunotherapy in malignant MCS tumors compared to benign MCS by reducing proliferation and enhancing the cytotoxicity in malignant MCS (Durek et al., 1999). Similarly, it has been observed that enhanced monocyte migration into fibroblastic tumor spheroids was due to high expression of CCL2 (monocyte chemotactic protein-1), which is under regulation of IL-6 compared to normal fibroblasts. Moreover, in vitro studies using spheroid adhesion assays suggest the potential role of T-regulatory cells to adhere and transmigrate through endothelial cells in tumor-derived EC spheroids (Nummer et al., 2007). However, the use of interferon-α (IFN-α) together with retinoic acid as a combinatorial immunotherapy has been shown to have a growth inhibitory effect in renal cell carcinoma (SN12C) spheroids (Rohde et al., 2004). Moreover as a part of cytokine therapy, treatment of the CD133 + colon stem cells derived spheroid cultures with anti-IL-4 antibodies rendered them more susceptible to apoptosis and enhanced the efficacy of the standard treatment regimens of colon cancer patients (Todaro et al., 2008). Another novel approach is the use of these immune cells as a carrier for gene therapy. Monocytes can be used as a carrier to deliver therapeutic genes; they are preloaded with magnetic nanoparticles and migrate across human endothelial cell layers into a 3D tumor spheroid in the presence of an external magnet (Muthana et al., 2008). Adenoviruses (ADVs) are also an attractive candidate for the gene therapy. Modification of ADV by inserting specific genes of tumor receptors provides significant improvement in gene transfer in glioma spheroids. In addition, targeting epidermal growth factor receptor (EGFR) by using specific anti-EGFR can also be used (Grill et al., 2001). Other selective approaches and new autologous cellular immunotherapies that may be more effective are currently being developed. For example, human cytotoxic T lymphocytes (CTL) are stimulated to study the interaction and to assess the cytotoxicity of CTL for the tumor spheroid. Similarly, monoclonal antibodies alone or in conjugation with drugs, toxins, and radioisotopes can be used for the treatment of cancer. For example, trastuzumab (Herceptin), a monoclonal antibody that specifically targets human epidermal growth factor receptor-2 (HER2), resulted in enhanced inhibition of proliferation of cancer cells in HER2 overexpressed spheroids. Interestingly, HER2 exist as homodimers in the 3D model compared to heterodimers in 2D. This homodimerization leads to enhanced activation of HER2 and also results in a switch in PI3K to MAPK signaling (Pickl and Ries, 2009).

Besides monoclonal antibodies, trifunctional antibodies can also be used to kill cancer cells. These antibodies have binding sites for two different antigens, typically CD3 and tumor antigen, and an Fc part for accessory cells (macrophages, natural killer cells, and dendritic cells). The net effect is that this type of drug links T cells (via CD3) and monocytes/macrophages, natural killer cells, dendritic cells, or other Fc receptor expressing cells to the tumor cells, leading to their destruction. For example, Hirschhaeuser et al. (2010) have shown the anticancer potential of catumaxomab (a trifunctional antibody) in a coculture of human EpCAM-positive FaDu tumor cells spheroids with human peripheral blood mononuclear cells. The results suggest that catumaxomab showed a strong dose-dependent antitumor response with decreased tumor volume, together with a massive immune cell infiltration and decreased signals for cancer cell viability and clonogenicity. However, the tumor microenvironment is frequently immunosuppressive and contributes to a state of immune ignorance and thus results in the inability of vaccines/immunotherapy to break tolerance against tumor antigens. Factors such as lactic acid in the tumor microenvironment can inhibit the migration and differentiation of monocytes into dendritic cells and also alter their antigen presentation. High lactic acid concentrations also block the export of lactate in T cells and thus alter their function and metabolism (Gottfried et al., 2006). Thus, modulation of a tumor's metabolic environment can be used to enhance antitumor responses and thus improvisation in immunotherapy-based tumor killing.

Application of 3D cultures in other diseases

There are several examples where 3D cultures have been used for various diseases. For example, Hwang et al. (2012) demonstrated that a PDMS-based concave-patterned film could be used for designing islet spheroids with improved cellular functionality and size uniformity to cure diabetes mellitus. Similarly, primary human hepatocytes were used for the spheroid formation to study hepatitis C infection (Chong et al., 2006). Furthermore, generation of 3D human liver models by coculturing of primary human hepatocytes and human adipose-derived stem cells in concave microwell-based

structures were used to facilitate the studies of human liver diseases like cirrhosis. Similarly, in vitro cardiac tissue models have been principally developed for the study of cardiac diseases and in the prediction of drug cardiotoxicity (Franchini et al., 2007). Skin, corneal, oral, and vaginal tissue models are now commercially available and can be used in validated toxicological assays. These models are also useful in testing the penetration and sensitization potential of nondrug chemicals. This has been of significant interest to the cosmetics industry, which is increasingly using skin tissue models as in vitro substitutes for the murine local lymph node assay, the gold standard method for testing the safety and allergenic properties of cosmetics (Carlson et al., 2008).

Spheroids also have promising roles in tissue engineering where they can help in repairing and replacement of injured tissue. Growing evidence reports that multipotent mesenchymal stem cell differentiation into different lineages is markedly improved in spheroid cultures when compared to 2D cell monolayers (Laschke and Menger, 2017). Furthermore, heterospheroids offer a unique opportunity to study the multicellular nature of solid tumors such as the studies involving the role of tumor-associated macrophages. In such studies, a spheroid containing both tumor cells and macrophages can be formed, thus mimicking the tumor-associated population infiltrated into tumor mass (Tevis et al., 2016).

Conclusions

MCTSs are excellent 3D in vitro models that bridge the gap between monolayers and in vivo tumors and that facilitate mechanistic studies as well as evaluation of anticancer therapies, particularly relevant to solid tumors. With the advent of novel techniques for establishing MCTS from primary tumor cells and coculturing with different normal cells, their value in cancer research has increased significantly. Spheroids with a heterogeneous cell population, for example, support studies on tumor-specific processes such as angiogenesis, invasion, and metastasis, as well as assessment of differential responses. Since the MCTS can be conveniently employed in the high-throughput screening system with a variety of automated cell-based assays, it is expected to facilitate drug discovery and evaluation of new anticancer therapies. MCTS as a secondary in vitro screening system is expected to significantly shorten the time required for translational research, bridging the important and major gap between discovery and clinical studies. Thus, spheroids have the potential to enhance predictability of clinical efficacy and may minimize, if not replace, animal studies to a large extent in the near future. Together with novel and emerging biotechnological tools, this 3D in vitro model is expected to substantially reduce the cost of new drug discovery, thereby making anticancer therapies more and more affordable to the public at large.

Ethical issues

Certain guidelines are required by the Institutional Review Board/Independent Ethics Committee (IRB/IEC) of the institutions/hospitals of the country for the collection, storage, and use of biological materials (tissue/organs) in research and diagnostic investigations. Ethical guidelines are important when conducting research on stored/archived human biological tissues that had been collected for the purpose of biobanking during routine investigation/treatment. The important ethical principles are as follows:

1. Beneficence (doing good).
2. Nonmaleficence (preventing or mitigating harm).
3. Fidelity and trust within the investigator/participant relationship.
4. Personal dignity of study participants or subjects.
5. Autonomy pertaining to informed, voluntary, and competent decision-making (informed consent).
6. Privacy of personal information.

Translational significance

MCS culture model, which have been demonstrated and established to restore the functional and microenvironmental features of in vivo human tumor tissue in many ways (such as the expression of antigens, pH and oxygen gradients within its microenvironment, penetration rate of growth factors and distribution of proliferating/quiescent cells within the spheroid), has contributed considerably to our knowledge of in vivo tumor and tissue physiology. Moreover, this model is proven to be more functionally accurate, and its gene expression profile is more similar to clinical expression profiles than the monolayer model. Therefore, the 3D MCTS culture has been translated as a valuable model to provide more comprehensive assessment of tumors in response to therapeutic strategies for improved clinical efficacy predictions. Indeed, the potential of sophisticated, 3D culture systems to be incorporated into mainstream development processes for new anticancer therapeutic strategies is increasingly recognized; it is thought to improve the preanimal and preclinical selection of both the most promising drug candidates and novel, future-oriented treatment modalities (Mueller-Klieser, 1997). Besides serving as a

good model to test the anti-cancer drugs, this model can also be exploited significantly for understanding tumor physiology and drug penetration studies in 3D models.

World Wide Web resources

There are a few web sites (WWW) that provide information on 3D cultures—including MCSs—that can be easily accessed by readers. One of them is the companion web site at www.3dcellculture.com. Designed for students, this site provides a single information source for cell culture practices using 3D techniques. Features include the following:

1. Tracking of 3D cell culture publications, with weekly citations on the site. All information can be accessed by unique cell−matrix−stimulus search interface software using keyword/title/author.
2. A forum for 3D cell culture protocols, product reviews, and other information useful in advancing 3D culture technology. Readers can also obtain information on various aspects of spheroids and their application in biomedical research from the websites of research institutions and universities such as University of the West of England (www2.uwe.ac.uk/services/Marketing/business/pdf/3d-cell.pdf). In addition to these academic websites, readers can also find additional information on spheroids and related products from the website of research suppliers like Invitrogen (http://www.invitrogen.com) and Sigma (www.sigmaaldrich.com) or (www.microtissues.com). Online videos are also available for easy access of 3D culture protocols at Jove's server (http://www.jove.com/video/2720/a-simple-hanging-drop-cell-culture-protocolfor-generation-3).

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Glossary

Gompertz function A sigmoidal function in a mathematical model used to study the growth of tumors.
Scaffold A temporary structure of material, such as silicon, for the support of tissue/cells.
Spheroid Clump of cells grown together in tissue culture suspension.
Tori Plural of the word "torus," which is a type of 3D circular shape.

Abbreviations

2D	Two dimensional
2-DG	2-deoxyglucose
3D	Three dimensional
ADV	Adenovirus
BAL	Bioartificial liver
BMG-1	Human glioma cell line
Brdu	Bromodeoxyuridine
CCL2	Monocyte chemotactic protein-1
CD	Cluster of differentiation
DMEM	Dulbecco's modified eagle medium
DU 4475	Human breast cancer cell line
F-DG	Fluordeoxyglucose
Glut-1	Glucose transporter-1
HER2	Human epidermal growth factor receptor-2
IFN-α	Interferon-α
IL-1,2,4,6	Interleukin-1, -2, -4, and -6
MAPK	Mitogen-activated protein kinase
MCTS	Multicellular tumor spheroid
MDA-MB-231	Human breast adenocarcinoma cell line
MTI-MMP	Matrix metalloproteinase protein

MTT	3-(4,5-Dimethylthiozol-2-yl)-2,5-diphenyltertazolium bromide
PDMS	Polydimethylsiloxane
PDT	Photodynamic therapy
PNI-PAAm	Poly-*N*-isopropyl acrylamide
SN12C	Human renal carcinoma cell line
TAM	Tumor-associated macrophages
U-251Mg	Human glioblastoma cell line
VEGF	Vascular endothelial growth factor

Long answer questions

1. How do you generate spheroids containing a precise number of cells? Write a short explanation of (1) the hanging drop method and (2) the microfabricated microstructure-based method. Why do they have an edge over other methods?
2. Describe how spheroids can be useful in studying tumor immune cell interactions and therapeutics.
3. How are spheroids useful in studying combined therapies? Briefly discuss few therapies.
4. Discuss in brief the advantages and disadvantages of 3D over 2D cultures, with examples.
5. Discuss the advantages of coculturing spheroids and how it can be useful in studying the effects of different tumor-associated parameters.

Short answer questions

1. What are the important factors that should be considered while generating spheroids?
2. What are the factors involved in the differentiation of immune cells?
3. What are the major factors responsible for increased resistance of spheroids (over monolayer) to therapy?
4. What are the different parameters to monitor the drug efficacy in 3D cultures?
5. How does the cell–cell interaction alter the therapeutic response of multicellular tumor spheroids?
6. Mention three methods for the generation of spheroids.
7. Are cells in spheroid more or less resistant to ionizing radiation than monolayer cultures?
8. Are hypoxic regions found in spheroids?
9. Can spheroids be generated with heterogeneous cell types?
10. What is the latest technology used in generating spheroids?

Answers to short answer questions

1. Spheroid size, possible damage, and influence in cellular physiology, production efficiency.
2. Growth factors (cytokines), lactate, etc.
3. Hypoxia and reduced vascularization, etc.
4. MTT assay for cell viability, cell cycle kinetics by Brdu labeling, Annexin V assay for apoptosis.
5. Through bystander effects.
6. Hanging drop, liquid overlay, rotary flask.
7. More resistant than the monolayer cultures.
8. Yes.
9. Yes.
10. SAW cell manipulation method.

CHAPTER

14

Animal tissue culture principles and applications

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Summary

Animal cell culture technology in today's scenario has become indispensable in the field of life sciences, which provides a basis to study regulation, proliferation, and differentiation and to perform genetic manipulation. It requires specific technical skills to carry out successfully. This chapter describes the essential techniques of animal cell culture as well as its applications.

What you can expect to know

This chapter describes the basics of animal cell culture along with the most recent applications. The primary aim is to progressively guide students through fundamental areas and to demonstrate an understanding of basic concepts of cell culture as well as how to perform cell cultures and handle cell lines. This chapter gives insights into types of cell culture, culture media and use of serum, viability assays, and the translational significance of cell culture.

History and methods

Introduction

Cell culture is the process by which human, animal, or insect cells are grown in a favorable artificial environment. The cells may be derived from multicellular eukaryotes, already established cell lines or established cell strains. In the mid-1900s, animal cell culture became a common laboratory technique, but the concept of maintaining live cell lines separated from their original tissue source was discovered in the 19th century. Animal cell culture is now one of the major tools used in the life sciences in areas of research that have a potential for economic value and commercialization. The development of basic culture media has enabled scientists to work with a wide variety of cells under controlled conditions; this has played an important role in advancing our understanding of cell growth and differentiation, identification of growth factors, and understanding of mechanisms underlying the normal functions of various cell types. New technologies have also been applied to investigate high cell density bioreactor and culture conditions.

Many products of biotechnology (such as viral vaccines) are fundamentally dependent on mass culturing of animal cell lines. Although many simpler proteins are being produced using rDNA in bacterial cultures, more complex proteins that are glycosylated (carbohydrate-modified) currently have to be made in animal cells. At present, cell culture research is aimed at investigating the influence of culture conditions on viability, productivity, and the constancy of post-translational modifications such as glycosylation, which are important for the biological activity of recombinant proteins. Biologicals produced by recombinant DNA (rDNA) technology in animal cell cultures include anticancer agents, enzymes, immunobiologicals [interleukins, lymphokines, monoclonal antibodies (mABs)], and hormones.

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Animal cell culture has found use in diverse areas, from basic to advanced research. It has provided a model system for various research efforts:

1. The study of basic cell biology, cell cycle mechanisms, specialized cell function, cell−cell and cell−matrix interactions.
2. Toxicity testing to study the effects of new drugs.
3. Gene therapy for replacing nonfunctional genes with functional gene-carrying cells.
4. The characterization of cancer cells, the role of various chemicals, viruses, and radiation in cancer cells.
5. Production of vaccines, mABs, and pharmaceutical drugs.
6. Production of viruses for use in vaccine production (e.g., chicken pox, polio, rabies, hepatitis B, and measles).

Today, mammalian cell culture is a prerequisite for manufacturing biological therapeutics such as hormones, antibodies, interferons, clotting factors, and vaccines.

Development of animal cell culture

The first mammalian cell cultures date back to the early 20th century. The cultures were originally created to study the development of cell cultures and normal physiological events such as nerve development. Ross Harrison in 1907 showed the first nerve fiber growth in vitro. However, it was in the 1950s that animal cell culture was performed at an industrial scale. It was with major epidemics of polio in the 1940s and 1950s and the accompanying requirement for viral vaccines that the need for cell cultures on a large scale became apparent. The polio vaccine from a de-activated virus became one of the first commercial products developed from cultured animal cells (Table 14.1).

Basic concept of cell culture

Tissue culture is in vitro maintenance and propagation of isolated cells tissues or organs in an appropriate artificial environment. Many animal cells can be induced to grow outside of their organ or tissue of origin under defined conditions when supplemented with a medium containing nutrients and growth factors. For in vitro growth of cells, the culture conditions may not mimic in vivo conditions with respect to temperature, pH, CO_2, O_2, osmolality, and nutrition. In addition, the cultured cells require sterile conditions along with a steady supply of nutrients for growth and sophisticated incubation conditions. An important factor influencing the growth of cells in culture medium is the medium itself. At present, animal cells are cultured in natural media or artificial media depending on the needs of the experiment. The culture medium is the most important and essential step in animal tissue culture. This depends on the type of cells that need to be cultured for the purpose of cell growth differentiation or production of designed pharmaceutical products. In addition, serum-containing and serum-free media are now available that offer a varying degree of advantage to the cell culture. Sterile conditions are important in the development of cell lines.

Cells from a wide range of different tissues and organisms are now grown in the lab. Earlier, the major purpose of cell culture was to study the growth, the requirements for growth, the cell cycle, and the cell itself. At present, homogenous cultures obtained from primary cell cultures are useful tools to study the origin and biology of the cells. Organotypic and histotypic cultures that mimic the respective organs/tissues have been useful for the production of artificial tissues.

How are cell cultures obtained?

There are three methods commonly used to initiate a culture from animals.

Organ culture

Whole organs from embryos or partial adult organs are used to initiate organ culture in vitro. These cells in the organ culture maintain their differentiated character, their functional activity, and also retain their in vivo architecture. They do not grow rapidly, and cell proliferation is limited to the periphery of the explant. As these cultures cannot be propagated for long periods, a fresh explanation is required for every experiment that leads to interexperimental variation in terms of reproducibility and homogeneity. Organ culture is useful for studying functional properties of cells (production of hormones) and for examining the effects of external agents (such as drugs and other micro or macro molecules) and products on other organs that are anatomically placed apart in vivo.

Primary explant culture

Fragments exercised from animal tissue may be maintained in a number of different ways. The tissue adheres to the surface aided by an extracellular matrix (ECM) constituent, such as collagen or a plasma clot, and it can even happen spontaneously. This gives rise to cells migrating from the periphery of the explant. This culture is known as a primary explant, and migrating cells are known as outgrowth. This has been used to analyze the growth characteristics of cancer

TABLE 14.1 Milestones in cell cultures and microfluidics.

1878	Claude Bernard	Established that a physiological state of the cell similar to the live cell can be maintained even after the death of the organism.
1907	Harrison	Cell entrapment and frog embryo nerve fiber growth in vitro.
1912	Alexis Carriel	Initiated tissue culture of chick embryo heart cells using embryo extracts as cultural media passaged for a reported period of 34 years.
1913	Steinhardt, Israeli, and Lambert	Grew vaccinia virus in fragments of guinea pig corneal tissue.
1916	Rous and Jone	Used trypsin to suspend attached cells in culture.
1927	Carrel and Rivera	First viral vaccine against chicken pox.
1948	Sanford, Earle, and Likely	Single-cell culture using microscale glass capillaries
1949	Enders, Weller, and Robbins	Polio virus grown on human embryonic cells in culture.
1952	Gey	Establishment of continuous cell line from a human cervical carcinoma (HeLa cells).
1955	Eagle	Established nutrient requirements of cells in culture and defined culture media for growth.
1956	Little Field	HAT (hypoxanthin, aminopterin, thymidine) medium introduced for cell selection.
1961	Hayflick and Moorhead	Studied human fibroblasts (WI-38) and showed finite lifespan in culture.
1965	Ham	First serum-free HAMS's media.
1975	Kohler and Milstein	First hybridoma secreting a monoclonal antibody.
1977	Genetech	First recombinant human protein: somatistatin.
1979	Terry, Jerman, and Angell	Gas chromatography system using silicon-etched microchannels
1985	Collen	Recombinant tissue plasminogen activator (tPA) in mammalian cells. Human growth hormone produced from recombinant bacteria was accepted for therapeutic use.
1986	FDA approval	First monoclonal antibody was approved by the FDA for use in humans (Orthoclone OKT3).
1986	Genetech	First recombinant protein commercialized (interferon alpha-2a).
1989	Amgen	Erythropoietin (EPO) recombinant protein produced in CHO cells available commercially.
1992	Manz, Harrison, Verpoorte, Fettinger, Paulus, Ludi, and Widmer	Micro-machined glass chip electrophoresis for separating molecules
1992	FDA approval	First genetically engineered recombinant blood-clotting factor used in the treatment of hemophilia A.
1996	Wilmut	Production of transgenic sheep (Dolly) through nuclear transfer technique.
1997	Hadd, Raymond, Halliwell, Jacobson, and Ramsey	Microchip device for enzyme assay
1998	Duffy, McDonald, Schueller, and Whitesides	Creation of microfluidic systems using PDMS (polydimethylsiloxane)
2000	Panaro, Yuen, Sakazume, Fortina, Kricka, Wilding	Lab-on-chip system (bioanalyzer)
2002	Cloneaid	Claimed to produce cloned human baby named EVE.
2003	Zheng	Protein crystallization chip
2004	FDA approval	First antiangiogenic monoclonal antibody that inhibits the growth of blood vessels or angiogenesis (for cancer therapy).
2005	Sanford, Earle, and Likely	Single-cell culture in open microfluidic systems
2005	Birch	Reported antibody titers at an industrial scale of 5 g/L and more.
2009	Nathalie Cartier-Lacave	Combined gene therapy with blood stem cell therapy, which may be a useful tool for treating fatal brain disease.
2011	Melanie Welham, David Tosh	1 M molecule treatment causes stem cells to turn into precursors of liver cells.
2012	Willison and Klug	Single-cell and single-molecule proteomics utilizing nanospace technology
2012	Maria Blasco	First gene therapy successful against aging-associated decline in mice.

cells in comparison to their normal counterparts, especially with reference to altered growth patterns and cell morphology.

Cell culture

This is the most commonly used method of tissue culture and is generated by collecting the cells growing out of explants or dispersed cell suspensions (floating free in culture medium). Cells obtained either by enzymatic treatment or by mechanical means are cultured as adherent monolayers on solid substrate.

Cell culture is of three types: (1) precursor cell culture, which is undifferentiated cells committed to differentiate; (2) differentiated cell culture, which is completely differentiated cells that have lost the capacity to further differentiate; and (3) stem cell culture, which is undifferentiated cells that go on to develop into any type of cell.

Cells with a defined cell type and characteristics are selected from a culture by cloning or by other methods; this cell line becomes a cell strain.

Monolayer cultures

The monolayer culture is an anchorage-dependent culture of usually one cell in thickness with a continuous layer of cells at the bottom of the culture vessel.

Suspension cultures

Some of the cells are nonadhesive and can be mechanically kept in suspension, unlike most cells that grow as monolayers (e.g., cells of leukemia). This offers numerous advantages in the propagation of cells.

Cell passage and use of trypsin

Passaging is the process of subculturing cells in order to produce a large number of cells from preexisting ones. Subculturing produces a more homogeneous cell line and avoids the senescence associated with prolonged high cell density. Splitting cells involves transferring a small number of cells into each new vessel. After subculturing, cells may be propagated, characterized, and stored. Adherent cell cultures need to be detached from the surface of the tissue culture flasks or dishes using proteins. Proteins secreted by the cells form a tight bridge between the cell and the surface. A mixture of trypsin-EDTA is used to break proteins at specific places. Trypsin is either protein-degrading or proteolytic; it hydrolyzes pepsin-digested peptides by hydrolysis of peptide bonds. EDTA sequesters certain metal ions that can inhibit trypsin activity, and thus enhances the efficacy of trypsin. The trypsinization process and procedure to remove adherent cells is given in Flowchart 14.1.

Trypsinization

Rinse monolayer culture
↓
Add trypsin-EDTA
↓
Incubate
↓
Stop trypsinization
↓
Spin cells
↓
Add fresh medium
↓
Count cells
↓
Seed new flask
↓
Incubate at 37°C

FLOWCHART 14.1 Trypsinization of adherent cells.

Quantitation

Quantitation is carried out to characterize cell growth and to establish reproducible culture conditions.

Hemocytometer

Cell counts are important for monitoring growth rates as well as for setting up new cultures with known cell numbers. The most widely used type of counting chamber is called a hemocytometer. It is used to estimate cell number. The concentration of cells in suspension is determined by placing the cells in an optically clear chamber under a microscope. The cell number within a defined area of known depth is counted, and the cell concentration is determined from the count.

Electronic counting

For high-throughput work, electronic cell counters are used to determine the concentration of each sample.

Other quantitation

In some cases, the DNA content or the protein concentration needs to be determined instead of the number of cells.

Reconstruction of three-dimensional structures

Cells propagated as a cell suspension or monolayer offer many advantages but lack the potential for cell-to-cell interaction and cell–matrix interaction seen in organ cultures. For this reason, many culture methods that start with a dispersed population of cells encourage the arrangement of these cells into organ-like structures. These types of cultures can be divided into two basic types.

Histotypic culture

Cell–cell interactions similar to tissue-like densities can be attained by the use of an appropriate ECM and soluble factors and by growing cell cultures to high cell densities. This can be achieved by (a) growing cells in a relatively large reservoir with adequate medium fitted with a filter where the cells are crowded; (b) growing the cells at high concentrations on agar or agarose or as stirred aggregates (spheroids); and (c) growing cells on the outer surface of hollow fibers where the cells are seeded on the outer surface and medium is pumped through the fibers from a reservoir.

Organotypic culture

To simulate heterotypic cell interactions in addition to homotypic cell interactions, cells of differentiated lineages are re-combined. Co-culturing of epithelial and fibroblast cell clones from the mammary gland allows the cells to differentiate functionality under the correct hormonal environment, thus producing milk proteins.

Types of cell culture

Primary cell culture

These cells are obtained directly from tissues and organs by mechanical or chemical disintegration or by enzymatic digestion. These cells are induced to grow in suitable glass or plastic containers with complex media. These cultures usually have a low growth rate and are heterogeneous; however, they are still preferred over cell lines as these are more representative of the cell types in the tissues from which they are derived. The morphological structure of cells in culture is of various types: (1) epithelium type, which are polygonal in shape and appear flattened as they are attached to a substrate and form a continuous thin layer (i.e., monolayer on solid surfaces); (2) epitheloid type, which have a round outline and do not form sheets like epithelial cells and do not attach to the substrate; (3) fibroblast type, which are angular in shape and elongated and form an open network of cells rather than tightly packed cells, are bipolar or multipolar, and attach to the substrate; and (4) connective tissue type, which are derived from fibrous tissue, cartilage, and bone, and are characterized by a large amount of fibrous and amorphous extracellular materials.

Advantages and disadvantages of primary cell culture

These cultures represent the best experimental models for in vivo studies. They share the same karyotype as the parent and express characteristics that are not seen in cultured cells. However, they are difficult to obtain and have limited lifespans. Potential contamination by viruses and bacteria is also a major disadvantage.

Depending on the kind of cells in culture, the primary cell culture can also be divided into two types.

Anchorage-dependent/adherent cells

These cells require a stable nontoxic and biologically inert surface for attachment and growth and are difficult to grow as cell suspensions. Mouse fibroblast STO cells are anchorage cells.

Anchorage-independent/suspension cells

These cells do not require a solid surface for attachment or growth. Cells can be grown continuously in liquid media. The source of cells is the governing factor for suspension cells. Blood cells are vascular in nature and are suspended in plasma and these cells can be very easily established in suspension cultures.

Secondary cell culture

When primary cell cultures are passaged or subcultured and grown for a long period of time in fresh medium, they form secondary cultures and are long-lasting (unlike cells of primary cell cultures) due to the availability of fresh nutrients at regular intervals. The passaging or subculturing is carried out by enzymatic digestion of adherent cells. This is followed by washing and re-suspending of the required amount of cells in appropriate volumes of growth media. Secondary cell cultures are preferred as these are easy to grow and are readily available; they have been useful in virological, immunological, and toxicological research.

Advantages and disadvantages of secondary cell culture

This type of culture is useful for obtaining a large population of similar cells and can be transformed to grow indefinitely. These cell cultures maintain their cellular characteristics. The major disadvantage of this system is that the cells have a tendency to differentiate over a period of time in culture and generate aberrant cells.

Cell line

The primary culture, when subcultured, becomes a cell line or cell strain that can be finite or continuous, depending on its lifespan in culture. They are grouped into two types on the basis of the lifespan of the culture.

Finite cell lines

Cell lines with a limited number of cell generations and growth are called finite cell lines. The cells are slow growing (24–96 hours). These cells are characterized by anchorage dependence and density limitation.

Indefinite cell lines

Cell lines obtained from in vitro transformed cell lines or cancerous cells are indefinite cell lines and can be grown in monolayer or suspension form. These cells divide rapidly with a generation time of 12–14 hours and have a potential to be subcultured indefinitely. The cell lines may exhibit aneuploidy (Bhat, 2011) or heteroploidy due to an altered chromosome number. Immortalized cell lines are transformed cells with altered growth properties. HeLa cells are an example of an immortal cell line. These are human epithelial cells obtained from fatal cervical carcinoma transformed by human papilloma virus 18 (HPV18). Indefinite cell lines are easy to manipulate and maintain. However, these cell lines have a tendency to change over a period of time.

Commonly used cell lines

Nowadays, for the production of biologically active substances on an industrial scale, a mammalian cell culture is a prerequisite. With advancements in animal cell culture technology, a number of cell lines have evolved and are used for vaccine production, therapeutic proteins, pharmaceutical agents, and anticancerous agents. For the production of cell lines, human, animal, or insect cells may be used. Cell lines that are able to grow in suspension are preferred as they have a faster growth rate. Chinese hamster ovary (CHO) is the most commonly used mammalian cell line.

When selecting a cell line, a number of general parameters must be considered, such as growth characteristics, population doubling time, saturation density, plating efficiency, growth fraction, and the ability to grow in suspension. Table 14.2 shows some of the commonly used cell lines.

Advantages of continuous cell lines

1. Continuous cell lines show faster cell growth and achieve higher cell densities in culture.
2. Serum-free and protein-free media for widely used cell lines may be available in the market.
3. The cell lines have a potential to be cultured in suspension in large-scale bioreactors.

The major disadvantages of these cultures are chromosomal instability, phenotypic variation in relation to the donor tissue, and a change in specific and characteristic tissue markers (Freshney, 1994).

Growth cycle

The cells in the culture show a characteristic growth pattern, lag phase, exponential or log phase, followed by a plateau phase. The population doubling time of the cells can be calculated during the log phase and plateau phase. This is critical and can be used to quantify the response of the cells to different culture conditions for changes in nutrient concentration and effects of hormonal or toxic components. The population doubling time describes the cell division rates within the culture and is influenced by nongrowing and dying cells.

Phases of the growth cycle

The population doubling time, lag time, and saturation density of a particular cell line can be established and characterized for a particular cell type. A growth curve consists of a normal culture and can be divided into a lag phase, log phase, and plateau phase.

Lag phase

This is the initial growth phase of the subculture and re-seeding during which the cell population takes time to recover. The cell number remains relatively constant prior to rapid growth. During this phase, the cell replaces elements of the glycocalyx lost during trypsinization, attaches to the substrate, and spreads out. During the spreading process, the cytoskeleton reappears; its reappearance is probably an integral part of the process.

Log phase

This is a period of exponential increase in cell number and growth of the cell population due to continuous division. The length of the log phase depends on the initial seeding density, the growth rate of the cells, and the density at which cell proliferation is inhibited by density. This phase represents the most reproducible form of the culture as the growth fraction and viability is high (usually 90%–100%), and the population is at its most uniform. However, the cell culture may not be synchronized, and the cells can be randomly distributed in the cell cycle.

TABLE 14.2 Commonly used cell lines and their origins.

Cell line	Origin	Organism
H1, H9	Embryonic stem cells	Human
HEK-293	Embryonic kidney transformed with adenovirus	Human
HeLa	Epithelial cell	Human
HL 60	Human promyelocytic leukemia cells	Human
MCF-7	Breast cancer	Human
A549	Lung cancer	Human
A1 to A5-E	Amnion	Human
ND-E	Esophagus	Human
CHO	Ovary	Chinese hamster
Vero	Kidney epithelial cell	African green monkey
Cos-7	Kidney cells	African green monkey
3T3	Fibroblast	Mouse
BHK21	Fibroblast	Syrian hamster
MDCK	Epithelial cell	Dog
E14.1	Embryonic stem cells (mouse)	Mouse
COS	Kidney	Monkey
DT40	Lymphoma cell	Chick
S2	Macrophage-like cells	Drosophila
GH3	Pituitary tumor	Rat
L6	Myoblast	Rat
Sf9 and Sf21	Ovaries	Fall Army worm(*Spodoptera frugiperda*)
ZF4 and AB9 cells	Embryonic fibroblast cells	Zebrafish

Plateau phase

The culture becomes confluent at the end of the log phase as growth rates during this phase are reduced, and cell proliferation can cease in some cases due to exhaustion. The cells are in contact with surrounding cells, and the growth surface is occupied. At this stage, the culture enters the stationary phase and the growth fraction falls to between 0% and 10%. Also, the constitution and charge of the cell surface may be changed, and there may be a relative increase in the synthesis of specialized versus structural proteins.

Monitoring cell growth

The animal cell culture can be grown for a wide variety of cell-based assays to investigate morphology, protein expression, cell growth, differentiation, apoptosis, and toxicity in different environments. Product yields can be increased if monitoring of cell growth is managed properly. A number of factors affect the maximum growth of cells in a batch reactor. Regular observation of cells in culture helps monitor cell health and the stage of growth; small changes in pH, temperature, humidity, O_2, CO_2, dissolved nutrients, etc., could have an impact on cell growth. Monitoring the rate of growth continuously also provides a record that the cells have reached their maximum density within a given time frame.

Characteristics of cell cultures

Animal cell cultures show specific characteristics and differ from microbial cultures. The important characteristics of the animal cell are slow growth rate, requirement of solid substrata for anchorage-dependent cells, lack of a cell wall (which leads to

fragility), and sensitivity to physiochemical conditions such as pH, CO_2 levels, etc. Some of the fundamental bioprocess variables are as follows:

Temperature

Temperature is one of the most fundamental variables as it directly interferes with the growth and production processes. On a small scale, thermostatically controlled incubators can be used to control temperature. However, cell cultures grown on a large scale in bioreactors require more sensitive control of temperature. Different bioreactors use different methods to maintain the temperature of the cell culture. Temperature in a bioreactor is maintained by a heat blanket and water jacket with a temperature sensor.

pH

pH of the culture medium can be controlled by adding alkali (NaOH, KOH) or acid (HCl) solution. Addition of CO_2 gas to the bioreactor, buffering with sodium bicarbonate, or use of naturally buffering solutes help maintain the pH of the culture. A silver chloride electrochemical-type pH electrode is the most commonly used electrode in the bioreactor.

Oxygen

Dissolved oxygen is the most fundamental variable that needs to be continuously supplied to the cell culture medium. It is consumed with a carbon source in aerobic cultures (Moore et al., 1995). Diffusion through a liquid surface or membranes is one of the methods for providing dissolved oxygen to the medium.

Cell viability

The number of viable cells in the culture provides an accurate indication of the health of the cell culture (Stacey and Davis, 2007). Trypan blue and erythrosin B determine cell viability through the loss of cellular membrane integrity. Both these dyes are unable to penetrate the cell membrane when the membrane is intact, but are taken up and retained by dead cells (which lack an intact membrane). Erythrosin B stain is preferred over Trypan blue as it generates more accurate results with fewer false negatives and false positives.

Cytotoxicity

The toxic chemicals in the culture medium affect the basic functions of cells. The cytotoxicity effect can lead to the death of the cells or alterations in their metabolism. Methods to access viable cell number and cell proliferation rapidly and accurately is the important requirement in many experimental situations that involve in vitro and in vivo studies. The cell number determination can be useful for determining the growth factor activity, concentration of toxic compound, drug screening, duration of exposure, change in colony size, carcinogenic effects of chemical compounds, and effects of solvents (such as ethanol, propylene, etc.).

The assays to measure viable cells (viability assays) are as follows:

1. [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT)/MTS/resazurin assay.
2. Protease marker assay.
3. ATP assay.

The MTT assay allows simple, accurate, and reliable counting of metabolically active cells based on the conversion of pale yellow tetrazolium MTT. Nicotinamide adenine dinucleotide in metabolically active viable cells reduces tetrazolium compounds into brightly colored formazan products or reduces resazurin into fluorescent resorufin (Fig. 14.1). MTT and resazurin assays are widely used, as they are inexpensive and can be used with all cell types. The protease marker assay utilizes the cell-permeant protease substrate glycylphenylalanyl-aminofluorocoumarin (GF-AFC). The substrate, which lacks an aminoterminal blocking moiety, is processed by aminopeptidases within the cytoplasm to release AFC. The amount of AFC released is proportional to the viable cell number. This assay has better sensitivity than resuzurin and the cells remain viable; thus, multiplexing is possible. The ATP assay is the most sensitive cell viability assay. It is

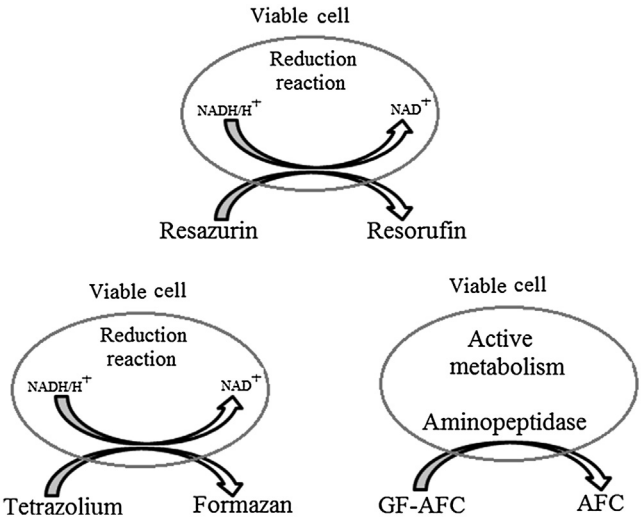
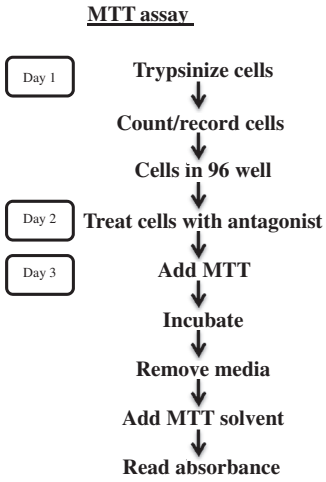


FIGURE 14.1 Schematic summary of biochemical events in different viability assays.



FLOWCHART 14.2 MTT assay.

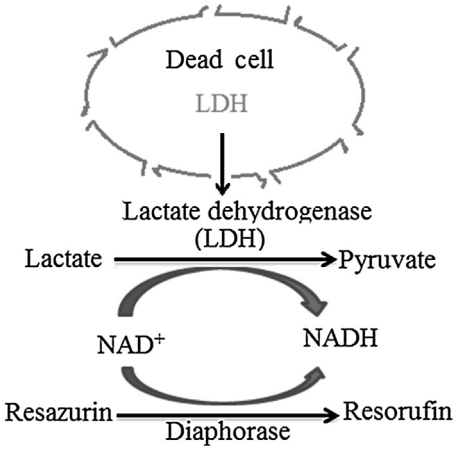


FIGURE 14.2 Principle of the LDH release assay.

measured using the beetle luciferase reaction to generate light. The MTT assay and procedure is given in Flowchart 14.2.

Assays to detect dead cells are as follows:

1. Lactate dehydrogenase (LDH) release.
2. Protease release.
3. DNA staining.

The viable cells in culture have intact outer membranes. Loss of membrane integrity defines a "dead" cell. The dead cells can be detected by measuring the activity of marker enzymes that leak out of dead cells into the culture medium or by staining the cytoplasmic or nuclear content by vital dyes that can only enter dead cells. LDH is an enzyme that is present in all cell types. It catalyzes the oxidation of lactate to pyruvate in the presence of co-enzyme NAD^+. In the damaged cells, LDH is rapidly released. The amount of released

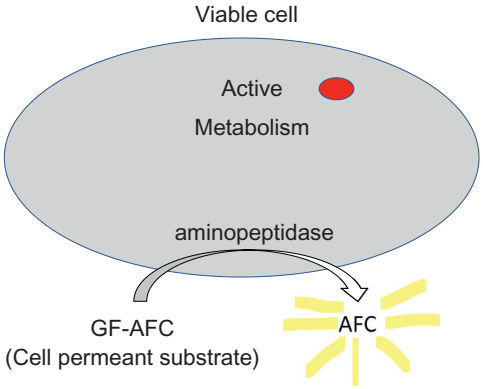


FIGURE 14.3 Principle of the luminescent protease release assay.

LDH is used to assess cell death (Fig. 14.2). This assay is widely used but has limited sensitivity as half-life of LDH at 37 °C is 9 hours.

The protease release assay is based on the intracellular release of proteases from the dead/compromised cell into the culture medium. The released proteases cleave the substrate to liberate aminoluciferin, which serves as a substrate for luciferase (Fig. 14.3) and leads to the production of a "glowtype" signal (Cho et al., 2008).

Hayflick's phenomenon

Hayflick limit or Hayflick's phenomena is defined as the number of times a normal cell population divides before entering the senescence phase. Macfarlane Burnet coined the term "c limit" in 1974. Hayflick and Moorhead (1961) demonstrated that a population of normal human fetal cells divide in culture between 40 and 60 times before stopping. There appears to be a correlation between the maximum number of passages and aging. This phenomenon is related to telomere length. Repeated mitosis leads to shortening of the telomeres on the DNA of the cell. Telomere shortening in humans eventually makes cell division impossible, and correlates with aging. This explains the decrease in passaging of cells harvested from older individuals.

Culture media

One of the most important factors in animal cell culture is the medium composition. In vitro growth and maintenance of animal cells require appropriate nutritional, hormonal, and stromal factors that resemble their milieu in vivo as closely as possible. Important environmental factors are the medium in which the cells are surrounded, the substratum upon which the

cells grow, temperature, oxygen and carbon dioxide concentration, pH, and osmolality. In addition, the cell requires chemical substances that cannot be synthesized by the cells themselves. Any successful medium is composed of isotonic, low-molecular-weight compounds known as basal medium and provides inorganic salts, an energy source, amino acids, and various supplements.

Basic components in culture media

The 10 basic components that make up most of the animal cell culture media are as follows: inorganic salts (Ca^{2+}, Mg^{2+}, Na^{+}, K^{+}), nitrogen source (amino acids), energy sources (glucose, fructose), vitamins, fat and fat soluble component (fatty acids, cholesterols), nucleic acid precursors, growth factors and hormones, antibiotics, pH and buffering systems, and oxygen and carbon dioxide concentrations.

Complete formulation of media that supports growth and maintenance of a mammalian cell culture is very complex. For this reason, the first culture medium used for cell culture was based on biological fluids such as plasma, lymph serum, and embryonic extracts. The nutritional requirements of cells can vary at different stages of the culture cycle. Different cell types have highly specific requirements, and the most suitable medium for each cell type must be determined experimentally. Media may be classified into two categories: (1) natural media and (2) artificial media.

Natural media

Natural media consist of naturally occurring biological fluids sufficient for the growth and proliferation of animals cells and tissues. This media useful for promoting cell growth are of the following three types:

1. Coagulant or clots: Plasma separated from heparinized blood from chickens or other animals is commercially available in the form of liquid plasma.
2. Biological fluids: This includes body fluids such as plasma, serum lymph, amniotic fluid, pleural fluid, insect hemolymph, and fetal calf serum. These fluids are used as cell culture media after testing for toxicity and sterility.
3. Tissue extract: Extracts of liver, spleen, bone marrow, and leucocytes are used as cell culture media. Chicken embryo extract is the most common tissue extract used in some culture media.

Artificial media

The media contains partly or fully defined components that are prepared artificially by adding several nutrients (organic and inorganic). It contains a balanced salt solution with specific pH and osmotic pressure designed for immediate survival of cells. Artificial media supplemented with serum or with suitable formulations of organic compounds supports prolonged survival of the cell culture.

The artificial media may be grouped into the following four classes: serum-containing media, serum-free media, chemically defined media, and protein-free media.

Serum

The clear yellowish fluid obtained after fibrin and cells are removed from blood is known as serum. It is an undefined media supplement of extremely complex mixture of small and large molecules and contains amino acids, growth factors, vitamins, proteins, hormones, lipids, and minerals, among other components (Table 14.3).

Advantages of serum in cell culture medium

1. It has basic nutrients present either in soluble or in protein-bound form.
2. It provides several hormones such as insulin and transferrin. Insulin is essential for the growth of nearly all cells in culture and transferrin acts as an iron binder.
3. It contains numerous growth factors such as platelet-derived growth factor (PDGF), transforming growth factor beta (TGF-B), epidermal growth factor (EGF), and chondronectin. These factors stimulate cell growth and support specialized functions of cells.
4. It supplies protein, which helps in the attachment of cells to the culture surface (e.g., fibronectin).
5. It provides binding proteins such as albumin and transferrin, which helps transport molecules in cells.
6. It provides minerals such as Ca^{2+}, Mg^{2+}, Fe^{2+}, K^{+} Na^{+}, Zn^{2+}, etc., which promote cell attachment.
7. It increases the viscosity of the medium, which provides protection against mechanical damage during agitation and aeration of suspension cultures.
8. It provides appropriate osmotic pressure.

Disadvantages of serum-containing medium

1. Expensive: Fetal calf serum is expensive and difficult to obtain in large quantities.
2. Variation: Batch-to-batch variation occurs in serum, and there is no uniformity in composition of serum. This can affect growth and yields and can give inconsistent results.
3. Contamination: Serum medium carries a high risk of contamination with virus, fungi, and mycoplasma.

TABLE 14.3 Serum components, their composition, and role in animal cell culture.

Component	Probable function
Protein and polypeptide	
Albumin	Major binding and buffering agent, antioxidant, transporter of insoluble molecules
Transferrin	Iron chelator and transporter
Alpha, beta, and gamma globulin fractions	Bind to iron and iron carrier and prevent infection
Regulatory proteins	Regulate gene expression
Growth factors	
Epidermal growth factor (EGF)	Proliferation and differentiation
Fibroblast growth factor (FGF)	Proliferation and differentiation
Hormones	
Insulin	Glucose and protein metabolism
Transferrin	Incorporation of iron by cells

4. Cytotoxic and inhibiting factors: The serum itself may be cytotoxic and may contain inhibiting factors, which in turn may inhibit cultured cell growth and proliferation. The enzyme polyamine oxidase in serum reacts with polyamines such as spermine and spermidine to form cytotoxic polyamino-aldehyde.
5. Downstream processing: The presence of serum in culture media may interfere with isolation and purification of culture products. Additional steps may be required to isolate cell culture products.

Serum-free media

The use of serum in culture media presents a safety hazard and source of unwanted contamination for the production of biopharmaceuticals. As a number of cell lines can be grown in serum-free media supplemented with certain components of bovine fetal serum, the development of this type of medium with a defined composition has intensified in the last few decades. Eagle (1959) developed a "minimal essential medium" composed of balanced salts, glucose, amino acids, and vitamins. In the last 50 years, considerable work has been carried out to develop more efficient culture media to meet the specific requirements of specific cell lines.

Advantages of serum-free culture media

1. Serum-free media are simplified, and the composition is better defined.
2. They can be designed specifically for a cell type. It is possible to create different media and to switch from growth-enhancing media to differentiation-inducing media by altering the combination and types of growth factors and inducers.
3. They decrease variability from batch to batch and improve reproduction between cultures.
4. Downstream processing of products from cell cultures in serum-free media is easier.
5. They reduce the risk of microbial contamination (mycoplasma, viruses, and prions).
6. Serum-free media are easily available and ready to use. They are also cost-effective when compared with serum-containing media.

Disadvantages of serum-free media

1. Growth rate and saturation density attained are lower than those compared to serum-containing media.
2. Serum-free media prove to be more expensive as supplementing with hormone and growth factors increases the cost enormously.
3. Different media are required for different cell types as each species has its own characteristic requirements.
4. Critical control of pH and temperature and ultra-purity of reagent and water are required as compared to serum-containing media.

Chemically defined media

These media contain pure inorganic and organic constituents along with protein additions like EGFs, insulin, vitamins, amino acids, fatty acids, and cholesterol.

Protein-free media

These media contain nonprotein constituents necessary for the cell culture. The formulations of DME, MEM, RPMI-1640, ProCHO TM, and CDM-HD are

examples of protein-free media. They promote superior cell growth and facilitate downstream purification of expressed products.

Characterization of cell lines

The characterization of cell lines is important to ensure the quality of cell-derived biopharmaceutical products. It helps in determining the cell source with regard to its identity and the presence of other cell lines, molecular contaminants, and endogenous agents. The characterization of mammalian cell lines is species-specific and can vary depending on the history of the cell line and type of media components used for culturing.

Mammalian cell line characterization can be done in four ways:

1. Identity testing.
2. Purity testing.
3. Stability testing.
4. Virological safety testing.

Identity testing

Identity testing can be carried out by isoenzyme analysis. The banding pattern of the intracellular enzyme (which is species-specific) can be determined by using agarose gels. DNA fingerprinting and karyotyping, and DNA and RNA sequencing are alternative methods to identity testing.

Karyotyping

Karyotyping is important as it determines any gross chromosomal changes in the cell line. The growth conditions and subculturing of a cell line may lead to alteration in the karyotype; for example, HeLa cells were the first human epithelial cancer cell line established in long-term culture, and they have a hypertriploid chromosome number (3n1).

Purity testing

Bacterial and fungal contamination of cell lines occurs due to impure techniques and source material. The occurrence of contaminants can be tested by a direct inoculation method on two different media. Mycoplasma infection is the contamination of cell cultures/cell lines with mycoplasmas, and it represents a serious problem. Detection by microscopy is not adequate and requires additional testing by fluorescent staining PCR, ELISA assay, autoradiography, immunestaining, or microbiological assay.

Stability testing

Characterization and testing of cell substrate (cell line derived from human or animal source) is one of the most important components in the control of biological products. It helps to confirm the identity, purity, and suitability of the cell substrate for manufacturing use. The substrate stability should be examined at a minimum of two time points during cultivation for production. In addition, genetic stability can be tested by genomic or transcript sequencing, restriction map analysis, and copy number determination (FDA guidelines, 2012).

Viral testing assays

Virus testing of cell substrate should be designed to detect a spectrum of viruses. Appropriate screening tests should be carried out based on the cultivation history of cell lines. The development of characteristic cytopathogenic effect (CPE) provides an early indication of viral contamination. Some of the viruses of special concern in cell production work are human immunodeficiency virus, human papilloma virus, hepatitis virus, human herpes virus, hantavirus, simian virus, sendai virus, and bovine viral diarrhea virus. For detection of viruses causing immunodeficiency diseases and hepatitis, detection of sequences by PCR testing is adequate. Cells exposed to be serum or bovine serum albumin require a bovine virus test. Some of the viral testing assays are XC plaque assays, S + L-focus assay, reverse transcription assay. XC plaque assay is utilized to detect infectious ecotropic murine retroviruses. S + L-focus assay is used to test cells for the presence of infectious xenotropic and amphotropic murine retroviruses that are capable of interacting with both murine and nonmurine cells. Real-time (RT) assays such as real-time fluorescent product-enhanced reverse transcriptase (FPERT) assay and quantitative real-time for fluorescent product-enhanced reverse transcript (QPERT) assay detect the conversion of an RNA template to cDNA due to the presence of the RT template when retrovirus infection is present in the cell line.

Advantages of animal cell culture

1. Physiochemical and physiological condition: Role and effect of pH, temperature, O_2/CO_2 concentration, and osmotic pressure of the culture media can be altered to study their effects on the cell culture (Freshney, 2010).

2. Metabolism of cell: To study cell metabolism and investigate the physiology and biochemistry of cells.
3. Cytotoxic assay: Effect of various compounds or drugs on specific cell types such as liver cells can be studied.
4. Homogenous cultures: These cultures help study the biology and origin of the cells.
5. Valuable biological data from large-scale cell cultures: Specific proteins can be synthesized in large quantities from genetically modified cells in large-scale cultures.
6. Consistency of results: Reproducibility of the results that can be obtained by the use of a single type/clonal population.
7. Identification of cell type: Specific cell types can be detected by the presence of markers such as molecules or by karyotyping.
8. Ethics: Ethical, moral, and legal questions for utilizing animals in experiments can be avoided.

Disadvantages of animal cell culture

1. Expenditure and expertise: This is a specialized technique that requires aseptic conditions, trained personnel, and costly equipment.
2. Dedifferentiation: Cell characteristics can change after a period of continuous growth of cells in cultures, leading to differentiated properties compared to the original strain.
3. Low amount of product: The miniscule amount of mAB and recombinant protein produced followed by downstream processing for extracting pure products increases expenses tremendously.
4. Contamination: Mycoplasma and viral infection are difficult to detect and are highly contagious.
5. Instability: Aneuploidy chromosomal constitution in continuous cell lines leads to instability.

In addition, this system cannot replace the complex live animal for testing the response of chemicals or the impact of vaccines or toxins.

Ethical issues

Despite considerable progress in the development of cell culture techniques, the potential biohazards of working with animal and human tissues presents a number of ethical problems, including issues of procurement, handling, and ultimate use of material. In most countries, biomedical research is strictly regulated. Legislation varies considerably in different countries. Research ethics committees, animal ethics committees for animal-based research, and institutional research boards for human subjects have a major role in research governance.

Some guidelines for the use of experimental or donor animals include assurances of proper conditions for housing animals and minimal pain or discomfort to any animal that is put to death or operated upon. These guidelines apply to higher vertebrates and not to lower vertebrates such as fish or other invertebrates.

Use of fetal bovine serum in animal culture of media

Fetal bovine serum (FBS)-supplemented media are commonly used in animal cell cultures. In recent years, FBS production methods have come under scrutiny because of animal welfare concerns. FBS is harvested from bovine fetuses taken from pregnant cows during slaughter. The common method of harvesting the fetus is by cardiac puncture without any anesthesia. This practice of harvesting FBS is inhumane as it exposes the fetus to pain and/or discomfort. In addition to moral concerns, numerous scientific and technical problems exist with regard to the use of FBS in cell culture. Efforts are now being made to reduce the use of FBS and replace it with synthetic alternatives.

In the case of human tissues, some considerations that need to be addressed are as follows (Freshney, 2011):

1. Consent: Patient's and/or relative's approval of tissue use.
2. Project summary: Explanation of the project, including the purpose, outcome, and medical benefits of the research.
3. Permission requests: Paperwork regarding possible use of the tissues.
4. Ownership: Establishment of ownership with regard to cell lines and their derivatives.
5. Patent issues: Commercial use of the tissues.

Translational significance

In biomedical research, the use of animal and human cell cultures has become beneficial for diverse applications. It provides indispensable tools for producing a number of products, including biopharmaceuticals, mABs, and products for gene therapy. In addition, animal cell cultures provide adequate test systems for studying biochemical pathways, intra- and intercellular responses, pathological mechanisms, and virus production. Some of the applications of animal cell culture are discussed below.

TABLE 14.4 Recombinant hepatitis B vaccines in eukaryotic cells.

Trade name	Manufacture	Recombinant protein	Expression host
Sci B-Vac	SciGen	HBsAgS, M, and L protein	Mammalian cells (CHO)
GenHevac B	Pasteur-Merieux Aventis	HBsAg S and M protein	Mammalian cells (CHO)
Enivac HB	Panacea Biotec Ltd	HBsAg S protein	Yeast (*P. pastors*)
Revac-B	Bharat Biotech International Ltd	HBsAg S protein	Yeast (*P. pastors*)

Antiviral vaccines

Animal cell culture technology has played an important role in the development of viral vaccine production. The establishment of cell culture technology in the 1950s and the consequent replacement of live animals for the development of antigens have led to considerable progress in bioprocess technology. With the advent of DNA technology, molecular manipulation of viruses has led to the development of a recombinant vaccine against hepatitis B virus (HBV) and several others potential vaccines that are in the final phase of clinical trials. Table 14.4 lists recombinant hepatitis B vaccines in eukaryotic cells.

Viral particles production by cell culture

Viral particle production by cell culture differs from the production of molecules such as proteins, enzymes, and toxins by bacteria or animal cells. The product formation may not be related to the development or growth of a cell and may occur through secondary metabolic pathways, unlike virus production, which does not result from secondary metabolic pathway. Virus production occurs after the viral infection directs cell machinery to perform viral particle production.

Two stages are involved in viral production:

1. Cell culture system: This requires the development of an efficient system for conversion of the culture medium substrate in the cell mass.
2. Virus production: This phase differs from the infection phase and has different nutritional and metabolic requirements. A number of immortalized cell lines are used for the industrial production of viral vaccines. Table 14.5 gives the cell lines used for vaccines.

TABLE 14.5 Cell lines used for viral vaccine production.

Cell lines	Origin
BHK-21	Kidney (hamster)
Vero	Kidney (African green monkey)
MDCK	Kidney (cocker spaniel)
CHO	Chinese hamster
Hela	Epithelial cells (human)

Production of virus-like particles

Most of the existing classical vaccines for viral disease are either altered or chemically inactive live viruses. However, incomplete inactivation of a virus or reversion of an attenuated strain can risk infection in vaccinated individuals. Viruses with segmented genomes with a high degree of genetic exchange can undergo re-assortment or recombination of genetic material with viruses of different serotypes in the vaccinated host, which can result in the production of new variants of the virus. Moreover, some live virus vaccines are teratogenic; for example, Smithburn neurotropic strain (SNS) (Smithburn, 1949) and MP12-attenuated (Caplen et al., 1985) vaccine strains of the Rift Valley fever virus. A new type of vaccine that does not present the typical side effects of an attenuated or inactivated viral vaccine has been made possible with the development of rDNA technology. Virus-like particles (VLPs) are highly effective as they mimic the overall structure of the virus; however, these particles lack the infectious genetic material. Capsid proteins can aggregate to form core-like particles in the absence of nucleic acids. These spontaneously assembled particles are structurally similar to authenticate viruses and are able to stimulate B-cell-mediated immune responses. In addition, VLPs stimulate a CD4-proliferative response and cytotoxic T-lymphocyte response (Jeoung et al., 2011).

VLPs resemble and mimic virus structure and are able to elicit a strong immune response without causing harm. The major advantage of VLPs is their simplicity and nonpathogenic nature. They are replication-deficient as they lack any viral genetic information, thus eliminating the need for inactivation of the virus. This is important as inactivation treatments lead to epitope modifications (Cruz et al., 2002). As the structural

morphology of VLPs is similar to the virus, the conformational epitopes presented to the immune system are the same as for the native virus particles. The immune response/antibody reactivity in the case of VLPs is significantly improved as VLPs present conformation epitopes more similar to the native virus. VLPs also induce a strong B-cell response. For broader and more efficient protection, it is possible to adapt one or more antigens to the multimeric protein structure. Another advantage offered by VLPs is that they significantly reduce vaccine costs as these can elicit a protective response at lower doses of antigen.

Vaccines based on virus-like particles

The FDA has approved VLP-based vaccines for HBV and HPV. The HBV vaccine was approved in 1986 and the HPV one in 2006 (Justin et al., 2011). To generate immunogenic VLPs, the S gene is cloned and expressed in a eukaryotic expression host such as yeast or mammalian cells (e.g., CHO cell line). The mammalian cell culture allows easy recovery because the cells are able to secrete the antigen HBsAg. The two companies producing CHO-based vaccines are the French-based Pasteur-Merieux Aventis (Gene Hevac B) and the Israeli-based SciGen (Sci-B-Vac). The Gene Hevac B vaccine contains the HBsAg S protein and M protein, whereas Sci-B-Vac contains the M and L proteins.

Human papilloma virus vaccine

Viruses of the Papillomaviridae family are known to induce lesions and warts and also cause cervical cancer. Fifteen strains of Papillomaviridae are known to cause cervical cancer. HPV-16 is considered a high-risk HPV type as the risk of cancer may be higher than for other high-risk HPV types. The two virally encoded proteins of HPV are L1 and L2. L1 is the main capsid protein that forms the outer shell of the virus. L2 is found in the interior of the viral particle and is less abundant. The recombinant L1 VLP is able to induce neutralizing antibodies in animals. Gardasil (the first HPV vaccine) was approved by the FDA in 2006. This vaccine is manufactured by Merck and Co., Inc. Ceravarix, another HPV vaccine (manufactured by Glaxo Smithkline), was approved by the FDA in 2009. It uses the Trichoplusia ni (Hi-5) insect cell line infected with L1 recombinant baculovirus (Jiang et al., 1998; Wang et al., 2000).

A number other VLP-based vaccines are in clinical trials. These include the anti-influenza A M2-HBcAg VLP vaccine (Clarke et al., 1987), two antimalarial vaccine nicotine-Qβ VLPs (Maurer et al., 2005), and an anti-AngIIQβ VLP. The VLP production in mammalian cell lines and Baculo cell lines of viruses infecting humans and other animals is summarized in Table 14.6.

Recombinant therapeutic proteins

Proteins play a major role in carrying out biochemical reactions, transporting small molecules within a cell or from one organ to another, formation of receptors and channels in membranes, and providing frameworks for scaffolding. The number of functionally distinct proteins in humans far exceeds the number of genes as a result of post-translational modifications. These modifications include glycosylation, phosphorylation, ubiquitination, nitrosylation, methylation, acetylation, and lipidation. The changes in protein structure as a result of mutation or other abnormalities often lead to a disease condition. Protein therapeutics offer tremendous opportunities for alleviating disease. The first therapeutic from recombinant mammalian cells was human tissue plasminogen, which obtained market approval in 1986. At present, 60%–70% of all the recombinant therapeutic proteins are produced in mammalian cells.

Main therapeutic proteins

The main therapeutic proteins can be divided into seven groups (Walsh, 2003):

1. Cytokines
2. Hematopoietic growth factors
3. Growth factors
4. Hormones
5. Blood products
6. Enzymes
7. Antibodies

Most of the proteins have complex structures and undergo chemical modification to insure full biological activity. Protein post-translation modifications (PTM) can happen in several ways. The most widely recognized form of PTM is glycosylation, which involves extensive sequence processing and trimming in the Golgi apparatus and endoplasmic reticulum. Eukaryotic cells are capable of carrying out this type of modification and are thus preferred in biopharmaceutical processes. Hamster, baby hamster kidney (BHK), and CHO cells are often the host cells of choice as glycosylation patterns generated from these cells are more similar to human patterns. Table 14.7 lists various therapeutic proteins produced in animal cell lines.

Cytokines

Cytokines are proteins of the immune system that play a central role in immune response. Cytokines are produced as a result of immune stimulus by various white blood cells. Interferons (IFNs) were the first family of cytokines to be discovered and used as biopharmaceuticals.

TABLE 14.6 VLP production in mammalian and baculo cell lines of viruses infecting humans and other animals.

Virus	Expression system	Cells	Recombination protein	Reference
SIV	B/IC	Sf9	Pr55 gag envelope protein	Yamshcikov et al. (1995)
Ebola	Human cells	HEK293T	VP40 and glycoprotein	Licata et al. (2004)
Rota virus	B/IC	Sf9	VP2, VP6, VP7	Parez et al. (2004)
SARS-CoV	Baculovirus-insect cell system	Sf21	E and M proteins	Ho et al. (2004)
SARS	Insect cell culture system	Sf-9	S protein, E protein, and M protein	Mortola and Roy (2004)
Marburg and Ebola virus	Mammalian cell culture system	293T cells	VP40 and glycoproteins	Swenson et al. (2005)
AAV	Insect cell culture system and mammalian cell culture system	Sf-9 and HEK 293	VP1, VP2, and VP3	Aucoin et al. (2007)
HIV-1	Baculovirus-insect cell expression system	Sf-21	HIV gag proteins like GagTN and GagRT	Pillay et al. (2009)
Influenza A	Baculovirus expression system	Sf-9	H1N1 protein, M1 protein, etc.	Krammer et al. (2010)
MERS-CoV	Recombinant baculoviruses (rBVs)	Sf9	Spike (S), envelope (E), and membrane (M) protein	Wang et al. (2017)
ZIKV	CHO	HEK293	rH7 antigens	Chen et al. (2019)

AAV, adeno-associated virus; *rBVs*, recombinant baculoviruses; *ZIKV*, Zika virus.

Applications of interferons

IFNα is used for treatment of hepatitis, and more recently has been approved for leukemia and other types of cancers. IFNβ is used for treatment of multiple sclerosis and is marketed under the names Avonex, Belaseron, and Rebif. IFNγ is used for the treatment of chronic granulomatous disease. Interleukin is another kind of cytokine that helps regulate cell growth, differentiation, and motility and is used as a biopharmaceutical. The recombinant form of IL-2 is used for the treatment of renal cell carcinoma.

Growth factors

Growth factors are proteins that bind to receptors on the surface of cells to activate the cells for proliferation and or differentiation. The different types of growth factors are TGF, insulin-like growth factor, and (EGF. The primary sources of PDGF are platelets, endothelial cells, and the placenta. Two isoforms of this protein are present in the human body and both of these have one glycosylation site and three disulfide bonds. Examples of growth factors used as biopharmaceuticals are the following:

1. Osigraft/Eptotermin alfa (bone morphogenetic protein) is used for the treatment of tibia fractures, is grown commercially in CHO cells, and was first approved in 2001 in Europe.
2. InductOS/Dibotermin (bone morphogenetic protein) is used for tibia fractures and in spinal surgery; it is also commercially grown in CHO cells. This product was first approved in Europe in 2002.

Hormones

Insulin, glucagon, gonadotropins, and growth hormones are the most well-known therapeutic hormones. The first biopharmaceuticals that obtained approval by regulatory agencies were insulin and recombinant human growth hormones. These were produced in microbial cells. The commercial recombinant forms of the gonadotropin family of hormones are Gonal-F, Luveris, Puregon, and Ovitrelle. All these are produced using CHO cells and are used for treating female infertility.

Therapeutic enzymes

A number of recombinant therapeutic enzymes are expressed in mammalian cells. Tissue plasminogen activator (tPA) is a thrombolytic agent involved in dissolving blood clots. Recombinant tPA is commercially is known as Alteplase and Tenectplase, which are used for the treatment of acute myocardial infraction.

Fabry disease, a genetic metabolic disorder, is characterized by a lack of enzyme α-galactosidase A. Fabrazyme (approved in 2001) is a recombinant

TABLE 14.7 Various therapeutic proteins produced in animal cell lines.

Cell line	Therapeutic protein	Potential application	Product name	Approval (FDA)
CHO	tPA	Acute myocardial infraction	Activase	1987
BHK	Factor VIII	Hemophilia A	Kogenate FS	1993
Sp2/0	Immunoglobulin GI	Rheumatoid arthritis, Crohn's disease	Remicade	1998
BHK	Factor VIIa	Hemophilia A + B	Novo Seven	1999
HEK-293	Activated protein C	Severe sepsis	Xigris	2001
Hybridoma CHO	Immunoglobulin G2a	Non-Hodgkin's lymphoma	Bexxar	2003
CHO	Immunoglobulin GI	Colorectal cancer	Avastin	2004
CHO	Humanized IgG	Cancer	Perjeta	2012
CHO	Humanized IgG1; DM1	Cancer	Kadcyla	2013
CHO	Humanized IgG4κ	Cancer	Keytruda	2014
CHO	Human IgG4κ	Cancer	Opdivo	2014
NS0	Humanized IgG1	Cancer	Empliciti	2015
CHO	Human IgG1κ	Cancer	Darzalex	2015
CHO	Humanized IgG1 Fab	Hemostasis	Praxbind	2015
CHO	Human IgG1	Cancer	Bavencio	2017
CHO	Humanized IgG1/κ	Asthma	Fasenra	2017
CHO	Human IgG1λ	Psoriasis	Tremfya	2017
CHO	Glycosylated IgG1	Multiple sclerosis	Ocrevus	2017
CHO	Human recombinant IgG1	Rheumatoid arthritis	Kevzara	2017

α-galactosidase A and is produced by genetically modified CHO cells.

Blood coagulation factors

Hemophilia A is caused by the lack of blood-clotting factor VIII, hemophilia B is caused by deficiency of factor IX, and hemophilia C by lack of factor XI. Factor VIII and IX are proteins. The first recombinant factor VII products were Recombinate and Kogenate, which were expressed in CHO and BKH cells, respectively. Recombinant factor FIX is commercially sold as BeneFIX and is produced in recombinant CHO cells.

Antibodies

Therapeutic antibodies are used in the treatment of cancer, cardiovascular disease, infections, and autoimmune diseases. In 2004, the antibody Avasin (Bevacizeimab) was approved for the treatment of metastatic colorectal cancer. This antibody acts as an inhibitor of vascular endothelial growth factor. Zenapax, another commercially available antibody, is used during prophylaxis for preventing the rejection of transplanted organs. This is commercially grown in the NSO cell line and was approved for human use in 1997.

Gene therapy

Importance of cell culture in gene therapy

Gene therapy involves the insertion, removal, or alteration of a therapeutic or working gene copy to cure a disease or defect or to slow the progression of a disease, thereby improving the quality of life. The human genome map was the first major step toward a new way of addressing human health and illness. Gene therapy holds great promise, however, the task of transferring genetic material into the cell remains an enormous technical challenge and requires ex vivo cell cultivation and adaptation from the lab to a clinically relevant state. The development of animal cell culture technology is imperative for advances in gene therapy.

Monogenic diseases caused by single gene defects (such as cystic fibrosis, hemophilia, muscular

dystrophy, and sickle cell anemia) are the primary targets of human gene therapy.

The first step in gene therapy is to identify the faulty gene. This is followed by gene isolation and generation of a construct for correct expression. Integration of the gene followed by delivery of the genetic material in vivo or ex vivo is crucial to the success of gene therapy. In in vivo therapy, the genetic material is introduced directly into the individual at a specific site, and in ex vivo treatment, the target cells are treated outside the patient's body. These cells are then expanded and transferred back to the individual at a specific site. The ex vivo technique involves gene therapy in the cultured cells, which are expanded and subsequently transferred to the targeted tissue.

Clinical correlation

A number of clinical studies and trials for gene therapy have already been approved and are being conducted worldwide. From 1989 up to the present, about 500 clinical studies have been reported; 70% of these studies are intended for cancer treatment.

The first product designed for gene therapy was Gendicine, a medication produced by Shenzhen Sibiono Genetech, China. Gendicine is used for head and neck carcinoma treatment. The tumor 4 suppressing gene p53 in recombinant adenovirus expresses protein p53, which leads to tumor control and elimination. SBN-cel is a cell line that was subcloned from the human embryonic kidney (HEK) cell line 293 and has been used for the production of Gendicine.

Biopesticides

In recent years, biopesticides have gained importance due to increased concerns about agrochemicals and their residues in the environment and food. Biopesticides provide an effective means for the control of insects and plant disease, and they are environmentally safe. The biological control of insect pests by another living organism (in order to suppress the use of pesticides) is an age-old practice. Presently, a number of biological controls are being used as biopesticides. With the high cost of chemical-based pesticides and the development of resistance to multiple chemical pesticides, baculoviruses are one of the most promising biocontrols for insect pests and have been increasingly used effectively against caterpillars worldwide. However, the major impediment in the development of baculoviruses as biopesticides is the high cost and small volumes of in vitro methods. Development of an in vitro production process for large quantities of baculoviruses at comparable costs to chemical pesticides will help provide insect control that is safe, efficacious, cost-effective, and environmentally safe.

Baculovirus production in animal cell culture

A number of factors are important for a successful commercial production of bioinsecticides:

1. Large-scale production of viruses at competitive costs.
2. Economic production of viruses (i.e., low cost for the media and running the culture).
3. Effective cell line with high virus per cell productivity.
4. With passage of the virus into cells, there is a loss of virulence and an increased risk of mutant formation; this should be avoided.
5. The quality of the polyhedral produced in the cell culture should be comparable to those obtained from caterpillars.

The insect baculovirus cell system offers a number of advantages. It produces recombinant proteins that are functional and are immunologically active, as it is able to make post-translational modifications. The recombinant system uses a powerful promoter polyhedron.

Cell lines for biopesticide production

The most commonly used cell lines in biopesticide production are the Sf21 and Sf9 cell lines, which are derived from ovarian tissues of the fall army worm (*Spodoptera frugiperda*). Sf9 cells show a faster growth rate and higher cell density than Sf21 cells and are preferred. High Five cell lines (designated BTI-Tn-5BI-4) established from *Trichoplusia ni* embryonic tissue are also being used.

Viral mutant formation in cell culture

The continuous culturing of cells for virus production leads to virus instability and the so-called passage effect. This can result in a decrease of virulence and polyhedral production and a variety of mutations. All these changes affect commercial production in vitro. Two types of mutations are commonly seen in continuous passaging of cell cultures for viral productions: (1) defective infective particles (DIPs) and (2) few polyhedral (Fp) mutations.

Fp mutations are characterized by (1) reduced polyhedral, (2) enhanced production of BV, and (3) lack of

occluded virions in polyhedra. All these factors reduce the infectivity of the target pest.

Spontaneously generated Fp mutants have been reported in AcMNPV (*Autographa California* nucleopolyhedroviruses) (Wood, 1980), *Galleria mellonella* nucleopolyhedroviruses (GmMNPV) (Fraser and Hink, 1982), and *Helicoverpa armigera* nucleopolyhedroviruses (HaSNPV) (Chankraborty and Reid, 1999).

DIP mutations are the formation of DIPs. They occur due to serial passaging for long periods, which results in a decrease in the filtering of infectious virus. DIPs have been reported in a number of animal virus systems and in baculovirus systems. DIP formation can be avoided by low multiplicity of infection. This minimizes the probability of the defective virus entering the cell with an intact helper virion.

Monoclonal antibodies

The majority of antibodies available on the market today are produced in animal cell cultures (Van Dijk and Van de Winkle, 2001). Animal cells are preferred because they are capable of glycosylation and structural conformation, which is essential for a drug to be productive. Hybridoma technology has been the most widely used method for small- and large-scale production of mABs. However, these antibodies have limited therapeutic applications since they produce an adverse immune response on repeated use.

A number of cell lines are now being used for the production of recombinant antibodies. The CHO lines are the most commonly used. Other cell lines used are marine myelomas NSO, Sp 2/0, HEK-93, and BHK.

A number of factors influence the production of mABs. For a high concentration of mAB production, the cell line should have high productivity. For high protein productivity, it is important that the selected cell line be productive in order to avoid large reaction volumes and the high cost of protein purification. Cell lines with the capacity to grow without anchorage offer an advantage in terms of scaling up the process; it is much simpler than with those designed for the growth culture of anchorage-dependent cells. Sp2/0 and NSO cell lines can grow naturally in suspension; other cells such as CHO and BHK can be easily adapted to this form of cultivation.

Stem cells

Stem cells are unspecified cells that have the potential to differentiate into other kinds of cells or tissues and become specialized cells. The two characteristics that define stem cells are their ability of self-regenerate and to differentiate into any other cells or tissues. These cells have the capability to renew themselves to form cells of more specialized function. In recent years, stem cell research has been hailed as a major breakthrough in the field of medicine. This property of turning a cell into any other specialized function cell has made researchers believe that stem cells could be utilized to make fully functional, healthy organs to replace damaged or diseased organs.

Culturing embryonic stem cells in the laboratory

Human embryonic stem cells (hESCs) are grown on nutrient broth. These cells are traditionally cultured on mouse embryonic fibroblast feeder layers, which allows continuous growth in an undifferentiated stage. The mouse cells at the bottom of the culture dish provide a sticky surface to which the cells can attach. In addition, the feeder cells release nutrients into the culture medium. Researchers have now devised animal-free culture systems for hESCs and have used human embryonic fibroblasts and adult fallopian tube epithelial cells as feeder layers (in addition to serum-free mediums).

More recently, methods to subculture embryonic cells without the feeder layer have been developed. Martigel from BD Biosciences has been used to coat the culture plate (Hassan et al., 2012) for effective attachment and differentiation of both normal and transformed anchorage-dependent epithelioid and other cell types. This is a gelatinous protein mixture isolated from mouse tumor cells.

Microfluidics three-dimensional culture

A major milestone in the biological sciences was the establishment of the tissue culture technique that can both maintain and propagate the growth of living cells under sterile in vitro conditions. Traditional cell cultures, which are two-dimensional (2D), are grown as monolayer cultures on a flat and rigid surface. Since their development, several advancements have been made to improve cell culture media as well as the biological materials used for culturing. The improvements have proven valuable for cell-based study due to their amalgamation of various modern analytical techniques, such as fluorescence, electrochemistry, and mass spectroscopy. 2D cell culture does not provide an adequate in vivo environment, where other cells surround the cells in a three-dimensional (3D) ECM (Edmondson et al., 2014). Cells under in vivo conditions both produce and continuously consume oxygen nutrients and other molecules, and such dynamic distributions are not mimicked in conventional 2D cell cultures. Moreover, 2D cell cultures fail to recapitulate

the highly complex 3D environment, function, and physiology of living tissues, the multitudinous regulatory interactions from surrounding tissue cells, the ECM, and other systemic factors that lead to nonpredictive data of an in vivo response (Li et al., 2012). The limitations of 2D cell culture systems have recently become more evident. Recent standard protocol advances in the fields of quantitative and system biology and imaging technology have allowed analysis of individual cells and observation of live individual cells growing in a natural physiological 3D environment. Cells cultured in a 3D model system more closely mimic in vivo conditions. Thus, unlike 2D cell cultures, which can sometimes cause misleading and nonpredictive data of in vivo responses, 3D systems are realistic for translating study findings. Compared to the 2D cell culture system, the 3D cell culture system provides a physiologically relevant and closer biomimetic environment, promotes better cell differentiation, and improves cell function (Edmondson et al., 2014). The 3D culture system holds great promise for applications in various fields, such as cancer cell biology, stem cell research, drug discovery, and various cell-based analyses and devices. While this culturing model offers state-of-the-art technology for facilitating drug development and numerous other applications, several hurdles remain before a universal, standardized, and validated system can be established (Sung et al., 2014). Recent developments in the transition from 2D to 3D cell cultures indicate promising applications for many industries; however, the cost of automation and easy-to-use readout systems are still key concerns.

The 3D cell culture system has provided a powerful tool that mimics a highly complex and dynamic in vivo environment, and it has gained greater momentum with the integration of microfluidic technology. Microfluidics is a technology characterized by the manipulation of fluids at the micron-scale for the improvement of diagnostics and cell culture research. It uses microfluidic devices to manipulate fluids in the small capillaries or microchannels. Microfluidics is a science of manipulating, mixing, monitoring, and analyzing minute volumes of fluids or gases on the surfaces of chips and microfluidic chips. This technology is ideal because it recreates the microenvironment of the vasculature and has become a powerful tool in cell culture research. It encompasses knowledge of the biological sciences, chemistry, physics, and engineering applications (Xu and Attinger, 2008). The microfluidic 3D cell culture model also allows precise spatial control over the gradients and medium exchange. It not only mimics but also promotes several biologically relevant functions not seen in the 2D cell culture. Furthermore, it has been increasingly used to generate high-throughput cell culture models and has shown considerable promise for improving diagnostics and biological research (El-Ali et al., 2006).

Notably, microfluidic cell cultures are potential candidates for next generation cell analysis systems. Several 3D-based cell culture approaches have been created to provide a better biomimetic microenvironment for cells than those of 2D cultures. In addition, crucial liquid handling steps, including cell loading, nutrient supply, and waste removal—under physiologically relevant conditions—can be performed with real-time microscopy (Xu et al., 2014). Numerous microfluidic devices have been developed to not only provide nutrients and oxygen continuously for cell proliferation but also to investigate several characteristics of a dynamic 3D cell culture, such as differences in concentration, temperature gradients, and shear force conditions on cell transport and cultivation. Numerous microfluidic platforms for 3D cell culturing have been developed and based on the substrates used for microdevice fabrication, including glass/silicon-based, polymer-based, and paper-based platforms. Polydimethylsiloxane (PDMS)-based microdevices are the predominant form of microfluidic 3D cell culture systems because they are economical and allow permeability of O2, which is vital in cell proliferation. To provide an in vivo-like environment that resembles living tissues, several natural polymers, such as collagen, fibrin, and agarose, have been used to fabricate microfluidic devices (Li et al., 2012).

Applications

Microfluidics technology has emerged as a viable and robust platform for tissue engineering—a multidisciplinary field aimed at replacing and repairing damaged and diseased tissues and/or organs and developing in vitro models to mimic physiological conditions. Successful clinical applications include the development of organ-on-a-chip technology—a microfluidic perfusion device for regenerative medicine—and a chip-based platform for the culture of cells and toxicological studies.

Organ-on-a-chip technology

Scientists currently rely on in vitro cell culture platforms and in vivo animal models to study biological processes and develop therapeutic strategies, although informative have significant shortcomings (Ziółkowska et al., 2011). In vitro platforms may not simulate the intricate cell−cell and cell−matrix interactions that are vital to regulating cell behavior in vivo (Guillouzo & Guguen-Guillouzo, 2008). Organ-on-a-chip devices could offer biological relevance and be a requisite for high-throughput applications. An organ-on-a-chip is a microfluidic cell culture device comprising a microchip with continuously perfused chambers that are

infused with living cells that are arranged to mimic the 3D tissue microenvironment and physiology (Ghaemmaghami et al., 2012). These chips have the potential to significantly impact drug discovery and toxicity testing (Ghaemmaghami et al., 2012). The simplest functional unit of organ-on-a-chip devices consists of a single, perfused microfluidic chamber that is composed of a single type of cultured cell. These systems are utilized for studying organ-specific responses, chemical responses, such as drugs or toxins, and physical stimuli. In a complex system, two or more independently perfused parallel microchannels are connected by porous membranes to recreate interfaces between different tissues.

Tissue models on a chip

Numerous tissue models have been developed to mimic in vivo biological processes. On-chip tissue models include those for the liver, kidney, lungs, intestines, muscle, fat, and blood vessels as well as models of tumors.

Liver-on-a-chip

Various chemicals and drugs, when administered over a long period, result in adverse effects and acute liver toxicity, known as hepatotoxicity (Gershell & Atkins, 2003). In vitro models used for identifying drug-induced liver toxicity have drastically limited utility. Therefore, efficient and reliable tools for testing liver toxicity are required. Microfluidics devices for liver tissue and cells that can maintain metabolic activity and can be used for drug discovery and toxicity studies have shown great potential for solving this problem.

Bioreactors with a perfused multiwell plate device were developed by Domansky et al. (2010) to recapitulate both the physiological and mechanical microenvironments of hepatocytes that can support both growth and functional integrity for up to 1 week. Khetani and Bhatia (2008) developed microscale cultures of human liver cells in a multiwell micropatterned co-culture system that can maintain phenotypic functions of liver cells for up to several weeks.

Tumor-on-a-chip

A significant challenge for cancer research is the early detection and development of in vitro strategies for studying the role of drug-carrier design in tumor transport and therapies for targeting rapidly dividing cancer cells while leaving normal, healthy cells untouched. The microfluidics tumor-on-a-chip platform can be used for detecting circulating tumor cells (CTCs) in blood flow, which may lead to early diagnosis of cancer (Millner et al., 2013). A variety of designs for studying the microenvironment of microfluidic devices that culture solid and liquid tumors were reviewed by Young (2013). Tatosian and Shuler (2009) developed a novel microfluidic system to study the multidrug resistance of cancer cells to chemotherapeutic combinations. Jang et al. (2011) fabricated a microfluidic device with an active injection system that produced 64 of 100 combinations of different chemical solutions at various concentrations and stored them in isolated chambers. To optimize system parameters for varied types of cancer cells while requiring minute amounts of reagents and cells, Jedrych et al. (2011) generated a microfluidics system for photodynamic therapy-based measurements. This system allows light-induced photosensitizers to be delivered to the carcinoma cells, which—on reaction with oxygen—produce a chemical toxin that is lethal to tumor cells.

World Wide Web resources

1. http://www.fda.gov
   http://www.fda.gov/downloads/biologicsbloodvaccines/guidancecomplianceregulatoryinformation/guidances/vaccines/ucm202439.pdf
   http://www.fda.gov/BiologicsBloodVaccines/Vaccines/ApprovedProducts/ucm205541.htm
   The Food and Drug Administration (FDA or USFDA) protects and promotes public health through the regulation of all foods (except meats and poultry), the nation's blood supply, and other biologics (such as vaccines and transplant tissues). Drugs must be tested, manufactured, and labeled according to FDA standards before they can be sold or prescribed.
2. http://www.promega.com
   http://www.promega.com/~/media/files/products%20and%20services/na/webinars/mechanism%20of%20toxicitywebinar2.pdf?la=en
   Promega manufactures enzymes and other products for biotechnology and molecular biology.
3. http://www.who.int
   http://www.who.int/biologicals/publications/trs/areas/vaccines/cells/WHO_TRS_878_A1Animalcells.pdf
   The World Health Organization (WHO) is a specialized agency that is concerned with international public health. It is affiliated with the United Nations and headquartered in Geneva, Switzerland. WHO ensures that more people, especially those living in dire poverty, have access to equitable, affordable care, so that they can lead healthy, happy, and productive lives.
4. http://amgenscholars.com

http://amgenscholars.com/images/uploads/contentImages/biotechnology-timeline.pdf

Amgen Scholars provides hundreds of undergraduate students with the opportunity to engage in a hands-on summer research experience at some of the world's leading institutions.

5. http://monographs.iarc.fr
   http://monographs.iarc.fr/ENG/Monographs/vol90/mono90-6.pdf
   The IARC monographs identify environmental factors that can increase the risk of human cancer. These include chemicals, complex mixtures, occupational exposures, physical agents, biological agents, and lifestyle factors.
6. www.iptonline.com
   http://www.iptonline.com/articles/public/IPTFIVE76NP.pdf
   IPTonline publishes "The Pharmaceutical Technology Journal," which is designed to provide information on the latest ideas, cutting-edge technologies, and innovations shaping the future of pharmaceutical research, development, and manufacturing.
7. http://www.aceabio.com
   http://www.aceabio.com/UserFiles/doc/literature/xcell_appnotes/RTCA_AppNote07_ACEA_LoRes.pdf
   ACEA Biosciences, Inc. (ACEA) is a privately owned biotechnology company. ACEA's mission is to transform cell-based assays by providing innovative and cutting-edge products and solutions to the research and drug discovery community.

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Glossary

Antigen Any substance that causes your immune system to produce antibodies against it.
Aseptic Free from pathogenic microorganism.
Cell culture To grow in vivo
Cytotoxicity The degree to which an agent has specific destructive action on certain cells.
Differentiation A change in a cell causing an increase in morphological or chemical heterogeneity.
Immortalized Changing a cell type with limited lifespan in vitro into a cell type with unlimited capacity to proliferate; sometimes achieved by animal cells in vitro or by tumor cells.
In vitro Cell growth outside the body, in glass, as in a test tube.
In vivo Cell growth in a living organism.

Medium A buffered selection of components in which an organism naturally lives or grows.
Monolayer A single layer of adherent cells on substratum.
Passage The process of passing or maintaining cells through a series of hosts or cultures.
Primary culture A culture initiated from an explant of cells, tissues, or organs in media conducive to their growth.
Trypsinization Use of the enzyme trypsin to remove adherence proteins from a cell surface.

Abbreviations

AcMNPV	*Autographa California* nucleopolyhedroviruses
BHK	Baby Hamster kidney
CD4	Glycoprotein on the surface of Helper T cells that serve as a receptor for HIV
CDM-HD	Chemically defined medium
CHO	Chinese Hamster ovary
CPE	Cytopathogenic effect
DIP	Defective infective particle
DME	Dulbecco's modified eagle's media
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
Fp	Few polyhedral mutations
FPERT	Real-Time fluorescent product-enhanced reverse transcriptase assay
GF-AFC	Glycyl-phenylalanyl-amino-fluorocoumarin
GmMNPV	*Galleria mellonella* nucleopolyhedroviruses
HaSNPV	*Helicoverpa armigera* nucleopolyhedroviruses
HBcAg	Hepatitis B core antigen
HBV	Hepatitis B virus
HEK	Human embryonic kidney
HeLA	Established human epithelial cell line derived from cervical carcinoma
hESCs	Human embryonic stem cells
Hi-5	Cells (BTI-TN-5B1–4) derived from the parental *Trichopulsia ni* cell line
HPV	Human papilloma virus
HPV18	Human papilloma virus 18
IFN	Interferon
IL-2	Interleukin-2
L1 VLP	HPV with L1 major capsid protein
LDH	Lactate dehydrogenase
mAB	Monoclonal antibody
MEM	Minimum essential media
MMT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MP12	Strain invented by serial mutagenesis of RVF virus with Egyptian ZH501 and ZH548 strains
MTS	3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
PDGF	Platelet-derived growth factor
PTM	Post-translation modifications
QPERT	Quantitative real time for fluorescent product-enhanced reverse transcript assay
rDNA	Recombinant DNA
RT	Real-time assays
SNS	Smithburn neurotropic strain
STO	Mouse embryonic fibroblast cell line
TGF	Transforming growth factor
TGF-B	Transforming growth factor beta
tPA	Tissue plasminogen activator
VLPs	Virus-like particles

Long answer questions

1. What are the components of serum and how do they help the cell culture?
2. What is the role of media in animal cell culture?
3. What are the advantages and limitations of animal tissue culture?
4. How can cell viability and cytotoxicity be tested in cell culture?
5. What is the role of cell culture in gene therapy and viral vaccines?
6. How can microfluidics revolutionize animal tissue culture?

Short answer questions

1. What is the Hayflick effect?
2. What is the source of cells for primary monolayer cell culture?
3. Serum is one of the basic components of cell culture media (true/false)?
4. What was the first recombinant human protein?
5. What are the different phases of the growth curve?
6. Is the VLP-based HPV vaccine approved by the FDA?

Answers to short answer questions

1. Limited replication capacity of cells in culture medium.
2. Organ/tissue of live animal.
3. False.
4. Somatostatin.
5. Lag phase, log phase, and plateau phase.
6. Yes, Gardasil (the first HPV vaccine) was approved by the FDA in 2006.

Yes/no type questions

1. Are cells obtained directly from organs and tissues in primary cell culture?
2. Is secondary culture used for studying transformed cells?
3. Is identity testing a way to determine purity of culture?
4. Is IFN-α used for the treatment of multiple sclerosis?

5. Is Bevacizumab approved for the treatment of colorectal cancer?
6. Does passage effect leads to an increase in the virulence of cultured viruses?
7. Do stem cells can not differentiate into other kinds of cells?
8. Microfluidic devices provide nutrients and oxygen for cell proliferation.
9. Living cells are used in organ-on-a-chip microfluidic cell culture.
10. Can embryonic cells be cultured without any feeder layer?

Answers to yes/no type questions

1. Yes—Mechanical, chemical, or enzymatic disintegration of tissues and organs is required in primary cell culture.
2. Yes—Secondary cultures are used in the study of transformed cells as these cultures maintain their cellular characteristics.
3. No—For testing the purity, one should use fluorescent staining PCR or ELISA.
4. No—IFN-β is used in the treatment of multiple sclerosis.
5. Yes—It is an inhibitor of vascular endothelial growth factor.
6. No—Passage effect leads to viral instability.
7. No—Stem cells can differentiate into other kind of cell types.
8. Yes—Microfluidic devices also help in investigating characteristics of 3D cell culture.
9. Yes—Chambers of organ-on-a-chip devices are continuously infused with living cells.
10. Yes—Martigel from BD biosciences can be used to coat the culture plate.

CHAPTER

15

Concepts of tissue engineering

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Summary

Tissue engineering is a multidisciplinary science aimed at improving the lost functions of injured or damaged tissue. It offers a new hope for balancing the need and availability of organs for transplantation. It is a unique approach that utilizes the combined knowledge of various disciplines like biology, engineering, and so on, to develop new tissues and bio-artificial organs.

What can you expect to know?

Tissue engineering is a new and evolving branch of the life sciences. It uses a multidisciplinary approach to achieve its goals. Transplantation biology has evolved to a great extent to solve the problems related to injured or lost organs. The major limitation to reaping the benefits of transplantation biology is the limited availability of organs for transplantation. It is assumed and hoped that one day it will be possible to exploit the technology of tissue engineering to develop new organs as needed.

The present chapter introduces the elementary concepts of tissue engineering. The initial part focuses on the fundamental principles of tissue engineering and introduces the materials and techniques for the fabrication of scaffolds; the latter part discusses applications in selected tissues, issues and challenges, and a future course for the technology. This chapter also discusses the salient features of some of the issues related to the ethical aspects of tissue engineering as well as the translational significance of this developing discipline.

Introduction

Wear and tear of tissues and organs is a natural process that becomes rapid with age. Apart from this, in accidental cases and diseases, patients may either lose organs or face malfunction of tissues/organs, causing a severe threat to life. Conventional treatment options of such conditions include organ transplantation, surgical repair, artificial prosthesis, mechanical devices, and drug therapy. Tissue engineering has emerged as a new concept that promises the regrowth of tissue structures using cells and natural or synthetic materials.

Tissue engineering is comprised of two words: tissue and engineering. A "tissue" is a group of different types of cells that have different phenotypes but together perform a specific function; the term engineering refers to the application of knowledge to design and build. Thus, in a broad sense, tissue engineering is a multidisciplinary science that deals with the application of knowledge to design and construct tissues. Tissue engineering can be defined as *an interdisciplinary field that applies the principles of engineering and the life sciences to the development of biological substitutes that restore, maintain, or improve tissue functions*. The fundamental goal of tissue engineering is to create a three-dimensional mass of cells of a specific tissue that exhibit its some or whole characteristics, and which can be used to augment the desired function of a tissue. Tissue engineering can be categorized into (1) in vitro construction of bioartificial tissue from donor cells, and (2) in vivo modification of cell growth and function. The first category applies to the replacement or augmentation of malfunctioning tissues or organs, while the second category implies in situ regeneration. Tissue-engineered organs not only provide clinical solutions, but can also be used as "models" to study cell–cell or cell–tissue interactions, cell

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migrations, drug toxicity, and a number of other biological mechanisms.

History

The roots of the concept of tissue engineering can be traced back to depictions in early paintings. One of the earliest references in an artistic work is the famous painting known as "Healing of Justinian" in which St. Cosmas and St. Damien are shown doing a transplantation of a homograft limb onto a person, possibly a soldier. A better understanding of nature led to experiments in the regeneration of a living being. Paracelsus, a German-Swiss scientist who lived during the fifteenth century, tried to create life using a recipe of different chemicals. Goethe (eighteenth century) envisioned the creation of life by means of nonliving objects. The transformation of tissue regeneration from fiction to reality was made possible by the advent of clinical and basic sciences surgery, transplantation, cell biology, biochemistry, etc. The use of wooden legs and metallic dentures can be considered earlier biomaterials for clinical purposes. The use of skin grafts can be considered the modern phase of tissue engineering. In the eighteenth century, Dieffenbach performed some clinical experimental work on skin transplantation. Later, Bünger successfully performed autologous skin transplantation. In the twentieth century, Ullman performed the first kidney transplantation in animals and Merrill in humans. Green in the 1970s tried to regenerate cartilage by utilizing bone as a scaffold with chondrocytes. Yannas and Burke used skin cells on a collagen scaffold to make a skin equivalent for burn patients. The famous article of Dr. Robert Langer and Dr. Joseph Vacanti about tissue engineering in the journal science is considered the official beginning of this area of work.

Basic approach to tissue engineering: principles and methodology

In native tissues, the cells of different lineages are organized into three-dimensional forms and are surrounded by the natural extracellular matrix (ECM) that is secreted by them. Its basic components include collagen, elastin, proteoglycans, glycosaminoglycan, glycoproteins, and hyaluronic acid. The ECM creates a special environment in the spaces between cells and helps bind the cells in tissues together. It is also a reservoir for enzymes, inhibitors, cytokines, and many hormones and growth factors controlling cell growth and differentiation so that each tissue formed has specific characteristics and performs specific tasks in the body. Due to the diversity, organization, and distribution of constituents in the ECM, cells exhibit differential gene expressions in specific tissues. In tissue engineering, we try to recreate the artificial environment of ECM around the cells of specific tissues. For the simplest tissue engineering experiments, one requires cells, scaffolds, media, and sometimes bioreactors to scale up.

Cells

The source of the cells has a tremendous effect on the success of tissue engineering. The cells can be autologous, allogeneic, and xenogeneic. Autologous cells obtained from the individual into whom they will be implanted require prior harvesting followed by expansion in culture. Allogeneic cells are obtained from an individual of the same species other than the recipient. Xenogeneic cells are derived from individuals of different species. Allogeneic and xenogeneic sources of the cells pose challenges like host rejection, disease transmission, and ethical issues, although they are readily available in advance of need.

Scaffolds

The artificial architectures for biosynthetic ECMs are designed so that they can direct the cells to maintain a three-dimensional organization and lead the development of new tissues with suitable function. The cells should be induced by the scaffold and can replace it with newly synthesized cell products. A large number of natural and synthetic polymers have been tried in making scaffolds for tissue engineering. Details about the scaffolds are provided in the next section.

Media

Media supplement the nutrition to cells and also complement the scaffolds to provide a complete bio-artificial extracellular matrix. The choice of medium depends upon the type of cells and the aim of the culture. Media can be divided into (1) seeding media, (2) differentiation media, and (3) maintenance media. The seeding media is the media used to introduce the cells into the scaffolds. It may or may not contain the serum. Generally, the cells remain in seeding media from 6 to 24 hours. The differentiation media contains a number of growth factors, cytokines, etc., to allow the cells to differentiate into the desired tissue. The maintenance media has serum with basic components to support the cells in culture.

Bioreactors

The requirement of a large number of cells and bigger engineered tissues (cell–polymer constructs) make

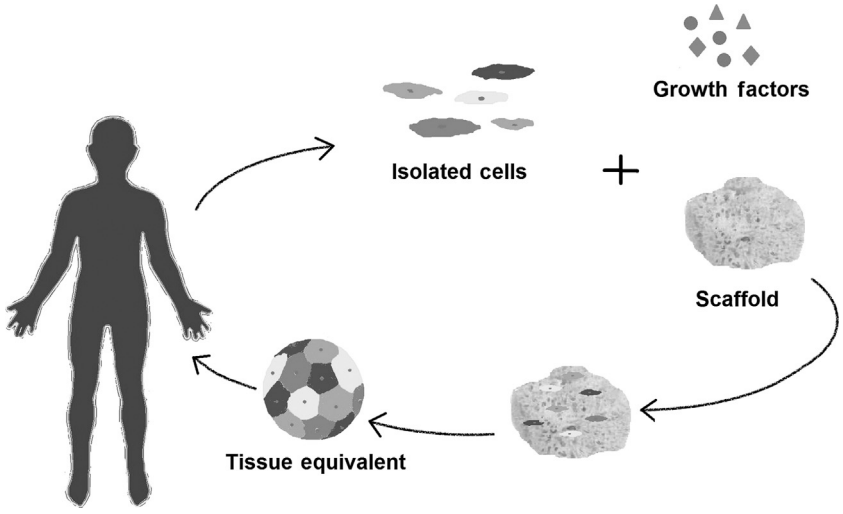


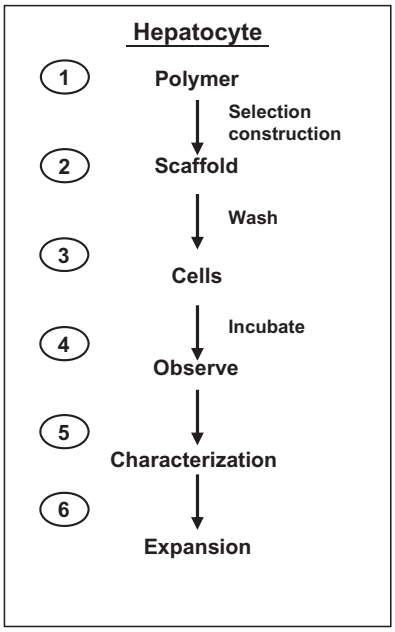
FIGURE 15.1 Basic approach to tissue engineering.

the use of bioreactors imperative in tissue engineering. Bioreactors provide a steady and controlled microenvironment for the development of functional tissues from cell–polymer constructs. Ideally, a bioreactor should help in the uniform distribution of cells in the scaffold. It should maintain the required nutritional and oxygen concentration. A number of bioreactor designs have been proposed, like simple static or mixed spinner flasks, rotating vessels, perfusion chambers or columns. Some bioreactors are also equipped with a mechanical stimulator for providing mechanical signals to the growing tissue. A number of modifications in the designing of bioreactors have been suggested by the researchers. Starting from miniature bioreactor where the tubular polymeric fibers suspended in media help to grow cells in 3D to hydrostatic pressure reactor.

Methodology

The simplest strategy for engineering any tissue is to isolate cells from the tissue of interest, followed by making a single-cell suspension, growing these cells onto a three-dimensional synthetic extracellular matrix (commonly known as a "scaffold") with appropriate growth factors, and allowing them to form a three-dimensional mass with definite biochemical or phenotypic characteristics of that tissue (Fig. 15.1). This type of cell culture is known as a *three-dimensional cell culture*. This is different from a *monolayer culture* where the cells occupy the culture vessel surface to form a cellular sheet-like structure.

The simplest workflow is to select the right polymer, right cells and media, and correct/reliable assessment of the characteristics of the tissue equivalent (Flow Chart 15.1). As given in Flow Chart 15.1, if one wishes



FLOW CHART 15.1 Crucial steps for using hepatocytes for tissue engineering.

to make a tissue equivalent of hepatocytes to model the liver, one has to fabricate the three-dimensional porous scaffolds of any suitable polymer (e.g., chitosan or alginate). The hepatocytes will be isolated from the liver biopsy or from the liver of experimental animals (e.g., rat or mouse). Hepatocytes are separated by a perfusion method. The perfusion medium or solution contains an enzyme cocktail. The most common is collagenase; alternatively, one can buy a readymade medium available from different vendors, like Gibco. The major purpose of perfusion is to loosen the cells so

that hepatocyte removal, collection, separation, and isolation become convenient. Cell death is a major concern during liver perfusion and can be optimized in various situations. Isolated and purified hepatocytes are seeded with scaffolds and incubated for a week. During incubation, cells start growing into the tissue-like structures known as spheroids. Such spheroids are characterized for the liver functions where both surface markers and metabolic functions are screened. Some of the common markers for hepatocytes are albumin, alpha-feto protein, glucose-6-phosphatase, tyrosine aminotransferase, etc. These hepatocyte markers can be confirmed for mRNA by either reverse transcriptase polymerase chain reaction or any other method that depends upon the expertise and availability of the resource. The protein levels of the same markers can be confirmed by various methods, like western blot, FACS and so on. If required, after confirmation, these cultures can be further expanded and used for the intended purpose. Toxicity testing is the one example that we would like to note here.

Scaffold design

For creating an engineered tissue, the choice of scaffold material is important, as it has to mimic the natural ECM. A number of properties have been recognized that are essential for the design of an ideal scaffold (Table 15.1). These include biocompatibility, an interconnecting porous structure, appropriate surface chemistry for the growth of cells, easy fabrication, mechanical strength, biodegradability, and bio-resorbability; they depend on the intended application. Biocompatibility of the material is the foremost requirement as the scaffold directly interacts with the cells in vitro and tissues in vivo after implantation. Thus, the material should not only be able to support the growth of the cells, but should also be immunologically the least reactive in order to be used inside the body. An interconnecting porous structure increases the surface area of the scaffold and allows more cells to be seeded on and inside it. The porous system maximizes cell-to-cell interactions and helps in the reorganization of a three-dimensional mass of the cells. In addition, the pores also allow diffusion of molecules that can be exploited to provide nutrients and gases to the cells entrapped within them. Another important consideration in scaffold design is surface chemistry. The surface of the scaffold material should have appropriate functional groups that can support the adherence of the cells to it. This property determines the overall architecture of the cell mass. In native tissues, a number of matrix proteins mediate cell adhesion by interacting with the appropriate cell adhesion molecules (CAMs) present on the surface of the cells. Among them, the integrin family is the most abundant and versatile. The integrin proteins interact with the RGD (R: arginine; G: glycine; D: aspartic acid) motifs of many matrix proteins, including vitronectin, fibrinogen, von Willebrand factor, collagen, laminin and are firmly anchored in the tissue. In the same manner, the materials should have cell-adhesive functional molecules to hold the cells on them. These groups can also direct the cells to grow into a specific tissue. Furthermore, the scaffold should also possess proper mechanical strength as it experiences biomechanical forces during cell adhesion and proliferation. In specific applications like vascular tissue engineering, scaffolds have to withstand hemodynamic forces due to the flow of blood. Biodegradation and bioresorbability of a material are the properties that are exploited during in vivo implantation of the scaffold. These properties cause the actual integration of the in vitro grown cells into the body and augmentation or replacement of the injured tissue. The scaffold material is gradually degraded by the enzymes of the body and replaced by natural ECM secreted by the cells. The products of the degraded scaffold material are removed from the site or are utilized as metabolites in other biochemical reactions.

TABLE 15.1 Few considerations for designing an ideal scaffold.

Biocompatibility
Interconnecting porous structure
Appropriate surface chemistry
Mechanical strength
Biodegradability and bioresorbability

Materials for scaffolds

In native tissues, the extracellular matrix promotes the development of proper tissue structure and function, provides mechanical support for the developing tissues, localizes cells to their respective places, and regulates the expression of tissue-specific genes. In tissue engineering applications, a successful strategy involves the appropriate design and fabrication of scaffolds that can serve these functions and promote new tissue formation from cultured cells. Among all the available biomaterials (Table 15.2), polymers remain the most widely used materials for scaffolds because their properties can be changed by altering their monomers. Both synthetic and natural polymers have been used in the construction of scaffolds. Both groups have advantages as well as disadvantages. Natural polymers are abundant, usually

TABLE 15.2 Materials for scaffolds.

S. No.	Material	Example
1	Natural polymers	Proteins−collagen−gelatin−fibrin−silk polysaccharides−cellulose- chitosan−alginate−agarose−hyaluronic acid
2	Synthetic polymers	Poly (lactic acid)−poly (glycolic acid) −poly (Є-caprolactones) −poly (ortho esters) −polyethylene−polyester urethane−polysulfone−poly(tetrafluoroethylene) −poly(vinyl chloride)
3	Ceramics	Hydroxyapatite−calcium−phosphates−alumina−bioglass−ceramics

biodegradable, and sometimes contain groups similar to natural ECM components that can interact with the cells; however, their complexity often renders modification and purification difficult. Their batch-to-batch variation may also pose a problem. They can guide cells to grow at various stages of development.

Synthetic polymers can be formed in various compositions and their properties can be adjusted, but they generally lack biocompatibility. Therefore, the choice of the material very much depends on the application. Synthetic polymers include aliphatic polyesters; for example, poly (lactic acid), poly (glycolic acid) and their copolymers, poly (ε-caprolactones), poly (ortho esters). Some important considerations that should be kept in mind while choosing the correct material for the fabrication of scaffolds according to different applications include biocompatibility, controlled degradability and bioresorbability, biomimeticity, mechanical stability, and sterilizability.

Another class of materials used for the fabrication of scaffolds is ceramics. These are the materials of metallic and non-metallic elements held together by ionic and/or covalent bonds. Ceramics have been widely used due to their biocompatibility and resemblance to the natural inorganic components of hard tissues like bone and teeth. Due to their high mechanical properties, they are used in load-bearing applications, but their disadvantage lies in their brittle nature.

Scaffold fabrication methods

Preferably, the fabrication process of a scaffold should not affect the biocompatibility of the material, should allow control over porosity and pore size, may give the desired shape and size, and should not alter the physical, chemical, and biological (if any) properties of the materials. In recent years, various methods have been developed to construct scaffolds of desirable properties. These methods range from traditional techniques like solvent casting or fiber bonding, to modern computer-based design and fabrication technologies like 3D printing. Each method has its own advantages and disadvantages (Table 15.3). The choice of method depends on the applications and requirements of the tissues as no technique is ideal.

Fiber bonding

As the name implies, the polymer fibers (e.g., polyglycolic acid) are bonded together in three-dimensional structures. The method forms highly porous scaffolds with large surface areas. Although fiber bonding forms porous scaffolds with interconnected pores, it requires a solvent, which if it remains in the scaffold, may be cytotoxic. To overcome this problem, the scaffolds are vacuum-dried and sometimes involve heating at high temperature. A number of techniques are used to generate polymeric fibers. These include wet spinning, melt spinning, and electro spinning. Wet spinning is used for fiber-forming substances that have been dissolved in a solvent. In melt spinning, the fiber-forming substance is melted for extrusion through the spinneret and then directly solidified by cooling. Electro spinning consists of spinning polymer solutions or melts in high electric fields. The process is based on the principle that strong electrical forces overcome weaker forces of surface tension in the charged polymer liquid. It is an inexpensive process that can be easily scaled up, employing multiple spinnerets. It is versatile in that almost any soluble polymer can be processed into nanofibers.

Solvent casting and particulate leaching

In this technique the polymer is dissolved into methylene chloride or chloroform and is poured into a petri dish filled with a porogen-like salt. The solvent is evaporated, and the polymer with salt is kept in the water, which dissolves the salt and leaves the porous scaffold. Poly (L-lactic acid) and poly (DL-lactic-co-glycolic acid) (PLGA) scaffolds have been formed by this method. The porosity and pore size can be controlled with a suitable porogen. Scaffolds created are of defined pore size, surface, volume ratio, and crystallinity. The technique is applied to polymers dissolvable in solvents like chloroform. The residual solvent in the scaffold may denature any incorporated bioactive molecules.

Melt molding

This technique utilizes the powdered polymer mixed with an aqueous soluble porogen, which is poured into

TABLE 15.3 Selected advantages and disadvantages of various fabrication processes.

S. No.	Fabrication process	Advantages	Disadvantages
1	Fiber bonding	Porous large surface area	Few polymers can be used toxicity of residual solvent poor mechanical strength
2	Solvent casting/particulate leaching	Control on pore size Crystalline	Few polymers can be used toxicity of residual solvent
3	Melt molding	No use of solvent Control on pore size	Functionality of natural polymers are compromised
4	Membrane lamination	Control on pore size	Poor mechanical strength, Poor interconnectivity
5	Phase separation	Incorporation of bioactive substances	No control over architecture
6	Freeze drying/ lyophilization	Incorporation of bioactive substances	Samples are hygroscopic
7	Gas foaming	No use of solvents	No control on pore size
8	Polymer ceramic composite foam	Good mechanical properties	Residual solvent and porogen
9	Steriolithography	Accuracy for imparting small features	Only for photopolymerizable and liquid polymers
10	Selective laser sintering	No use of solvents better compressive strength automated	Functionality of natural polymers are compromised
11	Fused deposition modeling	No use of solvents better compressive strength automated	Functionality of natural polymers are compromised
12	3D printing	Choice of materials automated and high resolution	Poor mechanical strength solvent toxicity
13	Pressure assisted micro-syringe	Automated and high resolution	Poor mechanical strength viscosity dependence

a Teflon mold. The mold is heated above the glass transition temperature (Tg) of the polymer for an appropriate amount of time. The polymer porogen composite is removed and kept in water to dissolve the porogen.

The porous polymeric scaffold is formed to the desired shape and size. PLGA scaffolds have been constructed using this technique with gelatin as the porogen. The technique does not employ any solvent and is carried out at a relatively low temperature; for non-amorphous polymers, high temperature is required, which prevents the incorporation of bioactive molecules. The porosity and pore size can be controlled, and the desired shape is obtained by choosing the correct mold.

Membrane lamination

In this method, porous polymeric membranes are used in the fabrication of contour plots of specific three-dimensional shapes. The shapes of the contours are cut from these membranes. The membranes are adhered to one another (using chloroform) in the desired three-dimensional shape. This method is useful only when the original porous structure is preserved, and the boundaries between two layers should not be differentiated. The technique offers independent control of shape and porosity, but the interconnectivity of pores is limited and there are generally poor mechanical properties.

Phase separation

In this technique, the polymer is dissolved into a suitable solvent at a relatively low temperature. Phase separation is triggered by altering the physical parameters (like temperature). This causes the formation of one phase rich in polymer and other in solvent. The resulting phases are then quenched to create a two-phase solid. The solidified solvent is then removed by sublimation, and porous polymeric scaffold is obtained. This method has been developed in order to deliver drugs, proteins, or growth factors without altering their functions. The advantage of this technique lies in its ability to incorporate bioactive substances and drugs without losing their functions; on the other hand, there is no control of the internal architecture.

Gas foaming

This technique does not use any solvent, but instead uses high-pressure gas. The polymer (PLGA) is compressed into a mold to form a solid structure. These

solid structures are saturated by high-pressure CO_2 gas. Gradually, the pressure is reduced, which causes pore formation in the scaffold. In another modification, a gas-foaming technique has been used in combination with a particulate leaching method. Here, PLGA was mixed with salt particles (porogen) and compressed to form solid disks. The porous structure was obtained due to gas foaming and particulate leaching.

Polymer ceramic composite foam

This technique was developed to form high mechanical strength scaffolds for hard tissues like bone. In this method, ceramics like hydroxyapatite fibers are incorporated into poly (α-hydroxy ester) polymer (Thomson et al., 1998). To evenly mix polymer and ceramic, a solvent-casting technique is used. The method offers excellent mechanical properties and control over porosity, but residual organic solvent and porogen pose problems.

Solid-free form techniques

In the traditional methods discussed so far, there is no precise control over shape, size, thickness, porosity, and internal architecture. To address these problems, computer-based approaches have been developed to form scaffolds of predefined architecture and geometry. In these solid free form fabrication techniques, the material is taken in powder or liquid form and solidified in layers defined by a computer program. These technologies include selective laser sintering, stereolithography, fused deposition modeling, and 3D printing.

Selective laser sintering

This technique is solvent-free and uses heat energy to form scaffolds of predefined shapes using polymeric particles. In this method, a laser beam is directed toward a polymeric powder layer; it increases the temperature of the powder, which causes fusion of the polymeric particles. In this way, a patterned architecture is formed. It has been used to make scaffolds of polymer-coated powdered calcium phosphate.

Stereolithography

This is based on a photo-polymerization process that requires a photo-initiator. The technique makes use of a laser light beam focused on predefined regions of a liquid polymer layer, solidifying the exposed regions. This has been used to generate scaffolds of diethyl fumarate and poly (propylene fumarate). Although the technique produces precise and accurate scaffolds, its use is limited to only photopolymerizable liquid polymeric materials.

Fused deposition modeling

In this technique, a 3D scaffold is deposited layer-by-layer through a nozzle that is attached to a device connected to a computer. The technique is solvent free, and the scaffolds show good compressive strengths. There is better control of the X and Y-axes than the Z-axis. Poly (ε-capro-lactones) scaffolds have been constructed using this technique.

3D Printing

This method uses solvent or adhesive materials to bind polymers. An adhesive solution is deposited onto a polymeric powder bed using an inkjet printer. This method has been combined with particulate leaching methods to form porous scaffolds. The method is automated and forms highly porous scaffolds, but resolution cannot be reduced below the particle size of the polymer. Few advantages of 3D bio-printing in the manufacturing of a scaffold for tissue engineering applications include rapid-fabrication, high-precision, and customized-production.

Pressure-assisted micro-syringe method

In this method, the polymer is dissolved in a solvent and is deposited through a syringe fitted with a 10- to 20-μm glass capillary needle. The thickness can be controlled by changing the syringe pressure, solution viscosity, and syringe tip diameter.

Freeze-drying

In this method, the polymer is homogenized in its solvent and water. The prepared emulsion is frozen at subzero temperatures. The frozen sample is put in a dedicated instrument (a lyophilizer), which sublimates the solvent with water in a vacuum. This not only creates highly porous scaffolds but the porosity can also be controlled. Another advantage is that since the whole process takes place at very low temperature, any biological molecules attached to the scaffolds do not lose their structure and function. Moreover, the prepared scaffolds can be stored at room temperature; however, since the scaffolds are hygroscopic, they have to be kept in a vacuum desiccator.

Freeze drying involves the removal of water or other solvent from a frozen product by a process called *sublimation*. Sublimation occurs when a frozen liquid goes directly to the gaseous state without passing through the liquid phase. In contrast, drying at ambient temperatures from the liquid phase usually results in changes in the product and may be suitable only for some materials. However, in freeze drying, the material does not go through the

liquid phase, and it allows the preparation of a stable product.

Examples of tissue-engineered organs

Skin

Skin is one of the most researched areas in tissue engineering as wound healing continues to be a major burden to patients, with the successful commercialization of several FDA-approved products. Examples of tissue-engineered skin products are Epicel (Genzyme Tissue Repair, MA), which is an epidermal cell sheet; Integra (Integra Life Sciences, NJ); Dermagraft (Advanced Tissue Sciences, CA); and Alloderm (Lifecell Corp., TX). Some of these are dermal substitutes. Organogenesis Inc., MA, has also introduced human skin equivalent (HSE) that has epidermal and dermal tissue components. The commercially available bioengineered skins are providing a great support in the healing of burns. Clinically, it has been observed that such skin products are effective in healing when the auto- or allografts fail.

Strategies to engineer skin tissue involve epidermal sheet fabrication, dermal replacements, or composite structures with both epidermal and dermal components. Collagen, being the major ECM component of skin, has been the most important natural polymeric scaffold for skin tissues. Recently, a new approach was suggested to produce a skin graft based on human acellular dermal matrix which was revitalized internally by human fibroblasts and keratinocytes on the surface (Labuś et al., 2019).

Pancreas

Diabetes is a major disease that causes morbidity and mortality worldwide. According to WHO, around 280 million people will have this disease by 2025. Most of the research work in pancreatic tissue engineering has been focused on encapsulating pancreatic islet cells. The polymeric encapsulation system should be able to protect the cells from the host's immune system by providing isolation from the host's immune system. This can be achieved by controlling the pore size of the polymeric capsule. There must be proper diffusion of oxygen and nutrients across the immunoisolated capsule for the cells. The capsules should be mechanically stable and biocompatible inside the body. Early work used alginate and poly(L-lysine) polymers for encapsulating islets. Another new technology is micro-physiological systems (MPS) or organ-on-a-chip technology which enables a closer approximation of the human organs and tissues by recreating tissue microenvironment. Lee et al. designed a three-organ MPS consisting of pancreas, muscle, and liver, to recapitulate glucose metabolism and homeostasis (Lee et al., 2019).

Liver

The liver is a vital organ that performs a large number of functions like metabolism and detoxification. The hepatocytes perform the functions of metabolism and detoxification. The liver has a very high regenerative capacity, and it fights continuous damage to hepatocytes due to their exposure to toxic substances. In a diseased condition, the regeneration process is compromised, leading to infection and bleeding risks, and ultimately liver failure.

The development of liver assist devices (LAD) is the major focus of liver tissue engineering. LADs consist of primary hepatocytes arranged similar to the liver on polymeric scaffolds in bioreactors. The design of the LAD should be such that it provides maximum hepatocyte viability and functionality. To meet these conditions, nutrients and gases should reach the cells, and toxins and waste should be effectively removed. LADs should be efficient in transportation of gases, nutrients, metabolites, and toxins.

Kidney

A tissue-engineered kidney should have a glomerulus and tubule to compensate for excretory functions. A synthetic membrane with suitable permeability is the key to the successful development of a bio-artificial kidney. A polymeric hollow fiber design has been shown to mimic glomerular filtrations. A bio-artificial renal tubule has been designed using epithelial progenitor cells and polymeric membranes. The combination of filtration devices and tubule has paved the way to a future bio-artificial kidney.

Bone/cartilage

Orthopedic defects usually hinder normal biomechanics and the structural strength of bone/cartilage. Many times, the correction of defects requires major surgical intervention in which healing is slow and there is a high chance of infection with no surety of complete reversal of the defect. Therefore, the search for surgical alternatives is critical. The approach to engineering bone tissue is to take a suitable polymeric scaffold with native tissue, and culture osteoblasts over it. Since bones and cartilage are hard tissues, the mechanical property of the material should match the native tissue. For implantation, the scaffold should support vascular networking, and the shape of the

scaffold should complement the defective site. Ideally, the body should resorb the material. Ceramics are the material of choice. Efforts are also made on the engineering of musculoskeletal joints by co-culturing skeletal muscles and bone (Wragg et al., 2019).

Nerves

Any injury of nerves is a serious clinical problem that may lead to major loss of nerve function, affecting the quality of patient's life. Presently, nerve autograft is commonly used to reconstruct the nerve gap. Such surgery has many disadvantages including donor site morbidity and limited availability. Neural tissue engineering focuses on the development of suitable scaffolds to support connecting a larger gap and facilitating nerve generation. The approach to engineering nerve tissue in situ is to fabricate a cylindrical structure using polymers and entubulate cut nerves. These cylindrical structures are known as nerve conduits or guidance channels. They guide the outgrowing nervous tissue to regenerate nerve cables. They also prevent the invasion of scar tissue. Regeneration of nerves can be improved by adding supporting cells like Schwann cells.

Blood vessels

The development of a reliable and efficient engineered blood vessel is important considering the large number of heart bypass surgeries that are performed each year. Early blood vessel constructs were built by co-culturing smooth muscle cells and endothelial cells using scaffolds of collagen.

The blood vessel should be elastic and should have enough mechanical strength to sustain the pulsatile flow of blood. It should be non-thrombogenic and should demonstrate patency for a considerable period of time. It should be well tolerated by the immune system. Grafts are influenced by the host tissue environment (including the hemodynamic environment); therefore, long-term resistance to hyperplasia is a major challenge (besides long-term immuno-compatibility and patency).

Tissue engineering using stem cells

Stem cells have the properties of self-renewal and differentiation. These properties have been used for engineering tissues like skin, bone, liver, pancreas, and cornea. In the skin tissue engineering approach, keratinocytes with epidermal stem cells were cultured to form the epidermal sheet. These sheets were kept on dermal substitutes composed of a scaffold seeded with dermal fibroblasts. Such skin tissue equivalents have the capacity to regenerate full thickness wounds.

In bone regeneration, skeletal stem cells can be isolated and cultured on three-dimensional scaffolds of ceramics that can be transplanted in vivo at the bone defect area. The adult mesenchymal stem cells (MSCs), which can be isolated from bone marrow, can be expanded in culture in an undifferentiated state. These MSCs have been used to engineer connective tissues. MSCs are considered good candidates for tissue engineering provided they are cultured with appropriate growth factors and cytokines that promote differentiation on suitable scaffolds. It has also been observed that MSCs secrete immunosuppressive molecules that help with immunological tolerance.

Embryonic stem cells (ESC) have both pros and cons. On the one hand, they can be generated in large quantities in the laboratory in an undifferentiated state, on the other hand, their ability to differentiate efficiently is questionable. Embryonic stem cells have been used to construct synchronously contracting engineered human cardiac tissue containing endothelial vessel networks. The constructed tissue consisted of cardiomyocytes, endothelial cells, and embryonic fibroblasts. The vessels were further stabilized by mural cells that originated from embryonic fibroblasts. There are numerous articles describing the potential of stem cells in tissue engineering. Stem cells not only provide a better cellular option that can be differentiated as per specific needs, but the molecules secreted by them have been found to be helpful in integrating the transplanted tissue into the body.

Issues and challenges

There are certain issues and challenges involved with the development and application of tissue engineering and its benefits to human health. The most significant challenge with tissue engineering is the shelf-life of the tissue-engineered product, along with the compatibility of the tissue-engineered product with the immune system of the recipient.

The use of a non-autologous source of cells has been an issue of great concern. The presence of fetal bovine/calf serum in the medium also poses risks of infection and a strong immune response in the host body. The cells designated for transplantation should be cultured in the xenofree media with either human serum components or synthetic components. Another challenge concerns the standardization of an isolation technique for the cells that ensures no contamination, but still produces of a large number of cells without compromising their functionality and phenotype. It is still a major obstacle in tissue engineering to produce cells of the same quality from lot to

lot. In the case of embryonic stem cells, and allogeneic and xenogeneic cells, there is also a high risk of immune rejection due to different genetic constitutions contributing foreign phenotypic expressions.

Quality control of the materials used for engineering tissue is also a matter of key concern. The material should be produced using strict manufacturing practices. The polymeric scaffolds used for making a tissue-engineering construct should be integrated into the implanted site. Their biodegradation should occur at an optimum pace, and the by-products should not produce any unwanted effects. Monitoring of the adverse effects of the by-products of biodegraded scaffold material should be closely observed, but might be challenged by the collective effects of the cells with scaffolds.

Clinical trials also have certain challenges. There is a strong possibility that the adverse events might not be detected for a long time during the clinical trials, so determining the end point might be costly. Data produced might be (to some extent) uncertain with respect to long-term effects. Post-trial follow-ups will be required for the observation of actual effects.

Ethical issues

Tissue engineering is advancing in the form of regenerative medicine. There is no doubt that tissue engineering can offer solutions for many degenerative disorders. On the one hand, it will improve the quality of life of those who suffer from these diseases, on the other hand, it raises various concerns about the ethics of this new and emerging branch of science. Some of the major issues are consent from the tissue provider. In other words, would it be ethical to give one person's tissue to another without his/her consent? Even the consent discloses the identity of the person, which could lead to various social, religious, and personal issues. Should personal and religious views be considered for someone who is in desperate need of health care or treatment? There is always the possibility that transfer of tissue from one human to another could lead to additional clinical conditions, like a fatal infection.

This leads to all sorts of questions for a profound technology such as tissue engineering. Did the procedure save the patient or create more trouble? Can the donor ask for property rights? Should the donor get a share from the commercialization of the product that originated from them? What if a downtrodden individual decides to sacrifice himself or herself and exploit this technology for profit? Where to stop? When to stop? What to stop? Who should stop? Why should we stop? There are bound to be numerous ethical issues. Hopefully, with time, they can be resolved.

Translational significance

Tissue engineering itself is a translational science that has innumerable applications. The information gathered through tissue engineering studies regarding the development and maintenance of any tissue or organ could be great, and could lead toward the development of new organs. There is even a possibility that some xenogenic tissue can be modified using different types of technology that make it acceptable to an entirely a different host. If this type of approach is made possible, it will most certainly be a great relief to most terminal patients in need of an organ. Tissue engineering has great potential to reverse the aging process, which would help reduce the cost of medication and would provide the elderly a better quality of life. This technology has the potential to treat deformities from accidents, burns, gun-shot wounds, etc.

World wide web resources

There are numerous resources on the web for information about tissue engineering and regenerative medicine. A simple web search (e.g., with Google) yields all sorts of information about tissue engineering, everything from the basics, to different methods, protocols, and specific applications. PubMed (maintained by NCBI) can be searched for more scientific and validated information. Apart from various vendors in the field of tissue engineering (who also provide useful information on their websites), the following three sites offer excellent information on this subject.

www.tissue-engineering.net:

A useful website that defines applicable terminology and provides the latest news, job postings, and various other updates in the field of tissue engineering.

http://news.discovery.com/tech/tags/tissue-engineering.htm:

Updated information about the current happenings in tissue engineering.

http://tej.sagepub.com:

The *Journal of Tissue Engineering* is an open access journal.

https://www.nibib.nih.gov/science-education/glossary: Glossary for useful terms.

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Glossary

Biocompatibility A measure of how a biomaterial interacts in the body with the surrounding cells, tissues, and other factors.
Biomaterial Any matter, surface, or construct that interacts with biological systems. Biomaterials can be derived from nature or synthesized in the laboratory using metallic components, polymers, ceramics, or composite materials. Medical devices made of biomaterials are often used to replace or augment a natural function. Examples include heart valves, hip replacements, and materials used regularly in dentistry and surgery.
Bioreactor A manufactured or engineered device that provides an environment that supports biological processes. Many bioreactors are used to grow cells or tissues for use in tissue engineering.
Extracellular matrix (ECM) The ECM is a collection of extracellular molecules secreted by support cells that provides structural and biochemical support to the surrounding cells.
Hydrogel A biomaterial made up of a network of polymer chains that are highly absorbent and as flexible as natural tissue. Hydrogels have a number of uses such as scaffolds for tissue engineering, sustained release drug delivery systems, and biosensors that are sensitive to specific molecules such as glucose.
Mesenchymal stem cells A term used to define non-blood adult stem cells from a variety of tissues.
Scaffold A structure of artificial or natural materials on which tissue is grown to mimic a biological process outside the body or to replace a disease or damaged tissue inside the body.
Tissue engineering An interdisciplinary and multidisciplinary field that aims at the development of biological substitutes that restore, maintain, or improve tissue function.

Abbreviations

3D	Three dimensional
CAM	Cell adhesion molecules
ECM	Extra cellular matrix
ESC	Embryonic stem cells
FACS	Fluorescence activated cell sorting
HSE	Human skin equivalent
LAD	Liver assist devices
mRNA	Messenger ribonucleic acid
MSC	Mesenchymal stem cells
PLGA	Poly-lactic and glycolic acid
RGD	R: arginine; G: glycine; D: aspartic acid
Tg	Transition temperature

Long-answer questions

1. What is tissue engineering? How is it important for human health?

2. What are scaffolds? Describe the various materials used to fabricate scaffolds.
3. Describe the various fabrication techniques for making scaffolds.
4. How are stem cells useful in tissue engineering?
5. What are the current challenges to taking tissue-engineered products from the bench to the bed-side?

Short-answer questions

1. Define the following terms:
   a. Biocompatibility.
   b. Biodegradability.
   c. Bioresorbability.
2. What are the different criteria for designing an ideal scaffold?
3. Give the name of markers for hepatocyte identification?
4. Why do we use collagenase and other enzymes to perfuse liver?
5. Write short notes on:
   a. Polymers.
   b. Ceramics.

Answers to short-answer questions

1a. Biocompatibility: The ability of any material to exist within a biological system without any harmful effect.

1b. Biodegradability: The ability of any material to be degraded by biological enzymes.

1c. Bioresorbability: The ability of any material to be absorbed by the biological system following degradation.

2. It should be porous with interconnecting pores, biocompatible, should have appropriate functional groups on its surface, appropriate mechanical strength, and, if required, should be biodegradable and bio-resorbable.

3. There are numerous markers that can be used for identification of hepatocytes. One can measure mRNA levels as well as protein levels of the same markers. Some of the most common markers are albumin, alpha-fetoproteins, glucose-6-phosphataase, *trans*-aminotransferse, etc.

4. The main objective of perfusion is to loosen the hepatocytes from the liver so that their identification, isolation, and culture become easier. It is always useful and helpful to use some enzyme during perfusion. Various enzymes can be included in the perfusion buffer. One of the most common enzymes used for the perfusion of the liver is collagenase.

5a. Polymers: These are the materials that have defined monomers linked together in a specific pattern.

5b. Ceramics: These are the materials of metallic and non-metallic elements held together by ionic and/or covalent bonds.

Kindly state yes or no against the following statements

1. In vivo modification of cell growth and function does not fall under Tissue Engineering.
2. ECM is a reservoir for enzymes, inhibitors, cytokines, and many hormones and growth factors.
3. Scaffolds should always be replaced by cell synthesized natural ECMs.
4. PLGA is a natural polymer.
5. Ceramics are used in load-bearing applications and are not brittle.
6. MSCs are better than ESCs for engineering connective tissues.
7. Xenogenic cells are best for transplantation.
8. Glass transition temperature is an important criterion for melt molding process.
9. Sublimation is the underlying cause behind freeze drying.
10. A synthetic membrane with suitable permeability is the key to successful development of a bio-artificial kidney.

Answers to yes/no statements

1. No
2. Yes
3. No
4. No
5. No
6. Yes
7. No
8. Yes
9. Yes
10. Yes

CHAPTER

16

Nanotechnology and its applications to animal biotechnology

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Summary

Nanotechnology is advancing at a fast pace with its ramifications felt in almost every field, including animal biotechnology and life sciences. Further growth in its applications to animal nutrition, health, disease diagnosis, and drug delivery is inevitable. This chapter provides an overview of the application of nanotechnology to the study of the structure, mechanics, and biochemistry of animal cells on a nanoscale.

What you can expect to know

Nanotechnology is advancing at a fast pace with its ramifications felt in almost every field, ranging from materials science to food, forensic, agricultural, and life sciences, including biotechnology and medicine. Nanotechnology is already being harnessed to address many of the outstanding problems in animal biotechnology. In the next decade we expect to see further growth in its applications to animal biotechnology (e.g., animal nutrition, health, disease diagnosis, and drug delivery).

The aim of this chapter is to provide an overview and related examples of the application of nanotechnology (which concerns the manipulation of matter at the atomic and molecular scale) to the study of the changes in the structure, mechanics, chemistry, and biochemistry of animal cells that occur on a scale of nanometers (1 nm = 0.000000001 m). The different nanotechnology techniques (such as atomic force microscopy (AFM), chemical force microscopy (CFM), fluid force microscopy (FluidFM), Near-field Scanning Optical Microscopy (NSOM), and Raman Spectroscopy and Imaging) used in biotechnology to visualize and manipulate cells, biomolecules, and proteins will be briefly explained and practical examples are given and discussed. In addition the main ethical issues related to the use of nanoparticles (NPs) in animal biotechnology will be addressed, and a few examples provided. After reading this chapter, the reader should be well equipped to explore further the fascinating and ever-expanding "nanoworld" and its multifaceted aspects. Nanotechnology is not only our nearest future but also an already existing daily reality (especially in the materials and cosmetics industries) for us all. It is therefore paramount for future generations of scientists to know about new developments in nanotechnology and nanobiotechnology (which is the intersection of nanotechnology and biotechnology).

History and methods

Introduction

The first use of the concepts in "nanotechnology," even before the word existed in the dictionary, was in a talk given by physics Professor Richard Feynman at an American Physical Society meeting at Caltech on December 29, 1959. This lecture was the birth of the idea and the study of nanotechnology. Albert R. Hibbs suggested to Feynman the idea of a medical use for Feynman's micromachines. Tokyo Science University Professor Norio Taniguchi first used the term

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"nanotechnology" in a 1974 paper. Eric Drexler popularized the potential of molecular nanotechnology in the late 1970s and 1980s. Scanning tunneling microscopy was invented in 1981. The first book on nanotechnology, *Engines of Creation* (Eric Drexler), was published in 1986. The atomic force microscope (AFM), which can be considered as being at the heart of nanotechnology, was invented by Binnig, Quate, and Gerber in 1986; and the first commercial AFM was produced in 1989. AFM is one of the foremost tools used for imaging, measuring, and manipulating matter at the nanoscale. What Feynman and Hibbs considered a possibility is becoming a reality 54 years later.

The functionality of biosystems is governed by their nanoscale structure and by the processes that occur at this scale. For a perspective on scale, one nanometer (0.000000001 m) is one-billionth of a meter, which is the same size as a human hair split 100,000 times width-wise; the width of a DNA molecule is ~2 nm. Nanoscale processes have evolved and been optimized in nature over millions of years. For all biological and man-made systems, the first level of organization, in fact, occurs at the nanoscale, where their basic properties and functions are defined.

Nanotechnology provides the ability to work (observe, move, and manipulate) at the atomic and molecular levels, atom-by-atom on a scale of ~1−100 nm to create, understand, and use new materials and devices with fundamentally new functions and properties resulting from their small scale. Nanobiotechnology, on the other hand, is a concourse of nanotechnology and biology where nanotechnology provides the tools and techniques to work from nanoscale principles to investigate, understand, and transform biological systems.

Professor Richard Feynman, a Nobel laureate, delivered his famous after-dinner speech, "*There is plenty of room at the bottom*," at a conference in 1959. Through his charisma and genius he inspired the conceptual beginnings of nanoscience and nanotechnology (while these words were still nonexistent) and laid the foundation for these fields. He talked about "maneuvering things atom by atom" and "doing chemical synthesis by mechanical manipulation." Thanks to the development of the scanning tunneling microscope (STM), the AFM, and other scanning probe microscopy (SPM) techniques, Richard Feynman's ideas have finally been realized. Not only it is now possible to understand the functions and properties of materials and biosystems at the nanoscale, but it is also possible to perform nanomanipulations on them for modern applications in drug delivery, nanosurgery, and nanomedicine. The nanoworld was discovered only about two decades ago, and it was driven mainly by the invention of AFM (Binning et al., 1986) and a variety of other methods for fabricating nanostructures.

Biological systems are in general very sensitive to their environment. For instance a cell's environment in vivo, being considerably detailed and complex, can react to objects as small as a few nanometers, which is ~5000 times smaller than itself. The requirement for cells to get closer to the object leads to many cell−cell adhesions with resulting biological and physiological responses/effects. When cells are taken out of the body to be cultured in vitro, or when external objects/devices such as prosthetics are introduced into the body, cells experience a strange new nanoworld that could be chaotic and detrimental for a cell's survival, but might offer a different or opposite signal to what the cell might normally receive. Similarly cells are exposed to a variety of environmental and age-related stresses resulting from disease, injury, and infection. Mechanisms involved in sensing stress, and the resulting changes in cell surface morphology and physiology, are thus crucial for understanding how cells adapt and survive adverse conditions. For the above reasons alone it becomes important to know even more about how cells react to this nanoworld (nanoenvironment) and how to control them to achieve better functionality of the biosystems.

It is now the right time to introduce the different length scales/regimes. In increasing order they are as follows: nanoscopic (~1−100 nm) < mesoscopic (~100−1000 nm) < microscopic (<1 μm) < macroscopic (~1 mm). While the macroscopic world (the world we see and perceive) can be scaled down through orders of magnitude to the microscopic scale with little or no change in the expected properties, it is not possible to do such scaling when we enter the nanoworld, the gateway to which is ~100−200 nm in dimension. Interfacial and quantization effects begin to play a major role at the nanoscale, and matter behaves differently at such small length scales. Physical properties of materials/biomaterials change as their size approaches nanoscale, where the fraction of atoms present at the surface of the material becomes significant. In contrast for bulk materials larger than 1 μm, the percentage of atoms at the surface is insignificant as compared with those present in the bulk of the material. When a macroscopic device is scaled down to mesosize, it starts to reveal quantum mechanical properties.

There is evidence now in the literature to show that a variety of animal cells do respond to their nanometric environment, which could also originate from nanopatterned substratum/surfaces required to nanofabricate a wide variety of devices (bioresponsive nanomaterials) for applications in animal biotechnology and biomedicine (e.g., nanobiosensors). Although nanofabrication also falls within the realm of the present chapter dedicated to "nanotechnology and its

applications to animal biotechnology," this does not constitute the major theme of this chapter. Nevertheless for completeness we will briefly digress into this topic in subsequent sections.

While macroscopic objects can be viewed with the naked eye, magnification becomes necessary to observe microscale objects (e.g., biological specimens) with high spatial resolution (i.e., how well it is possible to distinguish the fine features of the specimen). A variety of optical/light microscopes have been developed for this purpose, but among other factors, the wavelength of visible light determines the resolution limit. Resolution was improved and a gain in magnification achieved by designing different types of microscopes (e.g., compound microscope, phase contrast microscope, fluorescence microscope, confocal scanning optical microscope, polarized light microscope) or by using UV radiation (shorter wavelength than visible light) at the cost of image quality. The resolution limit reached in light microscopy could be further improved by using electrons with a shorter wavelength, as in scanning electron microscopy (SEM), but the requirements of ultrahigh vacuum and the need to use solid samples are severe limitations in the use of SEM for studying biological specimens without creating artifacts (e.g., due to desiccation). Additionally SEM did not permit the study of biosystems in their native state and under physiological conditions.

Although microscopes have traditionally been tools of vital importance in the biological sciences, the real breakthrough came with the invention of AFM, which is a SPM technique that has grown steadily since the invention of STM by Binning and Rohrer, for which they won the Nobel Prize for Physics in 1986. Because AFM, as opposed to other microscopic techniques, generates images based on the measurement of tiny forces ($\sim$pN between the scanning probe/tip and the sample), it became possible to achieve molecular-level resolutions. AFM can also be performed under fluids, thus permitting samples to be imaged in near native conditions. The ability to exchange and modify the fluid during imaging further allows real-time observing/monitoring of biological processes, something that electron microscopy is still unable to offer. In the meantime, environment scanning electron microscope (ESEM) was developed, which permits the study of biological samples under low vacuum conditions. Integration of different techniques such as AFM with Raman Spectroscopy has added further impetus to nanotechnology research for applications in biotechnology and medicine because it can yield not only the morphology and nanomechanical properties of the biological specimen obtained from AFM, but also simultaneously provides chemical identification (chemical fingerprints) of the samples under investigation. Similarly ESEM combined with energy dispersive spectroscopy (EDS) can simultaneously yield information on both the morphology and elemental composition of the biological specimens under investigation.

In the following section, we discuss methodologies of some relevant nanotools, nanotechniques, and nanodevices.

Methodologies

Nanotools and nanotechniques

In the era of fast developing technology, we are currently witnessing words like nanotools and nanotechniques that are becoming more and more familiar. However, do we really know what they mean? It is obvious that these two terms are related to the word "nanotechnology," which according to the Oxford dictionary means "the branch of technology that deals with dimensions and tolerances of less than 100 nanometers, especially the manipulation of individual atoms and molecules."

While *nanotools* are devices, molecules, and systems that function at the nanometer scale (e.g., nanopowders used to make inks, fuel cells and batteries; nanodots employed to produce plastics, polymers, and computer memories), *nanotechniques* (which stems from the merging of the terms nanotechnology and techniques) are methods used to fabricate (e.g., top-down and bottom-up fabrication techniques), visualize, manipulate (e.g. SPM family) and optically probe (e.g., Raman-based methods) at the nanoscale.

Top-down and bottom-up are two approaches used for the nanofabrication of materials. The top-down approach employs common microfabrication methods to cut, mill, and shape materials into the desired form. Examples are photolithography (i.e., use of light to transfer a photomask onto a light-sensitive coated substrate), a technique similar to the method used to make printed circuit boards routinely used in electronics, and ink-jet printing (i.e., the printer normally used to print out documents, letters, etc.). Conversely, the bottom-up approach uses the chemical and physical properties of molecules to obtain self-organized or self-assembled structures. Although nanofabrication has prevalently been used so far in solid-state physics (e.g., production of electronic components), it has also started to make an appearance in the fields of biophysics and bioengineering. Giving a detailed explanation of nanofabrication and nanotools in this chapter would be straying too far away from the main theme of this book.

SPM techniques

It is appropriate to say that the SPM family is probably the pillar on which the whole nanotechnology

world is built. The well-established techniques that belong to this family are about 30 in number. Among these, the most used techniques for animal biotechnology are AFM, CFM, FluidFM, and NSOM or SNOM.

Atomic Force Microscopy (AFM): It is a very high-resolution (down to a fraction of 1 nm, and thus 1000 times better than the optical diffraction limit) technique that is widely used to characterize the nanoscale surface of samples as different as whole cells or single molecules. In particular in the past 10 years AFM has emerged as one of the most powerful nanotechniques (Canetta and Adya, 2005; Ikai, 2010) in biology. The main reason for the huge success of AFM in biology and biotechnology lies in its ability to permit one to investigate the structure, function, properties, and interaction of biological samples in their culture media. Moreover the extremely high resolution of the AFM allows one to observe the finest details of the samples, such as lamellipodia and cilia in cells (Deng et al., 2010). Another advantage of AFM is the minimal sample preparation requirements, thereby making AFM a very attractive and versatile nanoimaging technique in biotechnology. AFM images can also be processed and analyzed in order to measure at the nanoscale the surface roughness, texture, dimensions, and volumes of biological systems. Hence AFM imaging does allow one not only to visualize a sample but also to measure its dimensions and determine its surface properties. Such measurements are not possible using standard light microscopes or even high-resolution electron microscopes.

We have so far discussed only about the imaging capabilities of AFM. Additionally however AFM can also measure forces between the AFM probe (which is the heart of the AFM instrument) and the sample surface. It is therefore possible to use an AFM in force spectroscopy mode to investigate the mechanical properties of biological samples, such as adhesion, elasticity (property of a material that causes it to be restored to its original shape after applying a mechanical stress), viscosity (resistance of a fluid to deform under shear stress), and viscoelasticity (when materials change with time they respond to stress as if they are combination of elastic solids and viscous fluids, e.g., blood is viscoelastic).

Having discussed about what an AFM can do, our next priority is to understand how an AFM works. The surface of the sample is raster scanned by a probe (the so-called AFM probe) and the local interaction between the probe and the sample surface is measured (Fig. 16.1). Thus the main component (or "heart") of an AFM is the probe, which is called a cantilever with a microfabricated tip. The cantilever obeys the famous Hooke's law, $F = k \bullet x$, where F is the force on the cantilever, k it's the spring constant, and x is its deflection. To measure the cantilever deflection, a laser beam is reflected off the backside of a reflective (gold- or aluminium-coated) cantilever toward a position sensitive detector (PSD, usually a four-segment photodetector).

One of the major applications of AFM in biology and biotechnology is imaging. The advantage of AFM over other high-resolution techniques such as electron microscopy is that a 3D image of the sample can be obtained. Moreover it is possible to analyze an AFM image in order to obtain the dimensions (section analysis, i.e., length, width, and height) of the sample as well as its surface characteristics, such as surface roughness (roughness analysis) and texture.

When the AFM is used in force spectroscopy mode, a force−distance ($F-d$) curve is obtained which shows that the force experienced by the cantilever when the AFM probe is brought in contact with the sample surface (*trace*, also called *approach* cycle) and when separated from it (*retrace*, also called *retract* cycle) (Fig. 16.2). Analyzing the $F-d$ curves by means of theoretical models allows one to study the elastic and adhesive properties of the sample.

An AFM can also be used in force mapping mode, which combines force measurements and imaging. The output of an AFM mapping experiment is a 2D map of

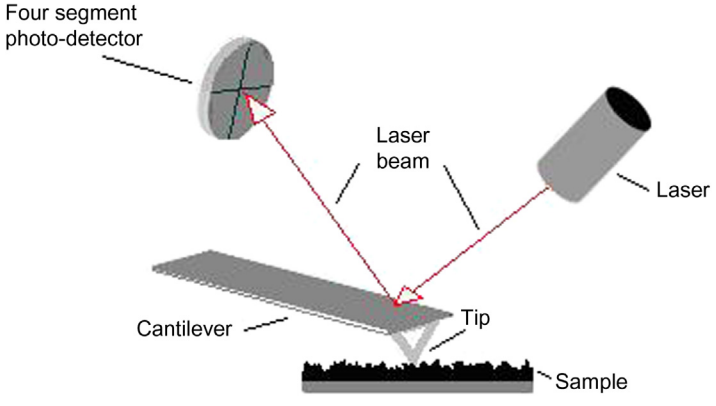


FIGURE 16.1 Operating principle of AFM.

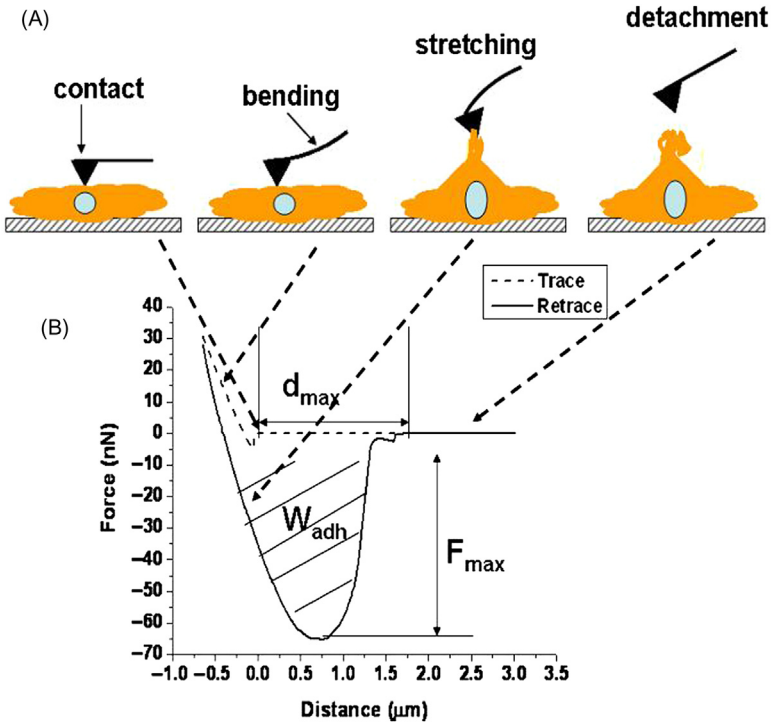


FIGURE 16.2 Sketch of the stages of an AFM-FS experiment: (A) contact between the AFM probe and the sample surface, and cantilever bending with AFM probe indentation occurs during the trace (approach) cycle of the AFM experiment. On the retrace (retract) cycle, the sample surface is stretched until the point of detachment. (B) An example of a typical experimental $F-d$ curve for a trace and retrace cycle is also presented; the stages are indicated by the arrows. The meanings of F_{max} (maximum adhesion force), d_{max} (contact point), and W_{adh} (work or energy of adhesion) are also identified. *Adapted from Canetta, E., & Adya, A.K. (2011). Nano-imaging and its applications to biomedicine, Proceedings of the International Conference on Image Analyses and Processing (ICIAP 2011). In Giuseppe Maino Prof. (Ed.).* Lecture Notes in Computer Science Series (LNCS) *(pp. 1–10). Springer.*

the elastic or adhesive properties of the sample. Fig. 16.3 shows the AFM height image and the corresponding elastic map of a red blood cell.

Flow Chart 16.1 depicts the protocol for AFM imaging, AFM force spectroscopy (AFM-FS), and AFM force mapping (AFM-FM) measurements. AFM data analysis for simple AFM experiments is also shown.

Chemical Force Microscopy (CFM): It (Noy, 2006) is a variation of AFM where the surfaces of the sample and the AFM probe are deliberately functionalized (see Chemical Modification of AFM Probes section) in order to obtain a well-defined tip–sample interface, and therefore to measure well-defined chemical interactions.

The main difference between AFM and CFM is that with AFM the morphology of the sample is visualized by exploiting the "natural" van der Waals forces between the nonfunctionalized AFM tip and the sample surface, whereas CFM uses the "well-defined" interactions between the functionalized AFM tip and the sample surface (which can also be functionalized, if needed).

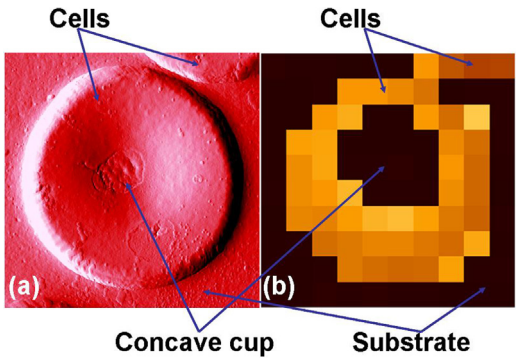
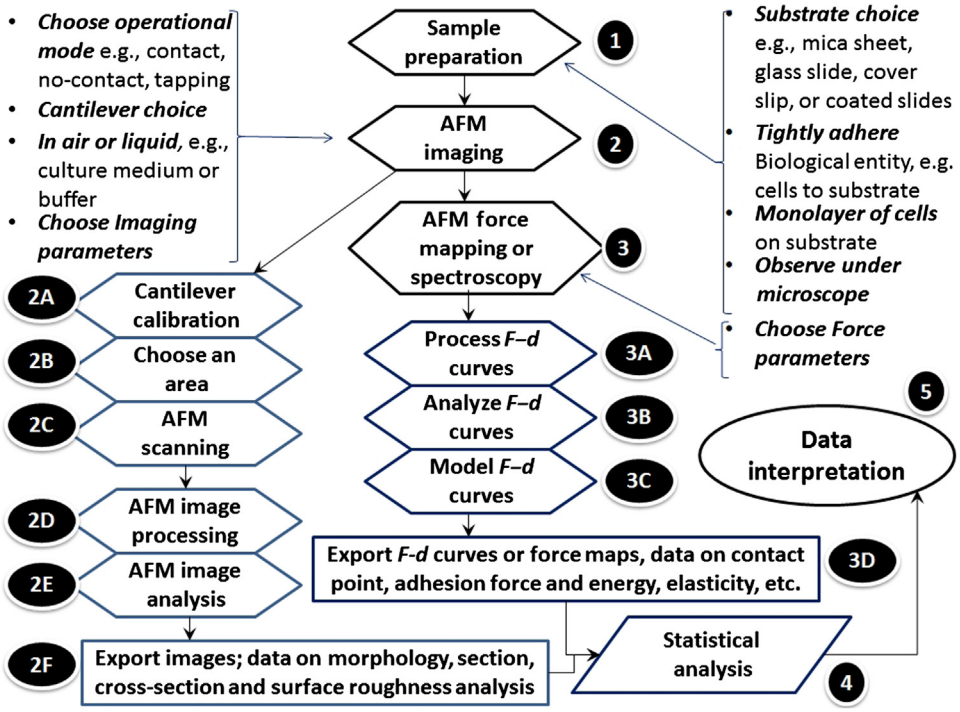


FIGURE 16.3 AFM force mapping on an individual red blood cell: (A) AFM height image of the cell on a glass slide. Note that the typical biconcave shape is clearly visible. (B) AFM elastic map of the cell shown in (a). The difference in elasticity of the glass slide (substrate) and the cell surface allows one to get an elastic map of the cell.

Fluid Force Microscopy (FluidFM): A very recent (2009) implementation of an AFM system is the so-called FluidFM (Doering et al., 2010). This advanced AFM combines the unique features and capabilities of nanofluidics with the high precision in positioning and



FLOW CHART 16.1 Protocol for AFM imaging, AFM force measurements, and data analysis.

the extremely high sensitivity to weak forces of an AFM (Meister et al., 2009). Now the introduction of a new concept: nanofluidics. But what does nanofluidics mean, and what is it used for? Nanofluidics is the study of the behaviors and properties of fluids that are confined in a nanoscale-size environment (for a review on nanofluidics, see Kirby, 2009). Because of the enhanced possibilities created by nanofluidics to control transport phenomena with unprecedented high precision, nanofluidic-based devices have been developed for cell sorting and analytical separations of DNA and proteins. Although nanofluidics is an extremely interesting and important field of research, it is not discussed here in order to keep on course with the main goals of this book. Notwithstanding it needs to be pointed out that the FluidFM system, resulting from a "marriage" of nanofluidics and AFM, has been designed to do the following: (1) "dispense and deliver" active agents from a solution inside a living cell; (2) "inject" NPs and other nanoscale-size materials (e.g. fluorescein isothiocyanate, FTIC dye) inside a living cell by perforating the cell wall, and "extract" genetic materials (e.g., DNA) from the cell nucleus and other cell compartments to be further manipulated; and (3) "pick and place" individual cells to move them from one place to another. This third task entails (for example) putting two cells in physical contact to investigate the phenomenon of cell–cell interaction, which can give invaluable information on how a cell adheres to another cell. This is extremely important information that can be used to better understand the phenomenon of cellular extravasation, which occurs during cancer metastasis, for example.

Near-field Scanning Optical Microscopy (NSOM or SNOM): The advantage of SNOM is that it breaks the far-field diffraction limit and uses the properties of evanescent waves (i.e., stationary waves whose intensity decays exponentially as a function of the distance from the boundary at which the wave was formed) to allow one to visualize nanoscopic structures, such as cells and biomolecules. First let us understand the meaning of diffraction limit and also what it means to break it. It is well known that the resolution of a microscope is limited by the quality of the lenses and also by their alignment or misalignment. However there is another element that affects the quality of the images obtained with a microscope and this is caused by the diffraction of the light. If a microscope can produce images with an angular resolution that is as good as the theoretical diffraction limit ($d = \lambda/2\,n\ \sin\theta$, where d is the size of a feature of the specimen that the microscope can resolve, λ is the wavelength of the light incident on the sample, n is the refractive index of the medium in which the sample is imaged, and θ is the half-angle subtended by the objective used) of the microscope itself, then the instrument is said to be "diffraction limited". Hence the resolution limit (i.e., the distance of the two nearest points) of an image is $\lambda/2$. SNOM can help increase this resolution to a lateral resolution of $\sim$20 nm and a vertical resolution of

~2–5 nm, thereby allowing the finest details of biological objects as small as biomolecules to be visualized. Moreover SNOM is an extremely "gentle" technique because it uses a narrow light beam coming from a fiber tip to "probe" the specimen; therefore there is no physical contact between the probe and the sample.

Although SNOM is a very powerful and nondestructive microscopy technique, its use is not as broad as that of AFM because of the many artifact issues (mainly due to tip breakage while scanning) as well as the very small working distance, the extremely shallow depth of field and the long scan times required for large sample areas.

Raman spectroscopy and imaging

Raman spectroscopy (RS), and more recently, Raman imaging (RI), are extremely powerful techniques for investigating the unique "chemical fingerprints" of solid and liquid samples. RS is a vibrational spectroscopy method (Gardiner and Graves, 1989) chiefly employed to investigate the vibrational, rotational, and other low-frequency modes in a specimen. In the last 25 years RS has been widely used to probe bonds in molecules, to provide characteristic chemical information about the effects of drugs on the cell biochemistry, to understand the mechanisms of cell death and differentiation, and to discriminate between different types of cells, the most common example being the use of RS (Gremlich and Yan, 2001) to distinguish normal and cancer cells. When a sample is shined with light (i.e., a laser) of a certain wavelength, the light interacts with the sample molecules and it gets either absorbed or scattered. Most of the light will be scattered elastically (Rayleigh scattering), namely, no wavelength change occurs, and only 1 photon (quantum of light) in 10^5–10^7 photons will be scattered inelastically, thus resulting in a wavelength change (Raman scattering) (Ferraro et al., 2003). This change in the wavelength of the photon scattered by the sample's molecule is called "Raman shift" and it is characteristic of the molecules the incident light interacts with. Another important point of Raman scattering is that the interaction of the incident photon with the molecule of the specimen under study results in an energy exchange, and thus the energy of the scattered photon can be higher or lower than the energy of the incident photon. This change in the photon energy is directly linked to the change in the rotational or vibrational energies of the molecule with which the photon has interacted. Because these vibrational or rotational energies are specific to a chemical bond or chemical constituent of the molecule with which the incident photon has interacted, it can provide a unique "signature" of that molecule. Please remember that only 1 in 10^5–10^7 photons will be a Raman photon (the one scattered inelastically). Therefore the intensity of the Raman scattering is significantly ($\sim 10^{-5}$–10^{-7} times) weaker than that of the Rayleigh scattering. What this means is that it is not always easy to distinguish the Raman peaks from the so-called "background" generated by the environment surrounding the sample. The background could be caused, for example, by the culture medium in which the cells are kept while investigating the sample with the incident laser beam of a Raman system. To minimize such background and see more clearly the Raman peaks (which are chemical fingerprints of the sample), near-infrared laser (e.g., 785 nm) should be used with enough acquisition time (on the order of minutes) in order to produce sufficient Raman-scattering photons and intense Raman peaks.

A typical Raman setup is built in a way that a laser beam generated by a narrowband laser (i.e., a laser with a linewidth <1 nm) is expanded through a telescope formed by two lenses to completely fill the back aperture of the objective lens, reflected from a 45° holographic notch filter (HNF) to remove the excitation laser light and focused on the sample by the objective lens (usually 50× or 100×). The Raman scatter from the sample is collected by the same microscope objective and filtered by the same HNF. It is then guided toward the spectrometer and acquired via a liquid nitrogen or thermally cooled CCD camera. For Raman imaging, a similar setup is used with the only difference being that the stage of the microscope is moved in the x and y directions by a motor that is synchronized with the spectroscopy CCD camera. In fact in Raman imaging, also known as hyperspectral or chemical imaging, hundreds to thousands of Raman spectra are acquired from all over the specimen and a chemical map recreated that shows the location and amount of different components of the sample. Raman imaging finds its major applications in the study of tissues and individual cells (Fig. 16.4).

The main advantages of Raman spectroscopy and Raman imaging are the very little sample preparation required and the "gentleness" of the "touch of light" on the sample. This makes RS a truly nondestructive and noninvasive technique. In addition, the ability of RS to work both in situ and in vivo, an essential factor when dealing with biological samples, has recently made this technique in great demand for animal biotechnology and life sciences. Moreover RS provides unique information about the molecular identity of the specimen: the "chemical fingerprints," the 3D structural changes in orientation and conformation of biomolecules (e.g., collagen; Bonifacio et al., 2010), the intermolecular interactions, and the dynamics of biological phenomena (e.g., cell death; Okada et al., 2012). The main disadvantages of Raman spectroscopy that need to be kept in mind are, however, the very small

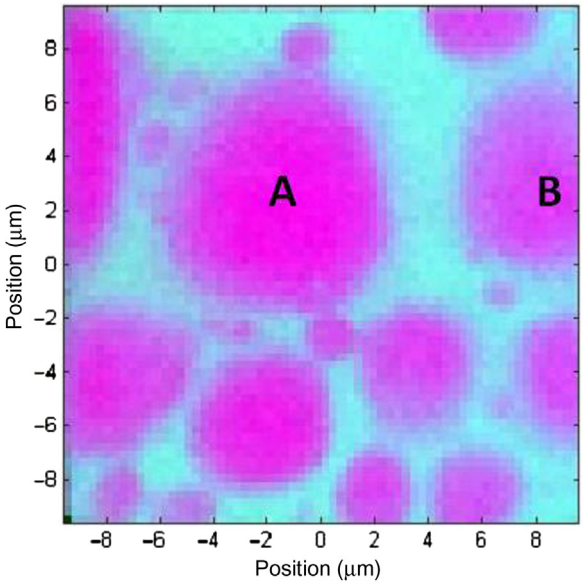


FIGURE 16.4 Raman image of a cluster of lipids in an adipocyte. The light blue color indicates the substrate, whereas the purple "blobs" are the lipid droplets. The different shadows of purple are related to the different amount of lipids inside the lipid droplets. For example, the lipid droplet labeled with (A) is richer in lipids (purple color is brighter) and of a quite homogenous composition (the intensity of the purple color is almost the same everywhere), while the lipid droplet labeled with (B) is less rich in lipids (purple color is faded) and less homogenous (different shades of purple are visible). This image is composed of 4096 Raman spectra, making Raman imaging a quicker method to obtain large data sets compared to Raman spectroscopy.

percentage of Raman scattered photons compared to the elastically scattered photons and the strong fluorescence background. The cumulative effect of these two detriments can sometimes almost completely hide the Raman peaks. To overcome these problems, different types of Raman systems have been developed. Confocal Raman Spectroscopy (CRS), Modulated Raman Spectroscopy (MRS), Surface-Enhanced Raman Spectroscopy (SERS), and Tip-Enhanced Raman Spectroscopy (TERS) are most broadly used for biological applications.

Confocal Raman spectroscopy (CRS): It has the advantage over standard RS of allowing specific spatial analysis deep within transparent samples, such as living cells, by focusing the laser beam at the exact point of interest. CRS offers high depth profiling and it greatly improves the rejection of fluorescence background. The high spatial resolution and precision in laser focusing can be easily achieved by positioning a pinhole of a few microns aperture in the back focal plane of the objective lens. The pinhole will allow only the contribution from the Raman photons to go toward the spectrometer while rejecting contributions from the background.

Modulated Raman spectroscopy (MRS): It (De Luca et al., 2010, Canetta et al., 2011) is a technique recently developed that is able to filter out the Raman spectra from the fluorescence background. The basic principle of MRS is very simple. When the laser wavelength of the excitation laser is continuously modulated, a wavelength shift of the Raman peaks occurs while the fluorescence background remains static, thereby permitting one to observe even very weak Raman features generally hidden by the fluorescence. Moreover MRS reduces the necessary spectra accumulation times and allows real-time applications that are crucial in life science and biology.

Surface-enhanced Raman spectroscopy (SERS): It is an extremely powerful variation of standard Raman spectroscopy (RS) in which the Raman signal of the molecules is amplified by up to 10^{10}–10^{11} times by absorbing the molecules under study on rough metal surfaces (Haran, 2010), thereby allowing easy detection of single molecules and hence making this technique an essential tool in single-molecule spectroscopy.

Tip-enhanced Raman spectroscopy (TERS): Similar to SERS, Tip-Enhanced Raman Spectroscopy (TERS) also takes advantage of the plasmon resonance effects of the surface on which the single molecules under study are absorbed. TERS, in fact, goes even further than SERS because it uses an SPM probe tip as the enhancing surface, thereby permitting Raman scattering to occur only from a nanoscale area. TERS therefore allows chemical analysis and imaging at the *nanoscale*, which holds huge promise in molecular biology (Elfick et al., 2010).

AFM–Raman confocal hybrid systems

Recently, AFM and Raman systems have been combined (integrated) in the so-called AFM–Raman hybrid. These instruments are extremely versatile because they allow one to perform AFM and Raman experiments simultaneously on the same sample. The AFM–Raman hybrid permits researchers to perform nanoscale visualization, manipulation, and determination of mechanical properties by using AFM while allowing chemical analysis (identification) at the submicron scale by performing Raman spectroscopy of specimens at the same time and location. Additionally, the configuration of the hybrid instrument allows one to collect TERS data and conduct SNOM experiments using just one instrument. This is extremely important in biology where the heterogeneity of the samples (e.g., cell populations) requires different experiments to be performed on the same position (e.g., the same cell) to obtain reliable and conclusive results. For example, the use of an AFM–Raman system has the potential to markedly improve the predictability of cell screening. In fact AFM is a surface characterization

technique that allows one to investigate the morphological and mechanical properties of cells and/or proteins expressed at the surface. Note that AFM cannot provide any information about the features of the proteins, lipids, and DNA "hidden" underneath the cell surface. Complementarity of AFM and Raman comes into play at this point because while Raman allows one to obtain information about the biochemical properties of the proteins, lipids, and DNA that lie underneath the cell membrane and inside the cell nucleus, the AFM provides surface characteristics, such as the morphology and mechanical properties of the same cell, through the AFM–Raman hybrid system.

Chemical modification of AFM probes

AFM is widely used to visualize at the nanoscale and to measure the nanomechanical properties of biological systems (see Nano-Structural Features of Animal Cells and Tissues and Nanomechanical Properties of Animal Cells and Tissues sections). To perform these measurements, standard and unmodified AFM tips are used. Recently however new types of experiments (Noy, 2006, Barratin and Voyer, 2008) have become possible via chemical functionalization of the AFM probes. Chemical modification of AFM tips allows one to study so-called "molecular recognition events" at a very high level of sensitivity. To make a complicated story simple if the goal is to investigate what happens on a cell surface at the single-molecule level, then the AFM probe needs to be functionalized by "attaching" to it either ligand or receptor molecules that will bind specifically only to certain types of cell surface molecules. The major problem encountered when chemically modifying the tip is that it is not possible to control precisely its functionalization, and this "randomness" can affect the measurement of single-molecule interactions.

The main applications of chemically modified AFM probes concern molecular recognition force measurements of cell–surface interactions (Li et al., 2011), which are extremely important in cell adhesion, cell migration, cell development, cell–cell communication and recognition, and ligand–cell membrane protein interactions; these are essential to gain a deeper knowledge of the molecular dynamics of biological processes.

Nanostructural features of animal cells and tissues

The most straightforward application of AFM is imaging. Additionally however the capability of AFM to work in liquid medium allows the opportunity to image live cells in their natural physiological conditions and under controlled culture systems (e.g., keeping the cells at 37 °C in a CO_2 environment and allowing continuous renewal of the culture medium via perfusion), thereby facilitating the imaging of cells in real time for long periods of time. Furthermore AFM images can be captured along with bright contrast, fluorescence, and confocal images with the final goal of obtaining as much information as possible from the same sample area. Recently the combination of AFM and Raman imaging (see AFM–Raman Confocal Hybrid Systems section) has allowed researchers to collect AFM images at the nanoscale and Raman chemical images on the same spot of the sample under study, thereby permitting one to obtain a complete "map" of the nanostructural and biochemical properties of the specimen.

A few examples of the type of information that AFM imaging can render are given. AFM has been used to investigate the effects of ionizing radiation on the nanostructures of pericardium tissues (Canetta et al., 2011) with the final goal to better understand what should be the maximum cardiac doses that a breast cancer patient should receive in breast radiotherapy (Fig. 16.5). Cross-sectional analysis of the AFM images showed that ionizing radiation caused an increment in the diameter of the collagen fibers that compose the pericardial tissue. Moreover the number of fibrils affected and the extent of swelling were found to increase with the radiation dose. Another example of the use of AFM in biotechnology is stem cell profiling. Undifferentiated and differentiated stem cells were visualized (Kiss et al., 2011) by AFM.

AFM images showed differences in the cytoskeleton organization of undifferentiated and differentiated stem cells. Cross-section and roughness analyses of the

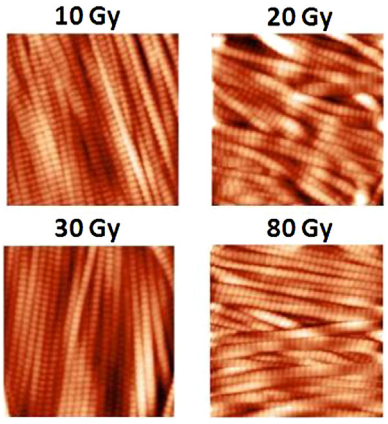


FIGURE 16.5 AFM height images ($2 \times 2\ \mu m^2$) of pericardial tissues exposed to 10 Gy radiation (height = 0–470 nm); 20 Gy radiation (height = 0–510 nm); 30 Gy radiation (height = 0–535 nm); and 80 Gy radiation (height = 0–650 nm).

AFM images have shown that undifferentiated cells are rounder and smaller in size, and have a smoother surface when compared with differentiated cells.

Nanomechanical properties of animal cells and tissues

AFM is widely used to investigate the nanomechanics of cells and tissues in physiological conditions. Knowing the differences in the elastic and adhesive properties of cells can be of great help in cell profiling. This is particularly true for stem cells where an appropriate differentiation protocol is still unavailable. AFM force spectroscopy was used to distinguish undifferentiated and differentiated stem cells (Kiss et al., 2011) based on the elasticity of their cell walls. Consistency of the mechanical properties across the cell wall of undifferentiated stem cells suggested their homogeneous nature. In contrast typical $F-d$ curves recorded at different positions on the cell wall of differentiated cells revealed different mechanical features, thereby indicating that differentiated cells have a heterogeneous nature. The observed higher elasticity variations between different cells than those on individual cells were interpreted to be arising due to significant structural differences between undifferentiated and differentiated cells. Undifferentiated cells were found to be more rigid with a higher Young's modulus (E) when compared with differentiated cells, which exhibited a lower E value.

Nanomanipulation

One of the more advanced uses of an AFM is nanomanipulation. AFM can be employed as a nanomanipulator, for example, to dissect (Rubio-Serra et al., 2005) metaphase chromosomes. Chromosomal dissection allows researchers to separate DNA from areas of the cell that have been cytogenetically identified in order to create genetic probes (Di Bucchianico et al., 2011). AFM can also be used as a low-invasive cell manipulation and gene transfer system to insert pieces of DNA inside single living cells.

Nanofabrication

A variety of nanotechnology tools can be used for the chemical synthesis of nanoscale structures, nanolithography, nanoscale surface patterning, synthesis, and characterization of nanomaterials (e.g., magnetic NPs for drug delivery and cell destruction, low and high adhesion surfaces for biomedical uses such as stents, carbon nanotubes, and fluorescent quantum dots). They offer a wide range of applications, such as in drug synthesis and delivery, cell therapy and cell surgery, early disease diagnosis and prevention, medical implants, biosensors, and nanoanalyzers. It is impossible to fathom the enormity of information required to cover all these aspects in this chapter. We can at best briefly introduce this topic here by including a few references for interested readers, and by giving an example or two in the Examples of Nanotechnology Applications to Animal Biotechnology section. Different nanotools can be used; for example, nanofabrication by using SPM techniques that have the additional advantage of simultaneous nanoscale imaging, surface modifications and nanolithography by using AFM, and soft lithography with self-assembled monolayers. Falconnet et al. (2006) reviewed a number of techniques for cell patterning to study cellular developments during cell culture assays that provide new insights into the factors that control cell adhesion, cell proliferation, differentiation, and molecular signaling pathways.

Examples of nanotechnology applications to animal biotechnology

This section presents some specific examples from the literature and discusses, in general, the applications of nanotechnology to animal biotechnology and biomedicine.

Nanoparticles in animal production

Recent research (Hill and Li, 2017) has shown the potential application of NPs in animal reproduction and in the production of animal products, such as eggs and milk. Quantum dots (semiconductor NPs whose color is determined by their size) are being used for elucidating the molecular processes occurring during animal fertilization (Feugang et al., 2015); whereas antimicrobial NPs are employed to protect cryopreserved animal sperm from bacterial agents (Bryla and Trzcinska, 2015).

Nanosurgery

Nanosurgery refers to the direct manipulation of living cells using either a laser or an AFM probe. The advantage of nanosurgery lies in its ability to intervene on single cells. Laser nanosurgery has been used on cardiomyocytes to selectively repair damages at their sarcomeric cytoskeleton (Müller et al., 2019). This nanosurgery technique could pave the way to future tailored medical treatment of heart diseases. AFM nanosurgery was performed on live keratinocytes, and

the intermediate filament network was dissected (Yang et al., 2015). Such surgery prevented cell–cell adhesion and could have a strong impact on a number of therapies (e.g., cancer therapy).

AFM as a diagnostic tool to identify orthopoxvirus in animals

During the 2005 natural outbreaks of Vaccinia virus (VACV) in dairy cattle in Brazil, samples of vesicles and crusts (dried scabs) from cattle and milkers' hands were collected and subjected to further analysis (Trindade et al., 2007). The purified samples used were intracellular mature virus (IMV) particles, which are the predominant viral forms produced during infection. The AFM nanotechnique was employed for viral identification and characterization from clinical samples and purified viruses. The results showed that AFM can provide a rapid and biosecure tool for the diagnosis of emerging orthopoxviruses with a potential for screening bioterrorism samples. In order to minimize the risk of spread of viral diseases and to adopt effective treatment strategies, rapid detection and identification are important.

Frictional response of bovine articular cartilage

The frictional response of bovine articular cartilage was investigated at the nanoscale by comparing micro- and macroscale friction coefficients of immature bovine articular cartilage (Park et al., 2004). Twenty-four cylindrical osteochondral plugs in pairs from adjacent locations in six fresh 4- to 6-month-old bovine humeral heads were harvested and divided into two groups for AFM (in physiological PBS medium) and macroscopic friction measurements. Surface roughness was acquired from surface topography, and elasticity modulus from indentation experiments with AFM. The AFM friction coefficient was found to be more representative of the equilibrium friction coefficient reported at the macroscale, which represents the frictional response in the absence of cartilage interstitial fluid pressurization. These results suggest that AFM friction measurements may be highly suited to providing greater insight into the mechanism by exploring the role of boundary lubricants in diarthrodial joint lubrication independent of fluid pressurization.

Microstructure and nanomechanical properties of cortical bone osteons from baboons

Recently the effects of tissue and animal age on mechanical properties, such as elasticity modulus, stiffness, hardness, and composition, through nanoindentation and Raman experiments on bone tissues of baboons (Burket et al., 2011) was examined. The results demonstrated that composition and mechanical function are closely related and influenced by tissue and animal age. Because tissue composition crucially determines the mechanical function, understanding these relationships will enhance the knowledge of normal and pathological bone function and enable the improvement of current therapies for skeletal diseases such as osteoporosis, which is known to alter the nanoscale heterogeneity of the material properties of bone tissue. Understanding aging-related material property changes at the tissue level is thus essential to predicting bone fragility, skeletal mechanical integrity, and age-related fracture.

Use of calf thymus DNA for cancer experiments

In 2011 a study of the effect of drug bioavailability of synthesized natural coumarin (SC) and nanocoumarin (NC) having potential drug value (by using DNA from calf thymus as the cellular target of therapeutic molecules) was carried out by using AFM, SEM, and a variety of other techniques and bioassays (Bhattacharyya et al., 2011). NC demonstrated greater efficiency of drug uptake and anti-cancer potential in melanoma cell line A375, as revealed by SEM and AFM. NPs now being increasingly used in bioapplications such as therapeutics, antimicrobial agents, transfection vectors, and fluorescent labels should be harmless to target organisms and organ systems. SC and NC showed negligible cytotoxic effects on normal skin cells and peripheral blood mononuclear cells of mice. NPs were found distributed in different tissues in mice, like heart, kidneys, liver, lungs, spleen, and brain. NC in mice brains crossed through the blood–brain barrier, but SC failed to cross the barrier, further suggesting that nanocoumarin was more active and potent than the synthetic coumarin in suppressing the p53-regulated expression of Cyclin D1, survivin, and Stat-3. This may have been because of the enhanced cellular uptake of the encapsulated nanoform of coumarin. These results imply that nano-coumarin may be superior to coumarin as an antitumor, antiinvasive, and anticarcinogenic agent. This in turn suggests it as a likely candidate for chemopreventive drug design, because of its small size, more rapid entry into target cells, and biodegradable nature.

Characterization of mitochondria isolated from normal and ischemic hearts in rats

AFM has been used to observe the morphological property changes in heart mitochondria isolated from a rat myocardial infarction model (Lee et al., 2011). The shape parameters from AFM topography images revealed that myocardial infarction caused the swelling of mitochondria. The biophysical properties determined from AFM force spectroscopy experiments showed that the adhesion force of heart mitochondria significantly decreased by myocardial infarction and

ischemic stimuli possibly induced the stiffening of mitochondria. The opening of channels on sufficient swelling can rupture the outer mitochondrial membrane and cause the release of cytochrome c, which leads to apoptotic cell death.

Polymorphism and ultrastructural organization of prion protein

AFM was employed (Anderson et al., 2006) to analyze the ultrastructure of amyloid fibrils produced from the full-length mouse prion protein (PrP). The fibrils displayed unprecedented variations in their morphologies. The results revealed extremely broad polymorphism in fibrils generated in vitro, which is reminiscent of high morphological diversity of scrapie-associated fibrils isolated from scrapie brains, suggesting that polymorphism is peculiar for polymerization of PrP, independent of whether fibrils are formed in vitro or under pathological conditions in vivo. Prion diseases are a group of fatal neurodegenerative brain disorders found in both animals and humans, and all of them are related to misfolding and polymerization of PrP.

Ultrastructural investigation of animal spermatozoa using AFM

While sperm morphology is considered to be a prognostic factor for fertilization and pregnancy, abnormal morphology is linked to male infertility. This is why morphological changes are targeted for developing contraceptives. Maturation and capacitation are some of the post-testicular processes that mammalian spermatozoa must undergo before becoming fully competent to fertilize an egg. During their passage from the testis to the site of fertilization, mammalian spermatozoa encounter a range of fluids of very different origins and compositions that greatly influence post-testicular processes. Morphological changes in the surface structure of bovine sperm heads during acrosome reactions have been reported (Saeki et al., 2005) by using AFM and SEM. AFM was combined with other techniques to study the supramolecular organization of the sperm plasma membrane during maturation and capacitation of bovine, porcine, and ovine spermatozoa (Jones et al., 2007). For a review of AFM imaging of spermatozoa, the reader can refer the study by Kumar et al. (2005). AFM has also been used to study the changes in plasma membranes overlying the heads of bull, boar, goat, ram, mouse, stallion, and monkey spermatozoa during post-testicular development, after ejaculation, and after exocytosis of the acrosomal vesicle (Ellis et al., 2002). To study the effect of prostegerone on sperm function, we recently investigated (Adya, 2012) the morphological and nanomechanical properties of boar sperm cells at different concentrations of prostegerone. Fig. 16.6 shows some of the AFM height images (unpublished results) of boar sperm cells on a scale of $10 \times 10\ \mu m^2$.

Multifactor analysis of living animal cells for biotechnology and medicine

Advances in AFM-based nanotechniques for investigating animal cells, with possible applications in animal biotechnology and medicine, have further expanded and opened new avenues for applications of nanotechnology. A particular emphasis has been to study living cells under physiological environments, assess cell morphology and the effects of environmental and age-related stresses, determine cell–substrate adhesion and cell–cell aggregation, investigate the nanomechanical/biophysical properties (e.g., stiffness and elasticity) of the cell membrane, map the cell surface by using chemically modified cantilevers, analyze the distribution of molecular/chemical components inside the cell by using micro- and nanosurgical approaches, and combine AFM with other techniques such as optical and confocal microscopy, and Raman spectroscopy. Most of these applications have already been discussed in the first "Methodologies" section. Raman et al. (2011) reviewed some of these.

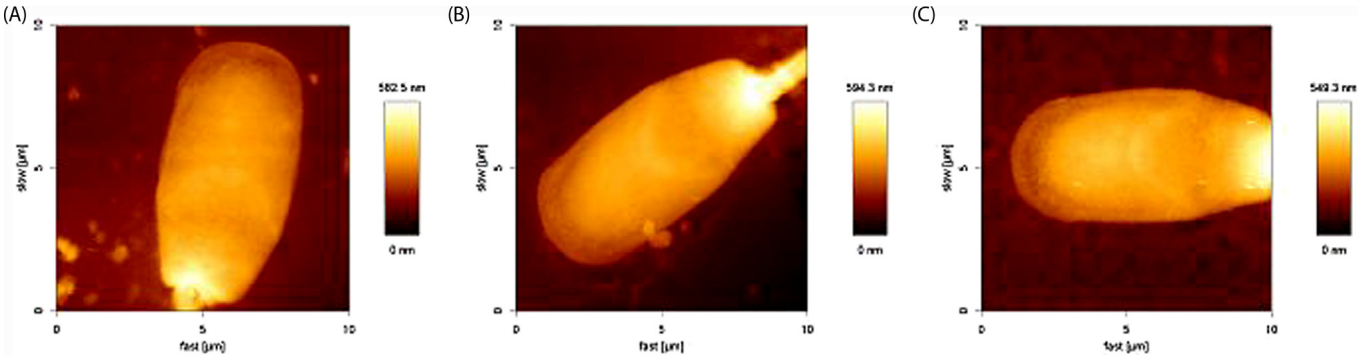


FIGURE 16.6 AFM height images ($10 \times 10\ \mu m^2$) of boar sperm cells (A) without prostegerone (B) with 100 nM prostegerone, and (C) with 1000 nM prostegerone. *Adapted from Adya AK (2012). Private communication (unpublished results).*

Ethical issues

In addition to the examples and applications given earlier, a variety of NPs in different formats (e.g., NPs embedded in a matrix, functionalized NPs, PEG-modified iron oxide NPs, polysaccharide-modified iron oxide NPs, polymer-coated NPs, nanostructured sol–gel silica–dopamine reservoirs, magnetic drug NPs, nanoscale oil bodies, surface-enhanced Raman-scattering NPs, silica or iron oxide-based magnetic NPs), shapes (e.g., nanorods, nanoshells, nanotubes, nanospheres, nanocages), and sizes are currently being used and further developed for targeted drug delivery with the final goal to cure cancer and other lethal diseases in mammals. Existing research has clearly demonstrated the feasibility of introducing nanoshells and nanotubes into animal systems to seek out and destroy targeted cells. NPs smaller than 1 μm have been used to deliver drugs and genes into animal cells. Such NPs can be toxic and/or carcinogenic, and thus hazardous to mammalian health and the environment.

One major problem faced by governments and many industries around the globe is the lack of information available about health hazards due to the use of different materials for the production of NPs. Some national and international agencies are working continuously to check and improve new techniques for testing toxicity and hazard limits of NPs before their production in companies. NP production companies should follow the regulations, which need to be continuously updated, and check all the hazardous side effects on the environment.

Apart from the risk and environmental and health concerns, there are other ethical issues involved with nanotechnology that need to be addressed. Several international bodies are already involved in this. Scientists are now faced with ethical limits in deciding what is acceptable to do. This is true with any new developing technology. As an example, researchers involved in the production and testing of "nanoscale oil bodies" as oil-based carriers for antitumor drug delivery had to carry out in vivo studies on mice to produce a good drug as well as good therapy for the cancer. Tumor creation in mice therefore became an ethical issue for them.

Translational significance

Nanotechnology itself is a translational science. Due to its multidisciplinary nature and wide-ranging scope (as discussed in earlier sections of this chapter), it drives the agenda for translational research, a "basic-to-applied" or "bench-to-bedside" approach to "translate" fundamental research results into practical applications, such as the development of new treatments for diseases in everyday practice. Scientific discoveries begin at the "bench" with basic research in which scientists study disease at a molecular or cellular level and then progress to the clinical level for the animals' well-being and the improvement of human health.

Clinical correlations

Nanotechnology plays a very important role in medicine, as shown by the fast-pace growing of nanomedicine. A recent application of nanotechnology to healthcare consists in the development of nanosensors for the detection of biomarkers related to chronic pain (Chakravarthy et al., 2018). Such a clinical tool would prove essential for the pain management. Clinical nanomedicine also opens up the possibility to develop personalized nanotherapies and nanodiagnoses via the use of NPs and nanomaterials for targeting individual pathogens and also for image-guided drug delivery (Theek et al., 2014).

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World wide web resources

R. Feynman's lecture "There is plenty of room at the bottom"

http://www.zyvex.com/nanotech/feynman.html

This is the transcript of the famous lecture that Professor Feynman gave on December 19, 1959 at the California Institute of Technology in which he formulated the basis of nanotechnology.

Principles of Atomic Force Microscopy

http://www.weizmann.ac.il/Chemical_Research_Support/surflab/peter/afmworks/

This is an easy-to-understand introduction to the principles of AFM.

Atomic Force Microscopy (Interactive tutorial)

http://medicine.tamhsc.edu/basic-sciences/sbtm/afm/principles.php

This website gives a comprehensive introduction to the main principles of AFM, and also allows the reader to "see" how an AFM works.

Applications of Nanotechnology to Animal Biotechnology

http://www.veterinaryworld.org/Vol.2/December/Nanotechnology and its applications in Veterinary and Animal.pdf

This is a good review of the applications of nanotechnology to livestock, biotechnology, and veterinary medicine.

Nanowerk

http://www.nanowerk.com

The Nanowerk Nanotechnology Portal is one of the most comprehensive nanotechnology and nanoscience resources. It includes a nanomaterial database (nanoBASE), an exhaustive "Introduction to Nanotechnology," daily news coverage and spotlight articles (nanoNEWS), and several links related to nanotechnology (nanoLINK).

Dedicated Nanotechnology Website

http://nanotechweb.org

This is another very comprehensive website with the most up-to-date nanotechnology discoveries and news. It gives ample information on the business side of nanotechnology, and the most recent nanotechnology events, showcases, and publications. It also has a good blog and a comprehensive list of useful links to journals, research centers, professional societies, government initiatives, books, etc.

The Institute of Nanotechnology (IoN)

http://www.nano.org.uk

This site provides deep insight on the latest research activities and events related to nanotechnology in the United Kingdom.

The European Nanotechnology Gateway

http://www.ece.umn.edu/users/hjacobs/publications/Nanoforum-European Nanotechnology Gateway.htm

This site gives an exhaustive overview of the main on-going research activities in Europe.

Understanding Nanotechnology

http://www.understandingnano.com

This is a basic, introductory website dedicated to nanotechnology. It is the most appropriate website for beginners.

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Glossary

Biomimetic	Synthetic imitation of natural processes and systems
Carbon Nanotube (CNT)	Nanomaterial formed by a rolled up carbon layer
Fullerene	Allotrope of carbon
Graphene	Monolayer of carbon atoms
Nanocomposite	Multiphase nanomaterial
Nanomaterial	Material with one or more dimensions below 100 nm
Nanotoxicity	Toxicity of nanomaterials
Quantum dot	Semiconductor nanoparticle

Abbreviations

AFM	Atomic force microscopy
AFM-FS	Atomic force microscopy—force spectroscopy
AFM – FM	Atomic force microscopy—force mapping
CCD	Charge-coupled device
CFM	Chemical force microscopy
CRS	Confocal Raman spectroscopy
DNA	Deoxyribo nucleic acid
EDS	Energy dispersive spectroscopy
ESEM	Environmental scanning electron microscopy
FluidFM	Fluid force microscopy
FTIC	Fluorescein isothiocyanate
HNF	Holographic notch filter
IMV	Intracellular mature virus
μm	Micrometer
MRS	Modulated Raman spectroscopy
NC	Nano-coumarin
NP	Nanoparticle
NSOM	Near-field scanning optical microscopy
PEG	Polyethylene glycol
pN	Piconewton
PrP	Prion protein
RI	Raman imaging
RS	Raman spectroscopy
SEM	Scanning electron microscopy
SERS	Surface-enhanced Raman spectroscopy
SC	Synthesized natural coumarin
STM	Scanning tunneling microscopy
SNOM	Near-field scanning optical microscopy
SPM	Scanning probe microscopy
TERS	Tip-enhanced Raman spectroscopy
VACV	Vaccinia virus
UV	Ultraviolet

Long Answer Questions

1. Write an essay describing nanotechnology, its translational significance, ethical issues and risks involved, and explain its use in biology and biotechnology.
2. Describe the history, operating principles, and advantages and limitations of a variety of microscopic techniques ranging from optical microscopy to SPM.
3. Describe how nanotechnology has helped move the frontiers in animal biotechnology. Give specific examples.
4. By drawing suitable diagrams where possible, explain a variety of SPM and RS techniques that can be employed to investigate biological materials at the nanoscale.
5. Describe the various operational and application modes of an AFM and show how this nanotechnique has been used for a variety of applications in biotechnology. Draw and label suitable sketches (where possible) to illustrate your answer. Describe briefly a hyphenated technique of AFM.

Short answer questions

1 By drawing and labeling an outline sketch, describe:
- **i.** The operational principle of Atomic Force Microscopy (AFM).
- **ii.** The three operational modes of AFM.

Describe the operation of Atomic Force Microscope in Force Spectroscopy (AFM-FS) application mode by sketching a force−distance ($F-d$) curve and explain it fully.

List three different quantitative properties of a specimen that can be determined from these AFM-FS measurements.

Explain the differences between AFM-FS and AFM-FM. What additional advantages does AFM-FM offer relative to AFM-FS?

2 Describe the basic principles of SPM techniques and write short notes on:
- **i.** Chemical Force Microscopy (CFM).
- **ii.** Fluid Force Microscopy (FluidFM).
- **iii.** Scanning Near-Field Optical Microscopy (SNOM).

What are the differences between (a) Scanning Tunnelling Microscopy (STM) and AFM, (b) Scanning Electron Microscopy (SEM) and AFM, and (c) CFM and AFM?

3 Describe the two techniques, Raman Spectroscopy (RS) and Raman Imaging (RI), and explain why they have recently been in demand.

List the two main disadvantages of RS, and write short notes on how these detrimental effects have been overcome in:
- **i.** Confocal Raman Spectroscopy (CRS).
- **ii.** Modulated Raman Spectroscopy (MRS).

iii. Surface-Enhanced Raman Spectroscopy (SERS).
iv. Tip-Enhanced Raman Spectroscopy (TERS).

Briefly describe a confocal AFM−Raman hybrid system, and explain what potential it holds in animal biotechnology.

4 What is AFM nanomanipulation? By giving a couple of examples show how it can be used in animal biotechnology. Write a brief note on the use of nanofluidics in nanobiotechnology.

5 By giving a few examples of applications of nanoparticles, show how these nanomaterials are at the leading edge of the rapidly developing field of nanobiotechnology. What are the main health and ethical issues associated with the use of nanoparticles in animal biotechnology?

Answers to short answer questions

1 For a labeled outline sketch and description of the operating principle of AFM, and for the sketch of a force−distance ($F-d$) curve and operation of AFM-FS and AFM-FM application modes, see "Atomic Force Microscopy" in the "SPM Techniques" section. Three different quantitative properties of a specimen that can be determined from AFM-FS measurements are F_{max} (maximum adhesion force in nN), d_{max} (contact point in nm), and W_{adh} (work or energy of adhesion in J). AFM-FM has additional advantages over AFM-FS in providing two-dimensional property maps and phase distribution across a sample surface.

AFM can be operated in three different modes, namely contact, noncontact, and intermittent contact (also known as tapping mode). Because the tip is in hard contact with the surface in *contact mode*, the stiffness of the lever (measured by its spring constant, k) has to be less than that holding atoms together, which is $\sim$1−10 nN/nm. Most contact mode cantilevers have a spring constant $<$1 N/m. In *noncontact mode*, a stiff cantilever is oscillated in the attractive regime (tip quite close to the sample but does not touch it), hence, "noncontact" mode. The forces between the tip and sample are quite low ($\sim$pN). The detection is based on measuring changes to the resonant frequency or amplitude of the cantilever. In *tapping mode*, a stiff cantilever is oscillated closer to the sample than in noncontact mode. Part of the oscillation, therefore, extends into the repulsive region so that the tip intermittently touches or "taps" the surface. Very stiff cantilevers are used because otherwise these can get "stuck" in the water contamination layer.

2 All the SPM techniques are based upon scanning a probe, typically called the *tip*, just above a surface while monitoring some interaction between the probe and the surface. To write short notes on: (1) CFM, (2) FluidFM, and (3) SNOM, see appropriate headings in the "SPM Techniques" section.

a. The salient differences between STM and AFM are that while in STM it is the tunneling current, in AFM it is the van der Waals forces that are monitored between the tip and surface.
b. The salient differences between SEM and AFM are that in SEM it is the beam of electrons while in AFM it is the tip (sharp metallic tip microfabricated at the end of a cantilever or a bead, functionalized or nonfunctionalized) that scans line by line over the surface; also, contrast enhancing agents are often required in SEM while no contrast enhancement is required in AFM.
c. The salient differences between CFM and AFM are that while AFM exploits the "natural" van der Waals forces between the nonfunctionalized AFM tip and the sample surface, CFM uses the "well-defined" interactions between the functionalized AFM tip and the sample surface, which can also be functionalized, if required.

3 For RS and RI see the section "Raman Spectroscopy and Imaging." Requirements of very little sample preparation and the "gentleness" of the "touch of light" on the sample make them truly nondestructive and non-invasive techniques. Additionally, their ability to work both in situ and in vivo, an essential requirement when dealing with biological samples, has recently put them in great demand for animal biotechnology and life science work. The main disadvantages of RS are the small percentage of Raman scattered photons compared with the elastically scattered photons, and the strong fluorescence background. The cumulative effect of these two can sometimes almost completely hide the Raman peaks. For a description of how these detrimental effects are overcome in (1) CRS, (2) MRS), (3) SERS, and (4) TERS, see Raman Spectroscopy and Imaging section. For the description of a confocal AFM−Raman hybrid system, see AFM−Raman Confocal Hybrid Systems section.

4 AFM nanomanipulation is the use of an AFM tip as a nanoscalpel or nanoneedle to perform nanopatterning (i.e., modification of surface geometry to improve cell adhesion), to inject nanoparticles and other nanoscale-size materials (e.g., fluorescent dye) inside a living cell, etc. Nanomanipulation can be used in animal biotechnology, for example, to insert pieces of DNA into single living cells for nano-genetic manipulations, to produce single genetic probes that

target particular cells, and to nanodissect chromatidial and chromosomal DNA.

Nanofluidics is the study of the behaviors and properties of fluids that are confined in a nanoscale size environment. For a brief note on nanofluidics, see SPM Techniques section, sub-heading Fluid Force Microscopy (FluidFM). For a review of nanofluidics, see the study by Kirby (2009).

5 Nanoparticles (NPs) play a very important role in the molecular diagnosis, treatment, and monitoring of therapeutic outcomes in a variety of diseases. Their nanoscale size, large surface area, and unique capabilities make them highly effective for biomedical applications, such as cancer therapy, thrombolysis, and molecular imaging. Magnetic NPs (MNPs) with functionalized surface coatings can conjugate chemotherapeutic drugs, making them useful for drug delivery, targeted therapy, magnetic resonance imaging, etc. The drug-conjugated MNPs can be rapidly released after injection or their release delayed, resulting in targeting of low doses of the drug.

Nanoparticles can be toxic and/or carcinogenic, and thus hazardous to mammalian health. This is due to the fact that there are several entry routes in the animal body, such as the respiratory tract and skin. Once inside the animal body, nanoparticles can have potential toxic effects on the primary target sites or organs, such as the lungs and skin. Moreover nanoparticles can start traveling inside the animal body and also have toxic effects on distant organs, such as the kidney or liver. The toxic effects of nanoparticles can even cause death of the animal.

Yes/no type questions

1. A nanoparticle is any particle with a diameter larger than 100 nm
2. A nanomanipulator is used to move nanoparticles, nanomaterials, and proteins
3. Colloid is a suspension of nano- and macro-scale objects
4. AFM and SEM operative principles are similar
5. AFM nanolithography is used to manipulate object at the nanoscale
6. A nanoporous material is used only for separating particles by size
7. Molecular electronics is synonymous of nanoelectronics
8. Quantum dots are semiconductor fluorescent nanoparticles
9. Biomimetics studies natural phenomena in order to mimic them
10. DNA is a nanomaterial

Answers to "Yes/no" questions

1. No—A nanoparticle has a diameter of 100 nm or less
2. Yes—Proteins are nanoscale objects
3. Yes—Colloidal suspensions are made of nanoparticles and microparticles suspended in another medium. Examples are aerosols, gels, and emulsions
4. No—AFM is based on the use of physical probe to image and to characterize a surface, whereas a SEM uses an electron beam to image a sample
5. Yes—AFM nanolithography is used for rearrange nanoparticles, nanorods, nanopyramids, etc., and also for creating nanoscale patterns on surfaces
6. No—A nanoporous material is used to separate particles on basis of their sizes, but also to create scaffolds for tissue engineering applications
7. No—Molecular electronics encompasses any electronic circuit whose individual components are molecules; nanoelectronics deals with the miniaturization at the nanoscale of an electronic circuit
8. No—Quantum dots are semiconductor nanoparticles that emit light of a certain wavelength (color) depending upon their size
9. Yes—Biomimetics is the study and observation of natural phenomena and systems in order to engineer devices that mimic their functional principles
10. Yes—DNA is considered as one of the best functional nanomaterials that when combined can produce materials with unique properties

CHAPTER

17

Antibodies: monoclonal and polyclonal

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Summary

Antibodies are one of the most important components of humoral immune response. They protect host against infections. Antibodies can be either polyclonal or monoclonal depending on their method of production. Both polyclonal and monoclonal antibodies (MoAbs) have wide implications for human health and welfare. Several MoAbs are the preferred treatment modality for diseases like Cancer, Dengue, Ebola disease, etc.

What you can expect to know

Antibody production is a hallmark of the adaptive immune response. Antibodies are produced either to neutralize or to eliminate antigens or pathogens. Antibodies are produced by B-lymphocytes after differentiation of B-lymphocytes into plasma cells. Polyclonal antibodies (PoAbs) can be raised by immunizing animals against the antigen of choice. PoAbs have various applications of analytical, diagnostic, and therapeutic significance. PoAbs were used to protect against infections like diphtheria as early as 1894. The major limitations in using PoAb are the huge variations in affinity and specificity of PoAb from lot-to-lot. Animals have to be given booster injections each time before collecting PoAb. The solution to these drawbacks of PoAb was found with the discovery of MoAbs. MoAbs were discovered by Kohler and Milstein. MoAbs are a perpetual source of antibodies with consistent specificity and affinity. The discovery of MoAbs has not only revolutionized the area of immunodiagnostics but also medical science has enormously benefitted. MoAbs have found widespread applications in clinical treatment, biomedical research, and industry. Nowadays, different kinds of antibodies produced by camelids are also gaining importance as they are being used for diagnostics and other purposes.

History and methods

Introduction

Antibodies belong to group of globular proteins of serum/plasma; they are therefore also known as immunoglobulins. Antibodies react specifically with antigens, which are responsible for the production or induction of those specific antibodies. The words antibody and immunoglobulin are interchangeably used in the literature. The term immunoglobulin can be used to refer to any antibody-like molecule regardless of its antigen-binding specificity.

An antibody is a Y-shaped molecule produced by B-lymphocytes. The terminal stage of B-lymphocytes (i.e., plasma cells) is the major source of immunoglobulin production. Antibodies are the most diverse protein known so far. The most startling fact about antibodies is that these protein molecules have almost similar amino acids residues in $\sim$90%–95% of the polypeptide chain, whereas the remaining $\sim$5%–10% comprises a hypervariable region that shows huge variation in the amino acid residues. The variation in the variable region is up to the extent of millions of different combinations of amino acids. Each antibody has two major functions: (1) antigen binding that occurs at the antigen-binding fragment (Fab), and (2) the effector function of antibodies, which is due to the fragment crystallizable (Fc) portion of the immunoglobulin. In the Y-shape structure of the antibody, the arms of the Y confer the versatility and specificity of response that a host can mount against antigens, while

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the stem region of the antibody decides the biological activities (e.g., complement-mediated lysis, phagocytosis, allergy). The biological activity always starts with binding of the antigen to the antibody. Knowledge about the presence of this protective protein in serum was known even in 1890 and was confirmed by Tiselius and Kabat in 1939. It took almost 50 years to gain an insight about immunoglobulins.

Tiselius and Kabat's experiment

Tiselius and Kabat were the first to demonstrate that immunoglobulins are globular proteins; they also demonstrated the specificity of immunoglobulins against antigens. Their conclusion was based on the electrophoretic analysis of hyperimmune sera from rabbits.

Interestingly, Tiselius and Kabat used sera from hyperimmune rabbits that were immunized with ovalbumin. Tiselius and Kabat obtained four different bands after electrophoresis of unimmunized rabbit serum. These four bands belonged to four different types of proteins. During electrophoretic separation of serum, the fastest migrating protein was albumin, which was followed by alpha (α), beta (β), and gamma (γ) fractions of serum. When hyperimmune serum was electrophoresed under the same conditions, the same four bands were again observed (i.e., albumin, α, β, and γ). However, this time the γ-fraction was much higher compared to the unimmunized serum. It was concluded that when a rabbit was immunized with an antigen like ovalbumin, it led to induction or production of protein that belonged to the γ-fraction of serum. Later, in another experiment, hyperimmune serum was absorbed with ovalbumin and then electrophoresed. Upon electrophoresis, absorbed hyperimmune sera was separated into four bands of albumin, α-, β-, and γ-globulins. Surprisingly, after absorption the electrophoretic pattern of hyperimmune serum was similar to preimmune serum. This result led to the interpretation that the γ-globulin fraction remained bound to ovalbumin; hence, Tiselius and Kabat concluded that the γ-globulin fraction was specific against ovalbumin, which was used to immunize the rabbit.

This was the landmark experiment of immunology that successfully demonstrated that immunoglobulins belong to the γ-fraction of serum and have specificity against antigens.

History

The protective effect of serum has been known since the late 19th century. In 1890, Emil von Behring and Shibasaburo Kitasato developed an effective antiserum against diphtheria. They transferred serum from an animal immunized against diphtheria to animals suffering from diphtheria. This therapy cured animals suffering from diphtheria, and for this study. Behring was awarded the Nobel Prize in 1901.

The first documented record of passive immunization tried in a human being was on Christmas night in 1891. A young boy in Berlin was cured by injection of diphtheria antitoxins. This experiment was based on the work carried out by von Behring and Kitasato at Robert Koch's Hygiene Institute at Berlin, whose findings had been published on December 4, 1890.

Since diphtheria was very common at the time and often had fatal consequences, von Behring started working toward the production of large amounts of diphtheria antiserum. This work was carried out by the Farberwerke Hoechst Company using sheep as a source for the antiserum against diphtheria. The first large-scale trial took place in 1893 with promising results. Later, sheep were replaced by horses to raise antiserum production. With use of the antiserum, mortality in Paris due to diphtheria fell from 52% to 25% (Black, 1997).

The most common problem with this passive serotherapy was development of anaphylactic reaction among treated patients. Therefore, an ammonium sulfate precipitate of the antiserum was used for the treatment, which somehow reduced the side effects of serotherapy even though it failed to eliminate the problems related with serum therapy. The next trial was of pepsin-treated gammaglobulin, which further reduced the incidences of serum sickness. The same treatment was also applied to other infections (e.g., *Streptococcus pneumoniae* and *Neisseria meningitidis*). This method was commonly used as the only choice for treatment until the discovery of antibiotics by Alexander Fleming.

In the 1920s, Michael Heidelberger and Oswald Awery showed a quantitative precipitation reaction upon interaction of antigens with antibodies. It was realized that serotherapy was an excellent option to treat various diseases, and in 1939, Tiselius and Kabat for the first time reported the specificity of the globulin fraction in hyperimmune sera. In 1942, Merrill Chase successfully demonstrated transfer of immunity against tuberculosis by transferring white blood cells among guinea pigs. In 1944, a human immunoglobulin preparation was used to treat measles (which finally overcame the problems of serum sickness). This treatment was also demonstrated to be effective in treating patients with agammaglobulinemia and is still in use today. In 1948, Astrid Fagreaus completed his doctoral work by demonstrating the role of plasma B cells in antibody production.

In 1959, James Gowan demonstrated the role of lymphocytes for humoral as well as cell-mediated immune response. In the meantime, various theories for the immune system and its functions were proposed by different scientists like Paul Ehrlich, Niel K. Jerne, and F. Macfarlane Burnet. Finally, Burnet's theory of "clonal selection" was widely accepted by immunologists throughout the world (Burnet, 1959). Evidence for clonal selection theory was provided by Sir Gustav Nossal by showing that one clone of a B cell produces only one antibody. In 1960, Edelman and Porter elucidated the structure of antibodies, and in 1976, Susumu Tonegawa demonstrated somatic recombination in immunoglobulin genes.

In 1975, major advancements in the field of medical science came with the discovery of MoAbs (Kohler and Milstein, 1975). MoAb offered a new hope for the use of antibodies again as therapeutic agents. This hope soon vanished, however, as there were so many issues related to the use of MoAbs as therapeutics. Nevertheless, advancements in the area of protein engineering renewed interest in the use of MoAb for therapeutic purposes. Protein engineering tools have helped in the development of newer types of antibodies (e.g., chimeric, humanized MoAb, and human Ab).

Elucidation of immunoglobulin structure

In 1939, Tiselius and Kabat confirmed that immunoglobulins were globular proteins present in the γ-globulin fraction of serum (Tiselius, 1937; Tiselius and Kabat, 1939), but it took another ~20–30 years for scientists to elucidate the structure of immunoglobulin as it is known to the world today. The credit for elucidation of immunoglobulin structure goes to two scientists, Rodney Porter from the United Kingdom and Gerald Edelman from the United States. For their landmark work in elucidating the structure of immunoglobulin, they were awarded the Nobel Prize for Medicine or Physiology in 1972. The contributions of another scientist, Nisonoff, need to be mentioned for this discovery. Both Porter and Edelman were trying to deduce the structure of immunoglobulin molecules, but their approaches to this research problem were different from each other. This chapter discusses their individual experiments and how they combined their results to identify the antibody structure (Fig. 17.1).

Edelman's experiment

Edelman was using rabbit immunoglobulin-G (IgG). He treated rabbit IgG with different chemicals like DTT (a reducing agent), iodoacetamide (an alkylating agent that prevents the reassociation of disrupted

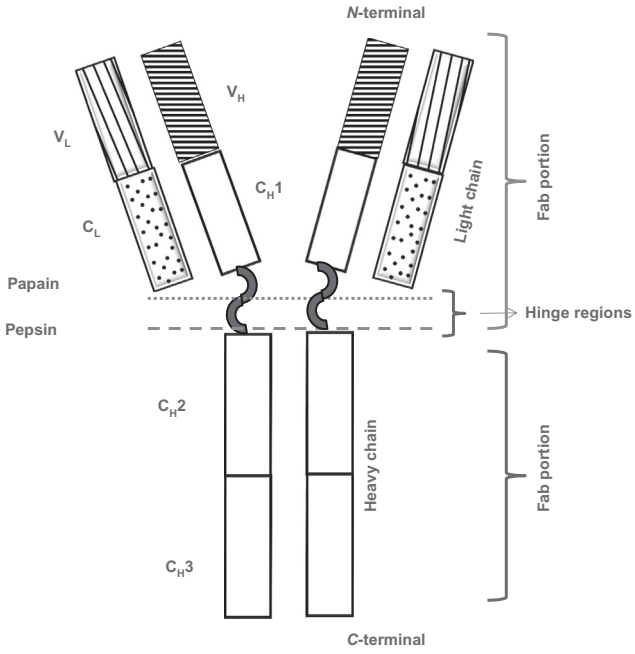


FIGURE 17.1 Structure of prototype immunoglobulins (IgGs). IgG has two heavy chains and two light chains. C_H1, C_H2, and C_H3 are constant domains of heavy chains (H-chain), and C_L is a constant domain of light chain (L-chain). V_H is the variable domain of the heavy chain, and V_L is the variable domain of the light chains. The hinge region has digestion sites for papain and pepsin enzymes. The lower portion of IgG that contains only constant domains of the heavy chain is known as the fraction crystallizable (Fc) portion. Fraction antigen binding (Fab) contains C_H1 and V_H domains of the heavy chain and C_L and V_L domains of the light chain. The antibody heavy chain and light chain are held together by interchain disulfide bonds, but for clarity, those bonds are not shown in the figure. The hinge region of immunoglobulin lies between the C_H1 and C_H2 domains of the heavy chain. It has a site for papain digestion and pepsin digestion.

disulphide bonds), and a denaturing agent (a substance that disrupts noncovalent interaction) (Edelman et al., 1961; Edelman, 1973). After digestion of antibodies with different chemicals, Edelman separated digested immunoglobulins by size exclusion chromatography and found two protein peaks in equimolar ratios. The molecular masses of the two protein peaks corresponded to 50 kD and 23 kD. The protein with a molecular mass of 50 kD was designated as heavy chain and the 23 kD protein was called light chain.

Conclusion of Edelman's experiment: The initial antibody molecular weight was 150 kD. So Edelman concluded that immunoglobulins could consist of two heavy chains and two light chains that are linked to each other by disulfide bonds and noncovalent interactions.

Porter's experiment

Porter was working on proteolytic digestion of immunoglobulins. He was using papain enzyme to

digest immunoglobulin in the presence of reducing agents like cysteine. Papain hydrolysis led to the generation of three fragments, I, II, and III. All three fragments were of the same size (50 kD), but had different electrical charges. Of these three fragments, two were identical and maintained their ability for antigen binding. Therefore, these fragments were called Fab. Porter knew that intact antibodies were bivalent and each Fab could bind antigen; so he concluded that Fab must be univalent in nature. However, the third fragment produced due to papain digestion did not bind with antigen; it crystallized upon cold storage. Porter named this fraction as the Fc fragment. The ratio between Fab and Fc fractions of immunoglobulins was found to be 2:1.

Conclusion of Porter's experiment: On the basis of observations of Porter's experiment, it was concluded that the ratio between Fab and Fc was 2:1. Fab could bind antigen, whereas the Fc portion could not.

Nisonoff's experiment

Apart from Edelman and Porter, Nisonoff was also working with immunoglobulins (Nisonoff et al., 1975). He was using pepsin (another proteolytic enzyme) to digest immunoglobulins. Pepsin digestion yielded a 100 kD fraction and numerous small fractions. The 100 kD fraction weight was double the weight of Fab as observed by Porter, so Nisonoff called this fragment F $(ab')_2$ (fragment antigen binding, divalent). The $F(ab')_2$ portion obtained from pepsin digestion could bind antigen and showed visible serological reaction (i.e., immunoprecipitation). The $F(ab')_2$ portion had both the antigen-binding sites of IgG. Amazingly, when the $F(ab')_2$ portion was treated with reducing agents, it gave two Fab-like fragments, as both fragments could bind antigens even after separation.

Conclusion of Nisonoff's experiment: Pepsin digestion lead to one $F(ab')_2$ portion and smaller fragments. The $F(ab')_2$ portion could bind to antigen and was divalent.

Conclusion from papain and pepsin digestion

Collectively, two enzymes (i.e., papain and pepsin) cleave in the same region of the immunoglobulin molecule. Papain cleaves at one side, while pepsin cleaves immunoglobulin on the other side of the bond that holds Fab fragments together.

The puzzle of immune structure revealed several facts:

1. Each Ig molecule has two heavy chains and two light chains.
2. Fab and Fc are produced by papain digestion.
3. Immunoglobulin molecules can split into $F(ab')_2$ and smaller fragments by pepsin digestion.
4. Fab and $F(ab')_2$ can bind antigen; Fc cannot.

Turning point

These landmark experiments helped in resolving the structure of immunoglobulin molecules. Still, the problem was to combine these facts to yield the final structure of immunoglobulin. The answer to this puzzle was resolved by Porter. He raised antisera against Fab and Fc of rabbit IgG. He found that antiserum against Fab reacted with both H-chain and L-chain. This observation confirmed that the Fab consists of both H-chain and L-chain, while the Fc portion contains only heavy chain.

Immunoglobulin G: a prototype for immunoglobulin

Antibodies are considered to be the workforce for humoral response, and they perform two major functions: (1) target recognition via the Fab portion and (2) antigen elimination/effector function via the Fc portion. Antibodies are classified as polyclonal and monoclonal. PoAbs are host proteins present in plasma and extracellular fluids and serve as the first response against attack by any pathogen or foreign molecule. The ultimate aim of antibody response is to neutralize or eliminate any threat encountered by the immune system. Antibodies are part of the adaptive immune response, and therefore, they are specific against the threat/infection; specific antibodies bind to antigens with high affinity. These two characteristics make antibodies unique tools for a variety of purposes in scientific and medical research. No other single material has contributed directly or indirectly to such a wide array of scientific discoveries. The application of antibodies for numerous diagnostic assays has had a profound impact on the welfare and health of humans and animals. PoAbs are produced in vivo, while biotechnological manipulations have been used to generate MoAbs.

The basic structure of immunoglobulin is made up of two different polypeptide chains. Ideally any antibody (IgG) contains two identical copies of heavy (H) chains (55 kb) and light (L) chains (28 kb). Heavy chains and light chains are held together by interchain disulfide bonds as well as other noncovalent interactions. In general, an IgG looks like a Y-shaped structure with molecular weight around 150 kD. The light chain contains two domains (i.e., variable and constant), while the heavy chain contains one variable domain and three or four constant domains. The Fab

portion is at the *N*-terminal end of the heavy and light chain and is responsible for antigen–antibody binding and interaction. The Fc portion is responsible for the effector or biological functions of an antibody, which occurs due to binding of the Fc portion to Fc receptors (FcRs) present on effector cells (Burton, 1987; Carayannopoulus and Capra, 1993; Frazer and Capra, 1999; Padlan, 1994). The Fc part is composed of constant region domains of heavy chain, and accordingly, heavy chain binds to FcR specific for that class of antibody. The flexibility of antibodies lies in the hinge region, which is present between C_H1 and C_H2 domains (Fig. 17.2).

Polyclonal antibody versus monoclonal antibody

PoAb and MoAb are terms commonly found in the immunology literature. Both of these antibodies have the same structure and function, but PoAb and MoAb differ from each other on the basis of their origin, production, and specificity. The basic difference between these two antibodies lies in the clonality of the cells that produce them. MoAbs are produced by a single clone, while PoAbs are produced by numerous clones together. Each of these antibodies has their own pros and cons (Lipman et al., 2005). Numerous advantages and disadvantages of PoAb and MoAb are presented in this chapter for a better understanding (Table 17.1).

Polyclonal antibodies

1. PoAbs are also known as antiserum, so PoAb and antiserum are interchangeably used in this chapter.
2. PoAbs are products of numerous B-lymphocytes.
3. Animals are the source of PoAb.
4. PoAb can be produced only in vivo, which means the requirement of an animal is essential.
5. PoAbs are specific in nature, but show cross-reactivity due to their polyclonal nature.
6. Production and repeated production of PoAb requires booster injection.
7. PoAb production does not require highly skilled manpower.
8. Sophisticated instruments are not needed to produce PoAb.
9. Sensitivity of PoAb varies from lot-to-lot, which is also attributable to their polyclonal nature.
10. Immunized animals (source of antiserum) are always vulnerable toward infections and other natural adversaries.
11. PoAbs are not homogenous in nature and so they cannot be easily characterized.

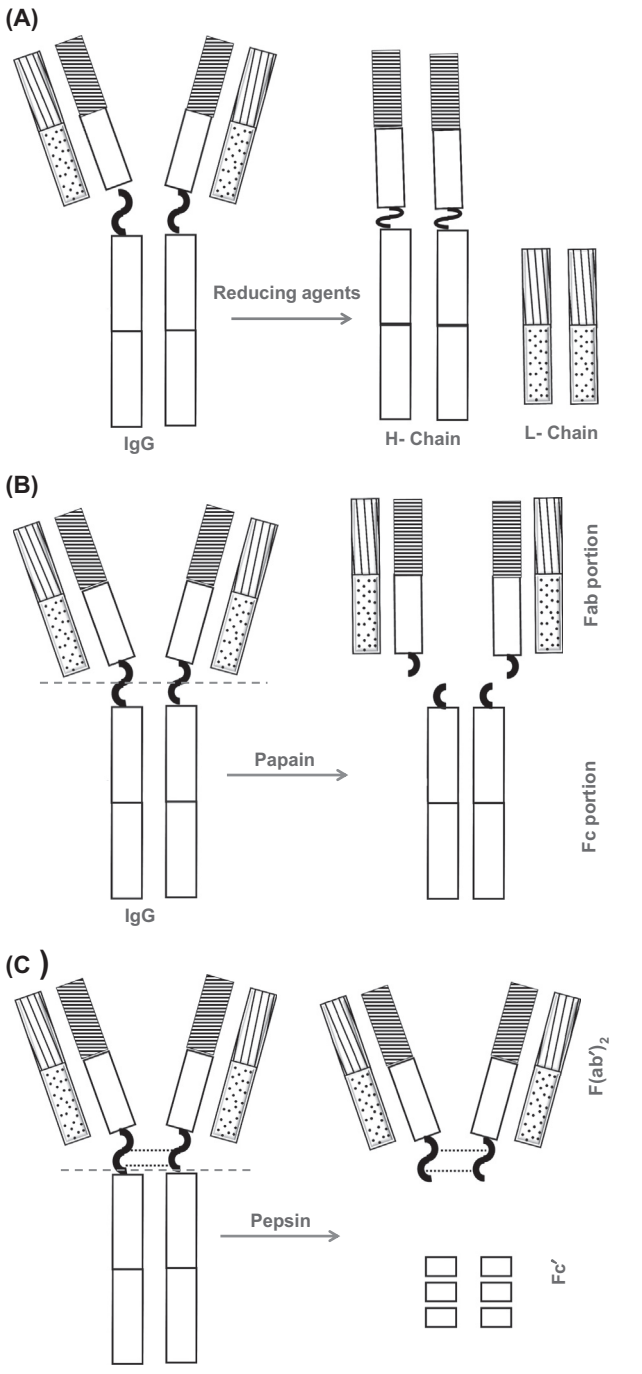


FIGURE 17.2 Elucidation of immunoglobulin structure. This figure shows the different fractions of immunoglobulin after digestion with different agents. (A) Edelman's experiment: digestion of immunoglobulin by reducing agents like 2-ME, which results in separation of two heavy chains (H-chain) and two light chains (L-chains). (B) Porter's experiment: Digestion of immunoglobulin with papain. Papain digestion site is present at the upper portion of the hinge region. After digestion, it gives two Fab fragments (fragment antigen binding) and one Fc fragment (fragment crystallizable). (C) Nisonoff's experiment: digestion of immunoglobulin by pepsin. The pepsin digestion site is present at the end of the hinge region of the antibody. Disulfide bonds present in the hinge region hold the F $(ab')_2$ together, accounting for its divalent nature. Smaller fragments (called Fc′) lose their ability to bind with FcRs on effector cells.

TABLE 17.1 Comparison etween polyclonal and monoclonal antibodies.[a]

Characteristics	Antibodies	
	Polyclonal	Monoclonal
Adverse Effect	Serum Sickness	HAMA[a]
Affinity Purified Product	Not homogenous	Homogenous
Antibody Purification	May/May Not	Not essential[b]
Booster Dose	Required	Not Required
Chemically Defined	Not Well	Well
Clonality	Numerous	Single
Cross-reactivity	High to low	Low to nil
Degradability	Low	High
Denatured Antigen[c]	Detect	May/ May Not
Epitope Detection	Multiple	Single
Homogeneity (Antibody)	No	Yes
Manpower Skills	Low skill	Highly trained
Origin	Any	Mouse (Mostly)
Production Cost		
i. Initial	Low	High
ii. Long-Term	High	Low
Production Requirement		
In vitro	–	+
In vivo	+	+
Specificity	Low	High
Variability[d]	High	Low

[a]*Human Anti-Monoclonal Antibody.*
[b]*Depends on application.*
[c]*Detection of antigen.*
[d]*Lot-to-Lot.*

12. PoAb are more tolerant toward minor variation in epitopes and still recognize and bind with specific antigens. Epitope variation could be due to various factors like glycosylation, polymorphism, etc.
13. PoAbs are less vulnerable to degradation.
14. PoAb can be used to detect degraded or denatured antigens (e.g., western blotting).
15. PoAbs are always a better choice to be used as capture antibodies in various immunological test [e.g., enzyme-linked immunosorbent assay (ELISA)].
16. PoAbs are not a good tool for affinity purification.
17. Serum sickness is the major adverse effect of PoAb.
18. Long-term production is expensive due to factors like maintenance of animal, animal deaths, etc.
19. PoAb can be used to provide passive immunity.

Monoclonal antibodies

MoAbs, as the name suggests, are products of a single clone. This single clone is selected after fusion of myeloma with B-lymphocytes. Pros and cons of MoAb are as follows:

1. MoAbs are a product of a single clone of cells.
2. MoAbs are produced by hybridoma cells by the fusion of B-lymphocytes with myeloma cells.
3. Initially, more time is required to produce MoAb.
4. Trained manpower is essential to produce MoAb.
5. Long-term production of MoAb is cheaper.
6. MoAbs are highly specific because they are the products of a single clone.
7. The range of specificity of MoAb is very very narrow (i.e., single epitope).
8. MoAb production requires both in vivo and in vitro systems.
9. Sensitivity of MoAb is high.
10. MoAb can be used either without purification or after purification.
11. Hybridoma cells that produce MoAb are perpetual sources of antibodies with the same specificity and sensitivity.
12. MoAbs are 100% reactive toward their target.
13. Mostly, MoAbs are highly specific and usually do not show cross-reactivity.
14. Since they are homogenous in nature, their chemical nature can be characterized easily; therefore, MoAbs are an excellent choice for conjugation to different probes.
15. When MoAbs are used as therapeutic antibodies, adverse effects caused by MoAbs are known as human antimonoclonal antibody response (HAMA).
16. MoAbs are used to produce chimeric antibodies as well as humanized antibodies.
17. MoAbs are an excellent choice or tool for affinity purification.
18. MoAbs are well established to give highly reproducible results.
19. For the reasons that MoAb have monospecificity and almost no cross-reactivity, they do not show any background during laboratory experiments or when used as probes.
20. Being homogenous in nature, MoAbs are vulnerable to degradation because of the same susceptibility of all antibody molecules present in solution.
21. Due to monospecificity of MoAb, they are not a suitable option for detection of denatured proteins.

Naming monoclonal antibodies

MoAbs have found numerous applications, and this technology has seen enormous improvement in

various aspects, including production and conjugation. These developments are the reason for production of new MoAb on a routine basis. As time progresses, more and more MoAbs are hitting the commercial market and being applied in research laboratories. Hopefully, the development of new MoAb will continue with this uninterrupted pace. Everyone producing MoAb started naming them as per their own rationale, convenience, and other factors such as personal preferences, which led to confusion about the names of MoAb. Sometimes these names became too complicated and the same antibody was available under different names and so differentiation and identification of MoAbs started getting difficult for different stakeholders. Another observation was that the same MoAb was found to be effective against more than one disease (e.g., tumors). Hence, a system to name MoAb with a systematic pattern became a necessity to avoid confusion among different stakeholders (e.g., clinicians, pharmacists, patients).

There are two main organizations that have developed guidelines for naming MoAb: (1) International Non-Proprietary Name (INN), Geneva, Switzerland; and (2) United States Adopted Names (USAN) Council Chicago, United States. The INN mode of naming MoAb is maintained under the umbrella of the World Health Organization (WHO), while the USAN system for naming MoAb is maintained by the USAN.

Currently, USANC has modified its scheme for naming MoAb in such a manner that their naming system harmonizes with the naming of MoAb at the international level. As per the USANC system, MoAb should have a two-word name. However, USANC believes that while naming MoAb, this system should not be effective retrospectively to change the preexisting names of MoAb. The rationale for USAN's decision is to avoid confusion among clinicians, pharmacists, patients, etc. with regards to the old and established names of same MoAb.

The nomenclature system uses a "stem." The stem for MoAb is "mab," while for a mixture of MoAbs, the stem is "pab." The PoAb here that is used is not the traditional PoAb, but represents a polyclonal pool of recombinant MoAbs. The naming system for MoAbs follows a pattern in which the name of each MoAb has four components: (1) prefix (1 in number), (2) infixes (2 in number), and (3) stem or suffix (1 in number). The new name, when given to a MoAb, should essentially contain following components (Table 17.2):

$$Prefix + Infix\text{-}1 + Infix\text{-}2 + Stem$$

These components and their significance are discussed in the following sections.

TABLE 17.2 USAN list of syllables used to Name Monoclonal Antibody.[a]

Meant for	Syllables	Examples
(A) Prefix	Variable	
(B) Infix-1		
Bacterial	-ba-	-bamab
Bone	-os-	-osmab
Cardiovascular	-ci-/-c-	-cixumab
Endocrine targets	-d(e)-	-demab
Antifungal	-fung-	-fungmab
Immune modulator	-li-	-liximab
Interleukin as target	-ki-	-kiximab
Miscellaneous tumor	-tu-	-tuzumab
Neurons	-ne-	-nemab
Serum amyloid protein	-ami-	-amimab
Skeletal muscle mass related growth factors and receptor	-gros-	-grosmab
Toxin target	-toxa-	-toxamab
Tumors	-ta-	-tamab
Veterinary	-vet-	-vetmab
Viruses and antiviral	-vi-	-vimab
(C) Infix-2		
Chimeric	-xi-	-liximab
Chimeric and humanized	-xizu-	-nexizumab
Human	-u-	-vizumab
Humanized	-zu-	-kizumab
Mouse	-o-	-bomab
Rat	-a-	-famab
Rat/mouse hybrid	-axo-	-caxomab
(D) Suffix	-mab	

[a]*Partial list as adapted from USANC; Infix-1 represents a syllable for target or disease; Infix-2 represents syllable for source of antibody.*

Prefix

The prefix is the first syllable in the name of any MoAb. The main purpose of the prefix is to create a unique name for each and every MoAb. The prefix is made up of a distinct and compatible syllable. The prefixes should be selected as per the USANC list for coining names of MoAb. It is recommended to use a small prefix to keep name of the MoAb as short as possible; in general, the syllable used as the prefix should

be two or more in number, which will be helpful in differentiating new MoAb from previously assigned names of other MoAb.

Infix-1

Infix-1 in the name of an MoAb is meant to provide information about the target of an MoAb. This is the reason that infix-1 is usually directed toward the target or to the disease class against which the MoAb is effective or targeted. USAN council has approved a list of syllables that are specific for the disease or for the target. If needed, an additional subclass can be added. In the case of tumors, an infix that has been recommended by USAN council is either "-tu" or "-t-," and so the name of the MoAb against the tumor would be "-tuzumab/-tumab," while in case of immunomodulators, the infix could be either "-li" or "-l-" and the name of the antibodies would be written as "-liximab/-lumab/-lixizumab."

Infix-1 can be truncated to a single letter to make it easy to pronounce. This kind of situation usually arises when the infix-2 (i.e., source infix) starts with a vowel (e.g., for humanized and chimeric antibodies). Infixes indicating tumor specificity have recently been discontinued because it has been observed that some antibodies may work for more than one type of tumor. This is the reason that nowadays infixes used for tumor specificity like -col-(colon cancer), -mel- (melanoma), -got- (testes), -gov- (ovarian), and -po- (prostate) have been dropped completely or are not in use any more for naming new MoAb.

Infix-2

Infix-2 is meant to identify the source of MoAb. Certainly, for therapeutic applications of MoAb, source information is crucial due to safety concerns. Sometimes MoAbs are based on the sequence of one species but manufactured in an entirely different source. In these cases, infix-2 provides information about the species that serve as the source of sequences of immunoglobulins. The use of infix-2 is in harmony with the INN nomenclature system (e.g., humanized (-zu-), mouse (-o-), rat (-a-)).

Additional words

Additional words are also used while naming some MoAbs. They are typically used as clarifying words for the nomenclature of MoAbs that have various applications and are conjugated with different types of payloads. These payloads can be of different types like radiolabels, toxins, etc. Then, it is very useful to add the name of the payload along with the name of the antibody. Addition of the payload name in the MoAb name is helpful in identifying the payload attached to the MoAb. For example, in radio-labeled payloads, different words used to identify the payload have to be in the following order: (1) name of isotope, (2) symbol of element, and (3) isotope number. This is followed by the name of the MoAb: (1) technetium Tc 99 m biciromab and (2) indium In-111-altumomab-pentetate, etc.

Similar rules also apply to the use of additional words for toxins when conjugated as the payload to the MoAb. In that case, the toxin is identified using a separate, second word ("tox") in the stem, and part of the name of the toxin has to be added to the stem (e.g., -aritox- means it contains a ricin A chain). If a drug or chemical is attached to the MoAb, then its name is incorporated into the MoAb name; for example, brentuximab vedotin, where vedotin is an anticancer drug conjugated with this antibody.

Antibodies as therapeutics: adverse effects

Use of antibodies as therapeutic agents is more than a century old practice and started with the use of antibodies/antiserum to provide passive immunity. Passive immunity provides immediate relief due to the neutralizing effect of antibodies because there is no time lapse in the production of antibodies; passive immunity does not leave immunological memory to protect against the next exposure. Unfortunately, PoAbs, when injected into a different host, leads to the generation of immunity against the antiserum. This occurs because the PoAbs are recognized as foreign proteins by the recipient. That is why second exposure of the same recipient with antiserum can cause adverse effects that could be severe. An example of using PoAb as protective agent is an antiserum against snake bites. Antiserum is an antidote for snake venom, and anti-venom gives immediate relief from the fatal effects of snake venom. This prevents immediate death, but leaves victims suffering with other complications that can be pretty serious in certain cases. The first injection of antidote is helpful to protect the victim, but it also generates immune memory against the anti-venom. On the next injection of antivenom, numerous problems arise that could have grave consequences.

Artificial induction of passive immunity was started with the use of hyperimmune serum. The discovery of MoAbs offered a new ray of hope for improvement in transfer of passive immunity. However, the application of MoAbs has its own adverse effects. In due course, various modifications of MoAb have been tried to decrease adverse effects caused by MoAb treatments. The adverse effects of therapeutic administration of antibodies are categorized into following four classes: (1) serum sickness, (2) HAMA, (3) human antichimeric

TABLE 17.3 Antibodies as therapeutics: a comparison

		Origin[a]		Human	Administration	effector
Antibody type	Adverse response	Constant	Variable	Immunoglobulin (%)	Frequency	Functions
Polyclonal	Serum sickness	Animal	Animal	0	+ + +	+
Monoclonal	HAMA	Mouse	Mouse	0	+ +	+ +
Chimeric	HACA	Human	Mouse	60–65	+	+ + +
Humanized	HAHA-1	Human	Human[b]	90–95	+	+ + +
	HAHA-2	Human	Human[b]	90–95	+	+ + +

[a]*Represent constant portion and variable portion of immunoglobulin; + , low;++, medium;+++ , high.*
[b]*Only CDR is of mouse origin.*
HACA, human antichimeric antibody response; *HAHA*, human antihumanized antibody response; *HAMA*, human antimonoclonal antibody response.

antibody response (HACA), and (4) human antihumanized antibody response (HAHA) (Table 17.3).

Serum sickness

Serum sickness is a condition that arises due to the immune reaction against different proteins present in antiserum. In certain clinical conditions, antiserum is administered to treat infections and other serious health risks like rabies, snake bites, etc. Immunologically, serum sickness is a delayed type of immune response. Generally, PoAbs (protective) are raised in different hosts or species (e.g., horse). Proteins present in antiserum, or even protective PoAbs themselves, are recognized as foreign or nonself-entities by the immune system of the patient. The infected patient's immune system starts producing antibodies against the antiserum; this immune response could be due to formation of immune complexes, complement-mediated immune reaction, etc.

Some of the most common symptoms of serum sickness are skin rashes, joint pain, fever, malaise, swollen lymph nodes, itching, wheezing, flushing, diarrhea, etc. Serum sickness is commonly observed within 2–3 weeks of the injection of antiserum, while in some patients, this response may appear within 3–4 days postinjection. A second exposure to antiserum leads to more severe and pronounced adverse effects compared to the first exposure.

Generally, hyperimmune serum contains additional serum proteins; therefore, to attain an effective concentration of protective antibodies, a greater amount of hyperimmune serum has to be administered. Higher amounts of serum contain higher concentrations of other nonspecific proteins; therefore, adverse effects are common, and the reaction is more severe with the use of antiserum. The bottom line of this treatment modality is that patients are saved from immediate threat, but their lives become miserable due to serum sickness. Nowadays, the use of antiserum is restricted and limited unless it becomes a necessity.

Human antimonoclonal antibody response

The discovery of MoAbs has been a great hope for the proponents of passive immunity for two reasons: (1) high specificity and (2) a consistent source of antibodies without variability. MoAbs used for passive immunity have two advantages: (1) lower amounts of antibodies have to be given because of their high specificity and (2) unnecessary proteins are not present in the preparation because MoAbs are purified before administration. It was fairly reasonable thinking that MoAb would resolve problems related to serum sickness caused by the therapeutic use of antiserum. Initially, MoAb had been a great hope among clinicians and scientists as a great modality for treatment. This belief was quickly shattered due to the HAMA response. Since MoAbs are of murine origin, when MoAbs are used to treat human patients, the immune systems of the patients recognize MoAb (protective) as a foreign particle and an immune response and immune memory are generated against MoAb (protective) too. This adverse response against MoAb is known as HAMA. Of course, the HAMA response has less severe adverse effects compared to serum sickness, but the pattern of the HAMA response was similar to the pattern of serum sickness (Tjandra et al., 1990; DeNardo et al., 2003). New modifications have been developed using MoAb to make MoAb better tools to generate passive immunity.

Human antichimeric antibody response

MoAb has an advantage to be a perpetual source of highly specific antibodies. This characteristic of MoAb can be further exploited by creating new modifications. Research on antibodies has delineated the fact that for

antigen–antibody binding or neutralization of the antigen–pathogen, only the Fab portion of the antibody is crucial. This information was exploited to graft the Fab portion of MoAb onto human antibodies. Now, the grafted antibody had the following advantages: (1) the Fab portion maintains high specificity against the antigen and (2) the majority of the antibody portion is of human origin. These manipulations can be achieved by the application of protein engineering, one of the latest tools that has allowed researchers to graft Fab of MoAb onto human antibodies. This modification of a human antibody is known as a "chimeric antibody." Chimeric antibodies contain only the Fab portion of mouse origin, while the rest of the chimeric antibody is of human origin. In a typical chimeric antibody, ~33% is of mouse immunoglobulin in origin and ~66% is of human immunoglobulin in origin.

With higher contents of human immunoglobulin, chimeric antibodies offer a great hope to raise passive immunity in patients. No doubt chimeric antibodies have turned out to be a better choice compared to either MoAb or PoAb as immunotherapeutic agents. At the same time, chimeric antibodies also have adverse effects because 33% of the chimeric antibody is still of mouse origin. The adverse response to chimeric antibodies is known as the human antichimeric antibody response (HACA). The intensity of the HACA response is comparatively less severe compared to the HAMA response or serum sickness. The HACA response turns out to be the limitation for wide application of chimeric antibodies as therapeutic agents. The severity of the HACA response depends on the immune status of the host (e.g., MABTHERA (rituximab) is a chimeric antibody raised against the CD20 receptor).

Human antihumanized antibody response

The HACA response again turns out to be limiting factor to using chimeric antibodies as therapeutic agents. It is a well-established fact that for antigen–antibody reactions, the most important part of the variable portion of the immunoglobulin molecule is the complementarity determining region (CDR). Therefore, in principle, one can graft only CDRs of MoAb to human immunoglobulins; if that is possible, one can reduce the adverse effects of HACA significantly because (1) CDR maintains high specificity for the antibody to neutralize the antigen and (2) the major portion of the hybrid immunoglobulin will be of human origin.

Protein engineering has helped in grafting CDRs of MoAb to human immunoglobulin; such a grafted antibody is known as a "humanized antibody." Humanized antibodies are similar to human antibodies, except for CDRs. Humanized antibodies contain only 6%–10% mouse proteins, while the other 90%–94% is human proteins. Humanized antibodies are far better than other antibodies (i.e., PoAb, MoAb, chimeric antibodies) as immunotherapeutic agents, even though just 6%–10% of the mouse portion of immunoglobulin could cause adverse immune responses in some patients (HAHA) (Nechansky, 2010).

The severity of HAHA is significantly lower compared to adverse effects caused by serum sickness, HAMA, or HACA. HAHA response is of two types HAHA-1 and HAHA-2. The severity of HAHA-2 is less than HAHA-1, and adverse effects due to HAHA are comparatively easier to manage.

One of the best tools to provide protection in the form of passive immunity is human antibodies. The best option to solve this problem is to develop recombinant human antibodies. Hopefully, one day there will be recombinant human antibodies readily available as a means to provide passive immunity without causing any ill effects.

Applications of antibodies

Primarily, antibodies are designed to protect the host by neutralizing invasive entities. If antibodies can protect one animal from invaders, it was hypothesized that in a similar fashion, antibody could protect other animals from the same infection if given exogenously. With time, other applications of antibodies were also recognized and tried, but the discovery of MoAb has revolutionized the applications of antibodies (Margulies, 2005). In general, antibody applications can be divided into three main categories: (1) therapeutic, (2) analytical, and (3) preparative.

Therapeutic applications

Antibodies are basically known to neutralize foreign molecules, toxins, pathogens, etc. These neutralizing antibodies are generated naturally by the host itself. The same rationale was used to raise antisera against pathogen, toxins, venoms, etc. Production of antivenom was one of the most useful applications of PoAb to protect humans after a snake bite. No doubt this strategy saved the host from death due to venom, but the host had to suffer the long-term adverse effects of antivenom (i.e., serum sickness). Serum sickness gets worse with the second use of antivenom; although the host is saved from immediate death caused by the snake bite, it can lead to lifelong illness. PoAbs have been successfully used to protect children against

infectious agents like *Corynebacterium diphtheriae*. Apart from serum sickness, another major drawback of immunotherapeutic application of PoAb is variation in specificity from lot-to-lot, so it is not easy to decide the concentration or dose of antidote.

MoAbs are a better choice as immunotherapeutic agents due to high specificity and the technical ability to produce same MoAb again and again without any alterations in characteristics. Therapeutic MoAbs under clinical trials and on the market have been extensively reviewed in several articles (Brekke and Sandlie, 2003; Chan et al., 2009; Francis and Begent, 2003). A list of various MoAbs used as therapeutic agents is given in Table 17.4. Another advantage of MoAb is that they can be conjugated with various payloads (e.g., drugs, radionuclides, toxins, markers). Conjugated MoAb can be used as therapeutics for the following reasons: (1) due to their specificity they can bind or affect specific targets, and (2) when conjugated with payloads, the payload can be delivered to specific targets. This is the reason why MoAbs found their therapeutic applications in cancer treatment. If someone uses toxin-conjugated antibody, then it can be defined as an immunotoxin. Similarly, using radiolabeled antibody is known as radioimmunotherapy. A new strategy has been adapted using MoAb to achieve direct cell toxicity, which is known as antibody-directed enzyme prodrug therapy.

Analytical applications

MoAbs, due to their high specificity, have turned out to be an excellent choice for various analytical applications. These applications range from the analysis of laboratory data to diagnostic tests used in the clinical or pathology laboratory. Since the advent of MoAbs and development of technology to conjugate various types of probes, MoAbs have revolutionized the field of immunodiagnostics. Nowadays, these tests have become so reliable that they are commonly used for diagnosis and confirmation of various diseases. Due to improvements in technology, these tests are so simple and user friendly that even a nontechnical person can perform them. Currently, immunodiagnostic tests can be directly used even in field conditions; some examples of field testing of immunodiagnostic tests are HIV tests, pregnancy tests, etc. Some of the most common examples of analytical applications of MoAbs in a laboratory environment are western blot, radioimmunoassays, Ouchterlony's double diffusion (ODD), radial immunodiffusion (RID), ELISA, fluorescent-associated cell sorting (FACS), immunohistochemistry (IHC), immunofluorescence, etc.

Preparative applications

The characteristic feature of reversible reaction of antigen–antibody interaction has turned out to be a blessing for preparative applications of antibodies. This is the reason a product of choice can be isolated and purified from a complex mixture using antibodies specific for the product. The complex mixture that contains product to be purified is mixed with antibodies attached to a matrix, and the product gets bound to the antibody. The bound product (antigen–antibody complex) can be dissociated by changing the pH or ion concentration of the buffer and the product can be recovered. This process is known as immune purification or immunoenrichment. It is used in the laboratory for various applications like FACS, MACS, etc. This method has enormous industrial applications.

Methodology, principles, and protocols

It is a well-known fact that PoAbs and the knowledge about them laid the foundation of immunology. Later, MoAbs were developed by Kohler and Milstein (it is more appropriate to use the term developed than discovered as MoAbs were developed by laboratory manipulations and not produced naturally). The significance of MoAbs cannot be denied because they have changed the face and scope of medical science in a number of ways. Since antibodies (either PoAb or MoAb) have enormous significance, what follows is a discussion of some of the common methods and principles involved in the production of these antibodies.

Polyclonal antibodies

The most important thing about the production of PoAbs is the requirement of an animal that can produce antibodies. In general, mammals are the best choice to raise PoAb, although birds can also be good models. Usually the criteria for the selection of animal models to raise PoAb are based on two important variables: (1) application of PoAb and (2) amount of antiserum required. If a larger volume or amount of antiserum is needed, larger animals or dairy animals are preferred. Commonly used animals for PoAb production are horses, buffalo, cows, sheep, goat, etc. If antibody is required in smaller quantities, then small animals are used to raise antiserum, such as rabbit, rat, or mouse. Mice are used mostly for investigative applications because the volume of blood or antibody that can be obtained from them is limited. Mice are a better choice when comparing differences between PoAb and MoAb raised against the same antigen, as most of the time MoAbs are developed using mice as a source of

TABLE 17.4 Therapeutic antibodies approved by the FDA[a]

Year	Name	Type	Target	Application
1986	Orthoclone OKT3	Mouse	CD3	Transplantation
1994	ReoPro	Chimeric	gpllb/gplla	Cardiovascular disease
1997	Rituxan	Chimeric	CD20	Non-Hodgkin's lymphoma
2000	Mylotarg	Humanized	CD33	Acute myeloid leukemia
2001	DigiFib	Ovine[b]	Digoxin	Digoxin overdose
2002	Zevalin	Mouse	CD20	Non-Hodgkin's lymphoma
2003	Humira	Human	TNF-α	Arthritis
2004	Erbitux	Chimeric	EGFR	Breast cancer therapy
2005	Lymphacide	Humanized	CD22	Non-Hodgkin's lymphoma
2007	HuMax-IL-15	Human	IL-15	Inflammation and arthritis
2008	HuMax CD4	Human	CD4	T-cell lymphoma
2009	Golimumab	Human IgG1	TNF	Rheumatoid and psoriatic arthritis, ankylosing spondylitis
2009	Ofatumumab	Human IgG1	CD20	Chronic lymphocytic leukemia
2011	Ipilimumab	Human IgG1	CTLA-4	Metastatic melanoma
2012	Pertuzumab	Humanized IgG1	HER2	Breast cancer
2014	Vedolizumab	Humanized IgG1	α4β7 integrin	Ulcerative colitis, Crohn disease
2015	Evolocumab	Humanized IgG2	PCSK9	High cholesterol
2015	Daratumumab	Human IgG1	CD38	Multiple myeloma
2016	Obiltoxaximab	Chimeric IgG1	Protective antigen of B anthracis exotoxin	Prevention of inhalational anthrax
2016	Reslizumab	Humanized IgG4	IL-5	Asthma
2016	Atezolizumab	Humanized IgG1	PD-L1	Bladder cancer
2017	Ocrelizumab	Humanized IgG1	CD20	Multiple sclerosis
2017	Emicizumab	Humanized IgG4, bispecific	Factor IXa, X	Hemophilia A
2018	Ibalizumab	Humanized IgG4	CD4	HIV infection
2018	Fremanezumab	Human IgG2	CGRP	Migraine prevention
2018	Cemiplimab	Human mAb	PD-1	Cutaneous squamous cell carcinoma
2018	Emapalumab	Human IgG1	IFN gamma	Primary hemophagocytic lymphohistiocytosis
2018	Ravulizumab	Humanized IgG2/4	C5	Paroxysmal nocturnal hemoglobinuria
2019	Caplacizumab	Humanized nanobody	Von Willebrand factor	Acquired thrombotic thrombocytopenic purpura
2019	Romosozumab	Humanized IgG2	Sclerostin	Osteoporosis in postmenopausal woman at risk of fracture
2019	Risankizumab	Humanized IgG1	IL-23p19	Plaque psoriasis

[a]*Food and Drug Administration; this is not an exhaustive list of therapeutic antibodies.*
[b]*Fab.*

B-lymphocytes. Birds are also used to raise PoAbs. One of the most commonly used avian models is the chicken. Animals are commonly employed to raise PoAb, but the procedure has the following limitations: (1) it takes time to complete immunization of animals, (2) it is expensive to maintain the herd of immunized

animals, and (3) immunized animals are vulnerable to threats such as infection, which can lead to enormous losses if antibodies are used for commercial scale.

Principle

The principle for the production of PoAbs is based on the observation that an animal has to be exposed to the antigen of choice. In laboratory circumstances, or even for commercial production of PoAb, animals have to be immunized with purified antigen (Carey Hanly et al., 1995; Coligan et al., 2005; Cooper and Paterson, 1995). There are various protocols for immunization of animals. Immunization requires multiple exposures of antigen at selected intervals to raise high-affinity antibodies. Affinity maturation of PoAb takes time, which is the reason for repeated antigen injections used in the immunization procedure. Repeated injections of antigen at specific time intervals induce both the primary immune response and the secondary immune response. It is advisable to use adjuvant along with antigen for immunizations. Adjuvant causes slow release of antigen, which supports generation of better immune response due to consistent release of antigen into the circulation. Finally, the decision to collect antibodies (serum or plasma) from immunized animals is made after the confirmation of antibody titer. A small volume of serum plasma from the immunized animal is used to confirm antibody titer. If antibody titer is not high, then booster doses of antigen are required.

It is always advisable to optimize conditions to raise PoAb (e.g., animal model, concentration of antigens). When antibodies are repeatedly required from the same immunized animal, it is advisable to give a booster injection before collecting antiserum.

Methodology and rationale for PoAb production

The methodology and rationale of different steps involved in PoAb production are discussed in this section. The method can be divided into steps: (1) antigen preparation, (2) immunization, (3) antibody titration, and (4) antibody isolation or purification.

Step 1: antigen preparation

Antigen has to be prepared for immunization so that one can produce good quality (high-affinity) antibodies. This is the reason antigen is usually injected with adjuvant. The most commonly used adjuvant is Freund's adjuvant. Freund's adjuvant contains components like mineral oil and emulsifying agents (mannide mono-oleate), which disperses the mineral oil into small droplets.

On mixing of antigen with Freund's adjuvants, the aqueous antigen preparation gets dispersed into small droplets that are surrounded by a film of oil, aiding slow release of antigen from the site of infection. Freund's complete adjuvant (FCA) contains heat-killed mycobacteria; routinely, it is recommended that the first immunization be done with antigen mixed or emulsified with FCA because heat-killed *Mycobacterium tuberculosis* is effective to induce nonspecific immune response. The second and other consecutive booster injection is given with antigen mixed or emulsified with Freund's incomplete adjuvant (FIA). FIA contains every other component of FCA except for heat-killed *M. tuberculosis*.

Step 2: immunization of animals

This is the second step for the generation of PoAbs. The major purpose of immunization is to expose animals with specific antigen and repeated exposure of antigen to the immune system to help in antigen recognition. Immunization causes immune activation, resulting in the production of antibodies against the antigen. The amount of antigen, site of injection, and duration between booster doses are decided on the basis of the animal used for antibody production.

If a rabbit is used to raise antibodies, one has to give an injection prepared with either FCA or FIA. A subcutaneous route of injection is commonly advised for immunization. To immunize a rabbit, the first step is to shave the hair (fur) from the rabbit's back with a hair trimmer. After trimming the hair, the skin has to be sterilized with 70% ethanol. Then, injection of the prepared antigen is given subcutaneously in the shaved skin area using a needle and syringe. If the injection volume is large, subcutaneous injections can be given at multiple sites. After the first injection of antigen, at least two more injections of booster doses have to be given to the same rabbit at an interval of 7 or 10 days. An immunization record has to be maintained, including name of antigen, concentration of antigen, volume of injections, route of injections, site of injections, dates of injection, as well as anything unusual that was noticed during the immunization process. (Note: Before immunization, it is important to check a serum sample of the rabbit to assure that serum does not have any antibody against the same antigen. It is also recommended that the rabbit be bled before each consecutive immunization.). The blood sample that is withdrawn is used to determine antibody titer as mentioned in the following text.

Step 3: antibody titer

During immunization and after immunization, it is important to know the titer of antibodies present in the immune serum. High-titer antibodies are always preferred over low-titer antibodies for therapeutic, analytical, or preparative applications. Antibody titration is done by applying various assays based on antigen–antibody reactions like agglutination, hemagglutination,

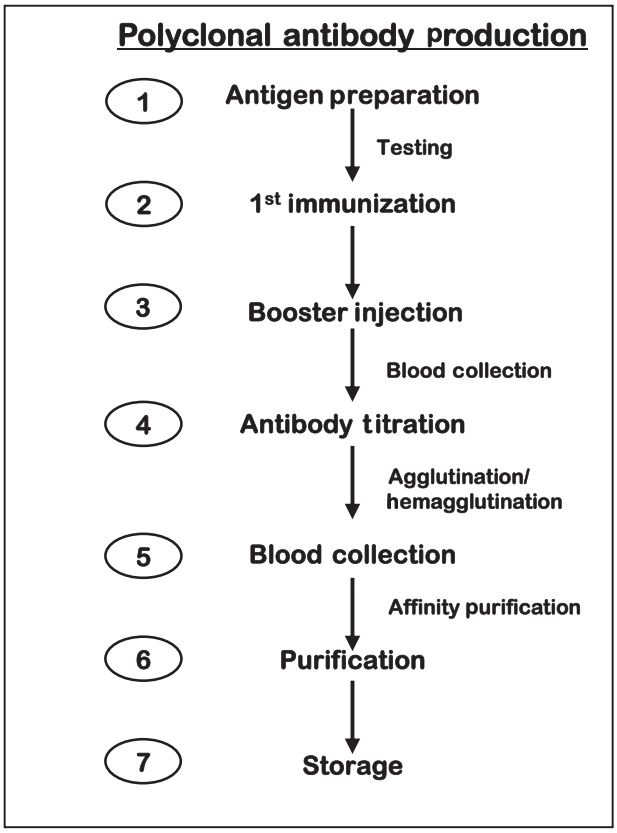
ELISA, RID, ODD, etc. Selection of the assay to quantify antibody titer is dependent on the facilities and expertise available. One of the most common and easy methods to determine antibody titer is by agglutination test. The advantage of this test is that it is convenient to perform, requires less time to perform, and can be used for particulate antigens as well as soluble antigen. [If the antigen is particulate in nature, it can be directly used for an agglutination reaction, e.g., red blood cell (RBC).] If the antigen is soluble, it can be performed with the use of beads (latex), sheep red blood cells (SRBCs), or even chicken RBCs (CRBCs). When RBCs are used for an agglutination test, the test is known as a hemagglutination test. When soluble antigens are coated on RBC (either chicken or sheep) for the agglutination reaction, then it is known as an indirect hemagglutination test. On the basis of the aforementioned tests, one can determine titer of antibodies. Antibody titer is the decision-making factor for further booster injections. Antibody titer also suggests possible dilution of antibodies to be used for further applications.

Step 4: purification and identification

Titration of antibodies provides information about specificity of antibodies. Antibody titration methods can also be used to test cross-reactivity of antiserum with closely related antigens. In certain circumstances, serum or plasma containing antibodies can be used directly. Yet in some other circumstances, it is essential to use purified antibodies. There are various methods for antibody purification. Selection of the method for PoAb purification depends on various factors like availability of resources, cost of purification, scale of purification, and downstream applications of the PoAb. The best and most convenient method for purification of PoAbs is affinity purification. Detailed protocols to raise PoAbs can be found in any standard protocol book or lab manual.

Crucial steps for PoAb production (Flow Chart 17.1) are as follows:

1. Antigen preparation
   Antigen has to be prepared with either FCA or FIA. Proper emulsification of antigen with adjuvant should be tested before injection (step 1).
2. Immunization
   The animal has to be injected subcutaneously with an antigen prepared with adjuvant. After initial immunization, booster doses have to be given at specific intervals. A serum/plasma sample has to be collected every time, before injecting the antigen (step 2 and 3).
3. Antibody titration
   Antibody titration provides the information about affinity maturity of specific antibodies.



FLOW CHART 17.1 Major steps for the production of polyclonal antibodies.

   Titration results are used as the criteria to decide booster doses and time of antibody collection. Agglutination or hemagglutination tests are used to quantify antibody titer (step 4).
4. Purification

If antibodies are showing high titer or expected titer, then PoAb can be used as antiserum. If unwanted protein of serum or plasma can interfere with downstream applications, then PoAb can be purified by various means (step 5–7).

Monoclonal antibodies

MoAbs are those antibodies produced from a single cell (i.e., one specific B cell). Antibodies with different specificities are present in antiserum as they are produced from numerous B cells or a clone. Undoubtedly, PoAbs are beneficial to the host, but have limited therapeutic, analytical, and preparative applications. It is easier to identify and purify antibodies of interest from a PoAb mixture; nevertheless, it is really difficult or next to impossible to identify unique B cells that produce that specific antibody. Even after tedious efforts to identify the B cell of interest, the B-lymphocyte

cannot be used as a continuous source of antibody because B cells have a limited lifespan. Therefore, to have a continuous source of consistent antibodies, the only option is to have immortalized B cells. The answer for this complicated problem came with the discovery of hybridoma technology, which is the principle applied in the production of MoAb. Now the major concern is that immortalization of B cells should neither change B cells characteristics of antibody production nor the specificity of the antibody after immortalization. MoAbs came into existence in the last quarter of the 20th century with the efforts of Kohler and Milstein, who designed an experiment to immortalize an antibody-producing B-cell by fusing it with tumor cells (i.e., myeloma cells). They had fused primary B cells (antibody-producing) with myeloma cells (tumor cells) and produced a hybrid cell that retained the characteristics of both cells. The method that Kohler and Milstein used exploited good characteristics of both cell types (i.e., hybrid cells or hybridoma cells were able to retain the antibody-producing quality of B cells and the immortalization properties of myeloma cells). The word hybridoma has evolved from the union of two English words, like the union of two cells (i.e., B cells and myeloma). For the word "hybridoma," "hybrid" came from the hybridization of two cells and "oma" came from myeloma cells. These hybridoma cells became a perpetual source of antibodies having the same specificity.

Principle

The principle for the production of MoAb relies on two important factors: (1) a source of antibody-producing cells (i.e., B cells) and (2) immortalization of these cells by fusion with tumor cells. After fusion, the cell mixture has three different cell types: (1) B cells, (2) tumor cells, and (3) hybrid cells. Although it sounds very simple to generate MoAbs, this method has had some inherent limitations (i.e., selection of correct hybrid cells). The issue of selection of hybrid cells was resolved with the use of selection medium. The rationale of the selection process is discussed under the Biochemical Pathway: Hybridoma Selection" section of this chapter. Hybridoma cells can be grown either in vitro (in tissue culture) or in vivo (in animals) as a tumor. Hybridoma cells can be frozen at ultra-low temperatures and can be retrieved and revived from frozen stocks for MoAb production when required. Generation of MoAbs requires working both under in vivo and in vitro conditions.

Methodology and rationale of MoAb production

This section discusses the methodology and rationale of different steps required for the production of MoAb using mice (Coligan et al., 2005). MoAb production can be divided into five major steps: (1) immunization of animal, (2) fusion of cells, (3) selection of clone, (4) expansion of clone, and (5) purification of MoAbs (Fig. 17.3).

Step 1: immunization of mouse

The prerequisite for the production of MoAbs is to immunize animals that can produce the antibody of choice. Immunization leads to antibody production because of the activation of B cells. After immunization, spleen is used as a source of lymphocytes. The spleen contains B-lymphocytes, which secrete antibodies against the antigen used for immunization.

For mouse immunization, the procedure followed is the same as mentioned for the production of PoAbs. The essential steps to immunize a mouse are (1) antigen preparation, (2) immunization, and (3) determination of antibody titer. These steps have already been discussed in "Methodology and rationale for PoAb production" section.

Step 2: preparation of splenocytes

In mice, a very limited volume of peripheral blood can be drawn, which can become a limitation. Therefore, the best source for lymphocytes is the spleen. Dissection of the spleen and preparation of splenocytes has to be performed under aseptic conditions. The mouse has to be killed by cervical dislocation, and its skin surface has to be sterilized with 70% ethanol before bringing it into the hood. The spleen has to be surgically removed under sterile conditions. Then, a single-cell preparation has to be prepared by lysing RBCs using RBC lysis buffer. After RBC lysis and washing, splenocytes are ready for fusion with myeloma cells.

Step 3: fusion of cells

For the fusion step, splenocytes and myeloma cells are mixed together in a specific ratio. A cell suspension of mixed cells has to be treated with agents that can induce fusion. There are various agents that are used for fusion of B-lymphocytes with myeloma cells. Some of the common ones are Sendai virus, polyethylene glycol, etc. Nowadays, the most convenient and cheapest method to fuse cells is by using polyethylene glycol. After fusion, cells become quite fragile, so further steps have to be performed with utmost care. No matter which method is used, a very common problem encountered is low cell viability during the fusion.

Step 4: selection of hybrid cells

When a mixture of fused cells is grown in hypoxanthine, aminopterin, and thymidine (HAT) medium, the aminopterin works as a folate antagonist and blocks the de novo pathway for the synthesis of the

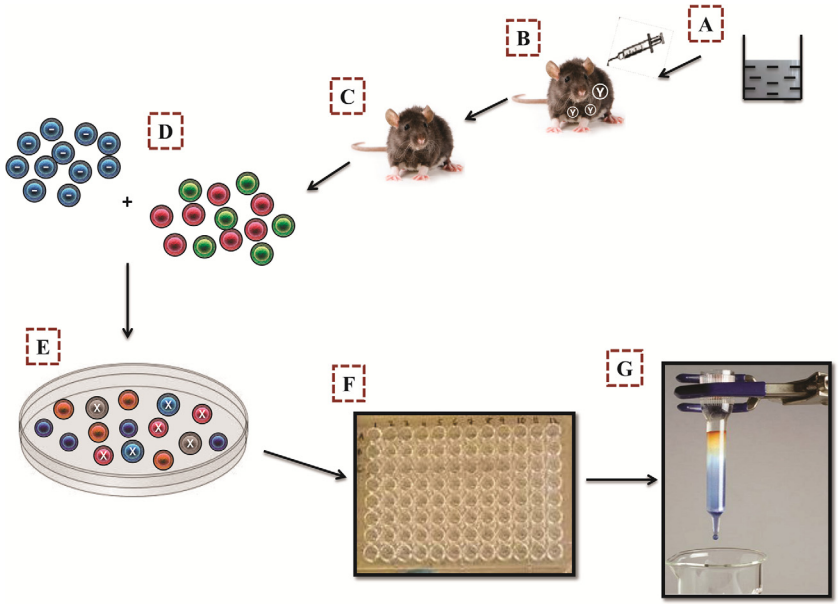


FIGURE 17.3 Schematic representation of production of monoclonal antibodies. This figure represents different crucial steps in the production of monoclonal antibodies. (A) Antigen preparations: antigen has to be prepared with Freund's complete adjuvant or Freund's incomplete adjuvant. (B and C) Immunization: mice have to be immunized by injecting antigen prepared with adjuvant; they also have to be given booster doses. (D) Preparation of splenocytes and fusion with myeloma: The spleen has to be removed from immunized mice and a single-cell suspension of splenocytes has to be prepared. Then, splenocytes have to be fused with myeloma cells in the presence of fusogenic agents. (E) Selection of hybridoma: after fusion, a hybridoma has to be selected from the cell population mixture. Cells have to be grown in HAT selection medium so that after selection only hybridoma cells can survive, while B-lymphocytes and unfused myeloma cells will die. (F) Screening of clone: after selection of hybridoma cells, a specific clone has to be selected. Different hybridoma cells are diluted in 96-well plates, and after a period of time, each clone has to be tested for specificity against the antigen. (G) Purification of monoclonal antibodies: after selection of a specific hybridoma clone, monoclonal antibodies can be purified. If downstream application requires purification of monoclonal antibodies, then the clone can be expanded and appropriate methods can be applied for purification of monoclonal antibodies. (Blue cells), myeloma cells; (Red and Green cells), lymphocytes; and (Grey cells), hybridomas. Cells marked with an "X" represent cell death in the selection medium.

nucleotide (i.e., purines and pyrimidines), so the cells that do not carry out the salvage pathway for nucleic acid synthesis eventually die in the selection medium.

In due course, primary cells (B cells) will die because they have a limited lifespan. Myeloma cells that do not get hybridized will die because of blockage of the de novo pathway and nonfunctional salvage pathway. Details are discussed under "Biochemical pathway: hybridoma selection" section. Finally, only those B-cells that are fused with tumor cells will get selected; these will be the only survivors. Even the selection medium leaves a mixture of cells producing antibodies with varying specificity. To identify hybrid cells producing the antibody of interest, screening procedures are required (as discussed in the next step).

Step 5: selection of clones

After selection of the fused hybridoma cell, one has to select a specific clone secreting the desired antibody from the population of hybridoma cells. This experiment involves plating of cells in a 96-well plate; the experiment is designed in a manner that after dilutions, each well contains a single antibody-producing cell. After incubation, cells start dividing and the cell number increases. The time eventually comes when they start producing detectable levels of antibody against the antigen. Ideally, each well of tissue culture should produce antibodies with single specificity because cells that have grown in each well should arise from a single cell (clones). During this step, culture supernatant from each well is screened for antibody production. Appropriate protocols can be applied for screening of clones, as mentioned in the production of PoAbs. Hybridoma cells that produce antibodies against the antigen are selected. This will be the desired clone, which has to be further expanded for the production of the MoAb.

Step 6: expansion of clone

After identification of the specific clone, it has to be expanded so that it can be used for larger antibody production. Expansion strategies are based on the need and amount of antibody required. Two methods commonly used for expansion of clones are the following:

1. In vitro method: In this method, as cells grow in number, they can be transferred to larger culture

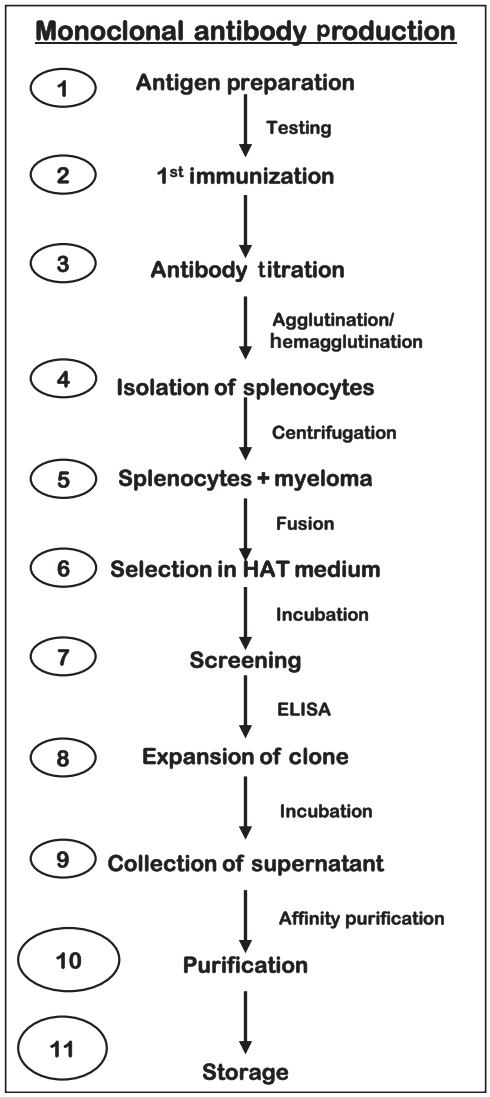
vessels stepwise (like 6-, 12-, 24-, 48-, and 96-well plates) so that they have enough medium and space to grow. Later they can be transferred to different sizes of tissue culture flasks. It is always a good practice to freeze some of these cells at ultra-low temperatures. These frozen cells can be retrieved and revived for further production of antibodies.

2. In vivo method: In this method, antibody-producing clones can be introduced into the peritoneal cavity of mice, where they start grow as a tumor. Antibodies will be secreted by the hybridoma (tumor) cells in ascites fluid in the peritoneal cavity of the mice. Further expansion of the clones depends on need, availability of facilities, and expertise.

Step 7: purification

Although it is not always essential to purify MoAbs as they are specific and produced from one clone, they can be used directly either in the form of tissue culture supernatant or ascites (if this does not interfere with further steps). Sometimes it might be necessary to purify MoAbs in certain conditions like when (1) antibody concentration is low, (2) other components of tissue culture medium or ascites fluid are interfering, (3) the antibody has to be used for therapeutic purposes, (4) the antibody has to be used for diagnostic purposes, or (5) the antibody needs further manipulation [e.g., conjugation with horse radish peroxidase (HRPO), alkaline phosphatase, fluorescent dye]. For these kinds of applications, it is always better to purify MoAbs from the tissue culture supernatant. MoAbs can be purified by various means; the most common method for purification of antibodies is by affinity column chromatography. Detailed protocols to raise MoAbs can be found in any standard protocol book or lab manual.

Crucial steps in production of monoclonal antibodies (Flow Chart 17.2)



FLOW CHART 17.2 Major steps for the production of monoclonal antibodies.

1. Antigen preparation: Antigen has to be prepared with the adjuvant of choice. The most common adjuvants are either FCA or FIA. Before injecting antigen, it is advisable to test the proper emulsification of antigen with adjuvant (either FCA or FIA) (step 1).
2. Immunization: Mice have to be immunized by injecting antigen prepared either with FCA or FIA. Usually injections are given subcutaneously, and a standard immunization protocol is followed (step 2).
3. Antibody titration: On the basis of antibody titer, a decision is made regarding booster doses and preparation of splenocytes (step 3).
4. Preparation of splenocytes: In mice, splenocytes are the source of B-lymphocytes (activated). A single-cell suspension is prepared for fusion with myeloma cells (step 4).
5. Fusion: Isolated splenocytes have to be fused with myeloma cells using fusogenic agents for the production of hybridoma cells (step 5).
6. Selection: After fusion, three different cell types are present in the mixture (i.e., splenocytes, tumor cells, and fused cells). From this mixture of cells, only fused cells are selected with the aid of selection medium (steps 6 and 7).
7. Expansion: After selection of fused cells, these cells have to be expanded and identified by confirming their ability to produce the antibody of interest. The unique clone has to be further expanded by different available methodologies (steps 8 and 9).
8. Purification: MoAbs can be purified by various methods. The most common method for purification

of antibodies is by affinity column chromatography (steps 10 and 11).

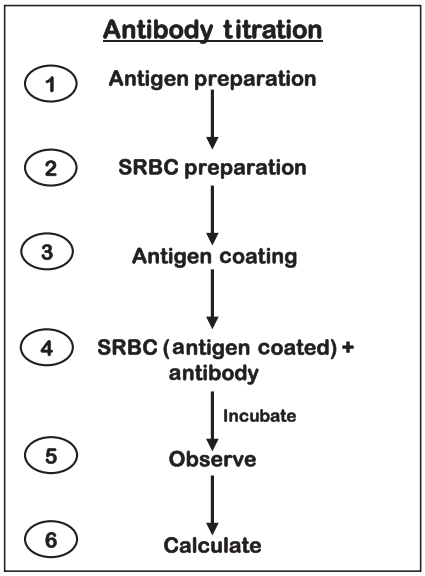
Antibody titration

PoAbs raised by immunization against an antigen need to be quantified for their specificity and titer. In a similar fashion, antibody titer is also required for the MoAb production. The quantity of antibodies for further application can be determined by knowing the titer of the antibodies. High-titer antibodies are also necessary for generation of good quality PoAb/MoAb. To ascertain titer of antibodies, one has to have an assay system to titrate antibodies either for production of PoAbs or for generation of MoAbs. Various methods to titer antibodies are ELISA, ODD, RID, agglutination, hemagglutination, etc. All these methods are based on the principle of antigen–antibody binding. The choice of method to be used is dependent on the facilities and expertise available, purpose, etc. One of the most common, convenient, and cost-effective methods for this purpose is the agglutination method, which is based on the principle of antigen and antibody interactions. When antigen and antibody are present in optimum concentrations, the antigen–antibody complex forms a three-dimensional, lattice-like structure.

Agglutination reactions can be performed with particulate antigens (e.g. RBCs, bacteria). The Widal test for *Salmonella typhimurium* or blood group testing is also based on the principle of agglutination reactions. When the antigen is soluble, one can coat the antigen on RBCs; this reaction is called as hemagglutination.

Methodology and rationale of antibody titration (Flow Chart 17.3)

1. Antigen preparation: A soluble antigen-like bovine serum albumin (BSA) has to be dissolved in appropriate buffer. This soluble antigen is used to coat SRBCs (step 1).
2. Preparation of SRBCs: SRBCs have to be collected and washed. A specific concentration of SRBC has to be prepared in buffer. After making the SRBC solution, it has to be coated with tannic acid. This tannic acid–coated SRBC can be used for coating any soluble antigen (step 2).
3. Antigen coating: After coating SRBCs with tannic acid, they have to be mixed with antigen and incubated for a certain time period. During incubation, antigens get attached to the SRBCs, and the coated SRBCs work as particulate antigens and can be used to titrate the antibody (step 3).
4. Antibody titration: Hemagglutination plates (which have 96 wells) are used. In these plates, one can use serial dilution of antibody. After preparing the dilutions, an equal volume of antigen-coated SRBCs are added in each well, and the plate is incubated at room temperature (step 4).
5. Observation: Positive agglutination is seen when cells form a continuous layer in the wells. In the wells where agglutination has not occurred, SRBCs settle down at the bottom of the well, resembling a red button (step 5).
6. Calculation: The maximum dilution showing positive agglutination, which can be observed with the naked eye or with the help of mirror, is recorded. This dilution is the antibody titer against the antigen (step 6).



FLOW CHART 17.3 Major steps for the titration of antibodies.

Biochemical pathway: hybridoma selection

Nucleotide synthesis is important and crucial for DNA synthesis and cell division. In any cell type, there are two pathways for nucleotide synthesis: a de novo pathway and a salvage pathway. In the de novo pathway (new; from scratch), nucleotides are synthesized from simple precursors that cells can procure from outside sources. In the salvage pathway, synthesis of nucleotides occurs by using intermediates found in the degradation pathway of nucleotides such as nucleotides and nitrogenous bases.

It has been studied and established that cells grown *in vitro* can make use of both the pathways for nucleotide synthesis. When we use splenocytes for fusion with myeloma, the fusion cell contains two different cell types: (1) splenocytes, which are primary cells, and (2) myeloma cells, which are tumor cells. Of these two cell types, primary cells have a limited lifespan, while

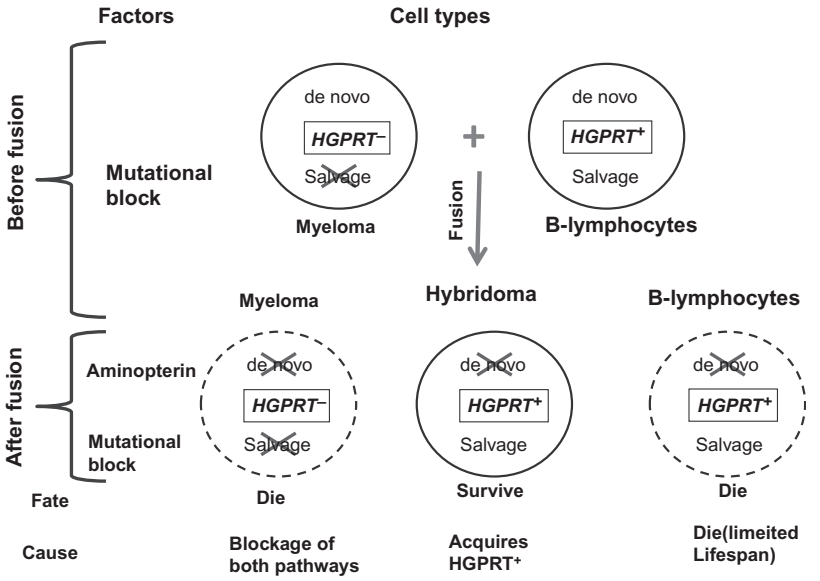


FIGURE 17.4 Biochemical pathways used for selection of hybridomas: for the production of monoclonal antibodies, myeloma cells and splenocytes are used, and after fusion, hybridoma cells are selected. Myeloma cells that are used for the production of monoclonal antibodies are HGPRT$^-$ (hypoxanthine-guanine-phosphor-ribosyl-transferase), which is an essential enzyme of the salvage pathway. These cells are unable to carry out the salvage pathway. After fusion, hybridoma cells acquire the *HGPRT*$^+$ gene from B-lymphocytes. When these cells are grown with selection medium (HAT medium), aminopterin present in this selection medium blocks the de novo pathway, but the hybridoma can survive by synthesizing nucleotides via the salvage pathway. Myeloma cells and B-lymphocytes die in the selection medium.

tumor cells are immortal and can be grown for an unlimited period of time. To select the hybrid cells, the nucleotide biosynthesis pathway turned out to be a rescuer. Mutation in enzymes related to salvage pathways is a common method used in mammalian cell cultures. The most common target enzyme is hypoxanthine-guanine phosphoribosyl transferase (HGPRT). HGPRT is an important enzyme of the salvage pathway. Since HGPRT is a part of nonessential pathway, HGPRT$^-$ cells can grow normally and perform DNA synthesis using a de novo pathway. It was speculated that B cells have a finite lifespan so they will die after some time, but myeloma cells have to be eliminated from the culture medium. Therefore, myeloma cells have to be made HGPRT deficient, and the culture should be done in a medium that favors the growth of HGPRT$^+$ cells only. This way myeloma cells will not survive in the selection medium. Using the same rationale, hybridomas produced after fusion of B cells and myeloma cells will be able to survive in the selection medium. HGPRT$^-$ can be selected by growing myeloma cells in the presence of purine analogs such as 8-azoguanine (8-AG). HGPRT uses 8-AG as substrate and converts 8-AG into nucleotide monophosphate. The 8-AG-containing nucleotides are further processed during DNA synthesis and get incorporated into DNA and RNA. Substitution of 8-AG is toxic, and therefore, cells that are HGPRT$^+$ (or normal) will die, and only HGPRT$^-$ cells will survive when grown in the presence of 8-AG. In this way, HGPRT$^-$ myeloma cells are selected. These HGPRT$^-$ myeloma cells are fused with B cells, which are HGPRT$^+$. After fusion, the mixture has different cell populations: (1) B-lymphocytes (unfused), (2) myeloma cells (unfused), and (3) fused cells (or hybrid cells). The major issue was to select only those hybrid cells that are producing the antibodies of interest. The solution to this problem came with the use of a selection medium that allowed only hybridomas to survive. The selection medium used for MoAb production is known as HAT medium. Aminopterin blocks the de novo pathway, whereas hypoxanthine and thymidine are required for nucleotide biosynthesis in the salvage pathway. In selection medium, the following things occur, which support the selection of hybrid cells: (1) B-lymphocytes (unfused), being primary cells, die due to limited lifespan; and (2) myeloma cells that are HGPRT$^-$ die in the selection medium because they do not have a salvage pathway. Only hybridoma cells can survive because now they are HGPRT$^+$ and can use the salvage pathway for nucleotide biosynthesis (Fig. 17.4).

Ethical issues

Currently, ethical issues have become significantly important, certainly keeping pace with antibody research and its commercialization. It has become a necessity to regulate experiments as well as industrial applications of antibodies and their modifications. Antibodies, either polyclonal or monoclonal, have to be harvested from animals. Harvesting antibodies is associated with various ethical issues regarding animal use. Animals either have to be bled or sacrificed to serve the purpose. Animal experimentation to raise antibodies involves the use of Freund's complete and FIAs; these adjuvants may cause wounds at the site of injection. Therefore, dose and volume of the immunization injection have to be carefully monitored for animal health and welfare. On the one hand, animals

serve as a source for antibody production, and on the other hand, they are good models for testing various aspects of antibodies.

These processes of animal experimentation need ethical clearance with due consideration to different aspects of antibody production (e.g., bleeding volume, bleeding site, choice of animals). The use of transgenics for antibody production raises various other ethical issues related to the use of recombinant DNA. Apart from animals, the use of human subjects is undoubtedly a serious concern for various ethical reasons, such as (1) the need for healthy volunteers to test therapeutic antibodies, (2) the trial of antibodies in terminal patients, and (3) the reliability of data obtained from such trials for further clinical utility and benefits. One of the glaring examples of antibody-related clinical trials took place in London in 2006. Six healthy individuals participated in a clinical trial for a new MoAb (TGN-1412). Within minutes of inoculation, all six volunteers were reported to have suffered multiple organ failure (Stebbings et al., 2007; Nechansky and Kircheis, 2010). These volunteers did survive, but the episode left many questions about the safest way to test antibodies in human subjects, and what the criteria should be in such a study. As time goes by, it is still difficult to find straight answers to some of these questions. New ethical issues will certainly develop over time, which will demand appropriate attention and resolutions.

Camelid nanobodies/single-domain antibodies/ variable domain of camelid heavy chain only antibody

Nowadays, biotechnology has reached unprecedented levels, and the field of recombinant antibody technology has highly progressed compared to that in antecedent time. Humanized, chimeric, and MoAbs are advanced examples of recombinant antibody technology. Nevertheless, all of these have some limitations of stability and clinical application like low tissue distribution, adverse responses related to non-human origin, and high cost scale up production. Overcoming to these problems, minimization of antibody size and antigen antibody binding site would be major advancements. Generation of monovalent fragment from IgGs, single domain antibodies, and single chain variable fragments provides hope to minimize the previous limitations related to recombinant antibody technology

In late 19th century, it was discovered that camelids (llama, dromedary, camel) produce two different types of antibodies (Hamers-Casterman et al., 1993). One is classical antibody having heavy chain with light chain and four Ig domains and another unique immunoglobulin having only a heavy chain. The only chain of camelid antibodies contains three domains and completely lacks the first CH1 domain. VHHs (variable domain of camelid heavy chain only antibody) are popularly also known as nanobodies. Nanobodies can bind antigens without requiring domain pairing. VHHs are similar to variable heavy chains of classical antibody, both are composed of three variable CDRs and four frame work regions. However, VHHs have some difference from VHs that is in their amino acid sequence. AA residues in VHHS are having substitutions at four (37, 44, 45, and 47) different places in the framework region. At position 37, valine is replaced with phenylalanine or tyrosine; at position 44, glycine is replaced with glutamate; at position 45, leucine is replaced with arginine; and at position 47, tryptophan is replaced with glycine or leucine. Substitution of amino acids and conversion of inter phase from hydrophobic to hydrophilic provide molecular stability to VHHs, which is important for its single-domain nature. In VHHs, CDR3 is long and extended, bearing average 18 amino acid residues in comparison to 14 amino acid residues in VHs. CDR3 is also stabilized by an additional disulfide bond with a cysteine residue in CDR1 or FR2, which resists the flexibility of the prominent loop and provides greater stability. Not only this, CDR3 contributes to covering the nanobody surface analogous to VL/VH interfaces.

VHHs have higher thermal stability in comparison to single-chain variable fragments (ScFv). Higher thermal stability of VHHs owing to replacement of hydrophobic amino acids is necessary for its efficient refolding after chemical or thermal denaturation. VHHs have capability of fast and dense tissue penetration due to their smaller size and extended CDR3 loop. Studies have revealed that these features play pivotal roles in receptor recognition by VHHs and are helpful in their binding to active site of enzymes or recessed antigenic sites. They have been used as diagnostic antibodies for trypanosome infections (De Genst et al., 2006).

Nanobody generation and selection:

- First step of nanobody generation is immunization of a llama (*Lama glama*) or a dromedary (*Camelus dromedaries)* with several antigens.
- Perform the Immunization through five to six injections (one/week) with approximately 0.5 mg protein.
- After last immunization, collect blood sample and separate the lymphocytes.
- Extract the total RNA from peripheral blood lymphocytes and perform cDNA synthesis with an oligo(dT)primer.

- Accomplish the polymerase chain reaction amplification from the cDNA and obtain VHHs encoding sequences.
- Then clone it into the phage display phagmid vector pHEN4.
- Obtain the nanobody library of approximately 10 independent transformants after three rounds of phage display.
- Perform the panning on solid-phase coated protein after assessing nanobodies by polyclonal phage ELISA.
- Perform the restriction fragment length polymorphism (RFLP) analysis and nucleotide sequencing of the nanobody sequences from the ELISA-positive colonies.
- This whole procedure takes 2–3 months from the starting of the procedure.
- However, in case of naive library preparation, immunization can be avoided and nanobodies are generated by collecting the blood of several nonimmunized animals. Amplification might be slower in case of naive library and extra round of panning would be required.

In 1975, Georges J. F. Köhler and Cesar Milstein reported the production of MoAb by hybridoma technique. For this discovery, they got the Nobel Prize in Physiology or Medicine for 1984.

Translational significance

The translational value of the immune system and immunity is only limited by the imagination. It is a Herculean task to decide where to start when considering the translational significance of antibodies because anything and everything about antibodies has translational significance. Applications of immunoglobulins are growing as more and more knowledge is gained, and numerous antibody applications are being realized and recognized by various stakeholders such as scientists, clinicians, etc.

von Behring demonstrated the first translational use of antibodies when he used them to treat infectious diseases. He probably used serum without knowing the details about antibodies, except from knowledge gained by observations in day-to-day life. Later, Edward Jenner's experiment with vaccines also implicated antibodies in the induction of immunity directly or indirectly. However, the discovery of MoAb opened so many avenues for their applications. MoAbs have been the main force for development of chimeric and humanized antibodies. Advancement in protein chemistry has introduced newer applications of antibodies (e.g., conjugated MoAb). Attachment of probes to MoAb has broadened the horizons for their versatile applicability in different areas of biomedical sciences. Currently, various probes can be conjugated with MoAb (e.g., alkaline phosphatase, HRPO, fluorescein isothiocyanate, magnetic probes). It is difficult to imagine ELISA, FACS, IHC, immunoelectron microscopy, or confocal microscopy without antibodies. What about purification of various products to homogeneity without immuno-affinity chromatography? With creative thinking, it will be possible to find many new applications for the specificity of antibodies.

As of now, protein engineering can be used to produce bi-specific antibodies (Presta, 2003). Employing recombinant DNA technology, it may be possible to produce multispecific antibodies in the near future. There is always a possibility that these antibodies may either have no side effects or may have minimal and manageable side effects when used as therapeutic agents. At this juncture, it is a possibility to produce completely human antibodies only by artificial means (i.e., outside the human body). These antibodies could be a boon to treat infections and other disease causative factors. Only time will tell if a magic bullet could be developed, as a solution for many illnesses, if not all.

Clinical correlations

Nowadays, Ebola virus disease is spreading like epidemic in several countries. MoAbs can be used as treatment therapy for Ebola virus, but the only problem is short-term nature of the immunity provided by MAbs. In a recent study, it was found that adeno-associated virus (AAV) was used to transfer *MAb* genes into the host to produce functional MAbs in vivo. This kind of process is also known as "vectored immunoprophylaxis." In vivo expression of non-neutralizing MAb 5D2 or 7C9 provided 100% protection and neutralizing MAb 2G4 provided 83% protection. A cocktail of both neutralizing and nonneutralizing MAbs AAV-2G4/AAV-5D2 provided complete protection when applied 7 days prior to challenge. AAV-MAb therapies provided sustained protection from challenge post 5 months following AAV administration (Van Leishout et al., 2018).

Zika virus infection is an epidemic disease associated with neurological complications in newborns and adults. For prevention of this disease, not many vaccines and antivirals have been developed till date. Only MAbs and PoAbs have shown potential in controlling and eliminating the Zika virus infection. Around 153 MAbs specific for Zika virus had been tested and ~63 could specifically bind to ZIKV E protein. Similarly some of the MAbs specific to ZIKV

protein were able to show 50% inhibition of the virus at concentrations of <1 mg/ml. This indicates that the ZIKV-specific MAbs can be explored further for their anti-Zika virus effects (Wang et al., 2017).

MAbs have also been helpful for asthma treatment. It has been studied that interleukin (IL)-5 and IL-13 cytokines are mainly responsible for asthma pathogenesis and progression. IL-5 cytokine was a key factor in maturation, recruitment, and survival of eosinophils associated for complexities of asthma. MoAbs mepolizumab and reslizumab directed against IL-5 prevent the cytokine from engaging its receptor. Another MAb benralizumab targeting the alpha chain of IL-5 receptor helps in bringing the lysis of target cells. Both the different kinds of MoAbs have been found to reduce the number of circulating eosinophils in human beings (Roufosse, 2018).

Nanobodies are better suited as alternative diagnostic tools and drugs for many diseases because of high thermal and denaturation stability, high affinity and specificity, smaller size, and low cost production in microorganism. For the prevention of *Escherichia coli* infection, VHHs are a good option as they have higher stability at extreme pH and at high concentrations of chaotropic agents (Dumoulin et al., 2002). VHHs have been used as therapeutic agents against neglected tropical diseases like Chagas disease, dengue, human African trypanosomiasis, leishmaniasis, rabies, schistosomiasis, cysticercosis, etc. (review by Fernades et al., 2017). VHHs have been used against cancer and tumor when coupled to toxic loads (Cortez-Retamozov et al., 2002). Camelid antibodies are used against upregulation of EFGR and STAT3 activation, which is common phenomenon of cancer and tumors growth (Singh et al., 2018; Rossotti et al., 2019). The smaller size of VHHs (about 15 kDa) is helpful in their rapid clearance from the renal filter. VHHs help in crystallization of conformational proteins by preventing domain mobility, hiding protein bound polysaccharides, and insertion in clefts or between interfaces, thus stabilizing loops or large complexes.

World Wide Web resources

The World Wide Web has become the most convenient and powerful tool for retrieving information on any subject, including scientific literature. As is well known, Google has turned out to be the most popular site for information retrieval. A search for the keyword "monoclonal antibody" at www.google.com returns more than 8.62 million hits, while "PoAb" returns 3.2 million hits. There is no doubt that Google is a gigantic resource for information regarding basic concepts on antibodies, immunoglobulin structure and function, immune response, animations, protocols for raising PoAbs and MoAbs, adjuvants, animal models for antibody production, rules and regulations, and guidelines on the use of animals for experimental studies. It is not an overstatement to say that any information related to MoAbs and PoAbs can be easily searched via Google.

Another important web resource for antibodies is the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov). This website is hosted by the National Institutes of Health (NIH), Bethesda, Maryland. It is one of the most authenticated web resources for scientifically validated and updated information. One can use various search options to retrieve information on research publications, gene sequences, and protein sequences; even some of the best books are available on this site.

Another useful web resource is the ImMunoGeneTics site (http://www.imgt.org). It covers different aspects related to immunoglobulins (e.g. genetics, gene sequences).

The Antibody Resource Page (http://www.antibodyresource.com) is one of the most comprehensive web pages dedicated to antibodies. This site provides information about antibody structure, function, production, research, and clinical applications, as well as great educational resources for immunology, antibody images and galleries, and names of journals and books related to the subject. This web resource also lists the suppliers of custom-made antibodies and contract service providers for antibodies, etc.

Other resources on antibodies are the web sites of Current Protocols in Immunology (http://onlinelibrary.wiley.com/book/10.1002/0471142735) and Current Protocols of Molecular Biology (http://onlinelibrary.wiley.com/book/10.1002/0471142727). These are some of the best resources for antibodies regarding principles, limitations, and benefits of a wide variety of protocols.

Currently, there is no way of getting around the use of animals for the production of antibodies. Therefore, there is always a need for information regarding animal welfare. For information of this nature, one can refer to the website maintained by the United States Department of Agriculture (USDA), Beltsville, Maryland. One can find numerous books and proceedings regarding antibodies at their site: http://awic.nal.usda.gov/awic/pubs/antibody/books.htm.

For MoAb naming conventions, the American Medical Association (AMA) provides good information at its site dedicated to naming biologics: http://www.ama-assn.org/ama/pub/physician-resources/medical-science/united-states-adopted-names-council/naming-guidelines/naming-biologics.page.

The antibody society is an international association hosting this site http://www.antibodysociety.org. It provides antibody related research and development information. It also gives updates about the conferences

related to Antibody Engineering and Therapeutics in Europe.

http://www.antibodysociety.org is the site where one can purchase antibodies, ELISA kits, and proteins for research work. http://www.ablynx.com is the official website of Ablynx, which is a commercial supplier of nanobodies for therapeutic uses.

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Glossary

Adjuvant Adjuvant is a substance that enhances the antigenicity of an antigen. Freund's adjuvants are the most commonly used adjuvant in laboratory.

Antigenicity Ability of antigen to combine/bind with the product of HM1 and/or CM1.

Antiserum Antiserum is the fluid component of clotted blood from an immune individual that contains antibodies against the agent used for immunization.

Camelid antibody Camelid antibodies are so called as they are produced by members of the family camelidae, commonly known as camelids. The camelid antibodies have only heavy chain and they do not have light chain. Thus these antibodies have a single *N* terminal domain which is functionally capable of antigen binding.

Chimeric This is derived from chimera, which means a substance that is created from the proteins or genes of two species, for example, chimeric antibodies contains the amino acid sequence of a different species in another species antibody, for example, antibody with human constant region and mouse variable regions.

Humanized antibodies These antibodies have CDRs from a species other than human beings although rest of the antibody production against specific antigen.

Immunization Process of artificially producing a state of immunity in an animal an antigen (commonly known as vaccination), but for laboratory purposes, immunization also means to induce antibody production against specific antigen.

Immunogenicity The capacity of host to induce an immune response is the characteristic of the host rather than the characteristic of an antigen. This is the reason that same antigen can cause immune response in one host, while not in the other one, for example, one cannot raise antibody against BSA in bovine but can raise antibodies against BSA in rabbit.

Monoclonal antibody Antibody produced by a single clone of B-cells having same antigenic specificity, for example, Humira.

Polyclonal antibody Polyclonal antibodies are antibodies that are secreted by different B-cell lineages and hence have different specificities.

Selection medium Media that favors the growth of a particular cell type, for example, HAT selection medium is used for the selection of hybrids B-lymphocytes and myeloma cells.

Abbreviations

Ab	Antibody/ies
8-AG	8-Azo guanine
CMI	Cell-mediated immunity
CRBC	Chicken red blood cells
DTT	Dithiothreitol
ELISA	Enzyme-linked immuno sorbent assay
Fab	Fragment antigen binding
$F(ab')_2$	Fragment antigen binding (divalent)
FACS	Fluorescent activated cell sorting/sorter
Fc	Fragment crystallizable
FCA	Freund's complete adjuvant
FDA	Food and Drug Administration
FIA	Freund's incomplete adjuvant
HAT	Hypoxanthine, aminopterin, and thymidine medium
HGPRT	Hypoxanthine guanine phospho ribosyl transferase
HMI	Humoral-mediated Immunity
HRPO	Horseradish peroxidase
Ig	Immunoglobulin/s
IgG	Immunoglobulin/s G
INN	International nonproprietary names
MACS	Magnetic-activated cell sorting/sorter
2-ME	2-Mercapto ethanol
MoAb	Monoclonal antibody/ies
ODD	Ouchterlony double diffusion
PoAb	Polyclonal antibody/ies
RBC	Red blood cells
SRBC	Sheep red blood cells
USAN	United States Adopted Names
USANC	United States Adopted Names Council
VHH	Single variable domain on a heavy chain antibody/ies
VH	Heavy chain variable domain

Long answer questions

1. Discuss the advantages and disadvantages of monoclonal antibodies.
2. Discuss the experiment of Porter and Edelman and how they deduced that antibodies have two heavy chains and two light chains.
3. Describe the nomenclature of monoclonal antibodies.
4. What are some applications of monoclonal antibodies as therapeutics?
5. Discuss serum sickness, HAMA, HACA, and HAHA. How are these symptoms or diseases are correlated with antibody structure?
6. What is the principle behind the selection of hybridoma cells for monoclonal antibody production?
7. How are polyclonal antibodies raised?

8. Discuss the translational significance of polyclonal and monoclonal antibodies.

Short answer questions

1. What is a monoclonal antibody?
2. What is serum sickness?
3. What are the different components of selection medium used for the production of monoclonal antibodies?
4. Name the cells whose salvage pathway is blocked during monoclonal antibody production.
5. Name the different fractions produced after papain digestion. Do papain-digested fragments of antibodies bind with antigens? Can any of these fragments be used for immunoprecipitation?
6. What are the benefits of the hinge region in an antibody?
7. What fractions are produced after pepsin digestion? Write one line for each fraction produced due to pepsin digestion.

Answers to short answer questions

1. Monoclonal antibodies are those antibodies produced by a single clone of B-lymphocytes.
2. Serum sickness is a disease that happens due to the use of polyclonal antibodies as therapeutic agents. The adverse effect is more pronounced when the same antibody is given again. Antivenom is one of the best examples for serum sickness.
3. Selection medium used for the production of monoclonal antibodies is known as HAT medium. The letters in "HAT" represent its individual components: H, hypoxanthine; A, aminopterin; and T, thymidine.
4. The salvage pathway is blocked in myeloma cells for the selection of hybridomas during monoclonal antibody production.
5. Papain digestion of antibodies gives Fab and Fc fragments. Papain-digested Fab fragments will bind with antigens, but neither of the two fragments will cause immunoprecipitation.
6. The hinge region of an antibody exists between the C_H1 and C_H2 domains. This region provides the flexibility for the antibody to bind with an epitope. In some antibodies, the hinge region is not present and is replaced by an extra domain of constant region. Antibodies having hinge regions are more vulnerable to degradation/digestion.
7. Pepsin treatment causes digestion of antibodies, producing $F(ab')_2$ and degraded Fc fragments. F $(ab')_2$ is divalent, binds with antigens, and is capable of immunoprecipitation. Fc fragments are extensively degraded so the Fc portion loses its ability to bind with FcR; therefore, the Fc portion produced due to pepsin digestion cannot initiate the biological functions of antibodies.

Yes/no type question

1. Is the first antiserum that was developed was effective against diptheria?
2. Is the Nobel Prize for Medicine or Physiology was given to Tiselius and Kabart in 1972?
3. Are polyclonal antibodies product of numerous B-lymphocytes?
4. Are mostly monoclonal antibodies highly specific and show cross reactivity?
5. Are monoclonal antibodies heterogenous in nature?
6. Is the adverse response against monoclonal antibody known as HACA response?
7. Do humanized antibodies contain 60%–64% of human proteins?
8. Are the only survivors after hybridoma experiments will be B-cell fused with tumor cell?
9. Is each antibody is made up of two heavy chains and two light chains?
10. Is hypoxanthine-guanine phosphoribosyltransferase enzyme is an important enzyme of purine salvage pathway?
11. Is camelid antibody highly molecular and thermally stable than classical antibody?
12. Are nanobodies more efficient for viruses?

Answers to yes/no type questions

1. Yes—In 1890, Emil von Behring and Shi-basaburo Kitasato developed an effective antiserum against diptheria.
2. No—The credit for elucidation of immunoglobulin structure goes to Rodney Porter and Gerald Edelman.
3. Yes—Polyclonal antibodies are product of numerous B-lymphocytes
4. No—Mostly monoclonal antibodies are highly specific and usually do not show cross reactivity.
5. No—They are homogeneous in nature.
6. No—Human HAMA is the adverse response against monoclonal antibody.
7. No—Humanized antibodies contain 90%–94% of human proteins.

8. Yes—B-cell fused with tumor cell is the only survivor after hybridoma experiment.
9. No—Camelid antibody is made up of only two heavy chains.
10. Yes—HGPRT plays an important part in salvage pathway to synthesis of nucleotide by attaching purine base to a sugar.
11. Yes—Camelid antibody has amino acid substitution and hence conversion hydrophobic to hydrophilic provides molecular and thermal stability.
12. Yes— Protruding nature and the binding with cervices make them more efficient for viruses.

CHAPTER

18

Molecular markers: tool for genetic analysis

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Summary

The development of molecular techniques for genetic analysis has led to a great augmentation in our knowledge of animal genetics and our understanding of the structure and behavior of various animal genomes. These molecular techniques, in particular the applications of molecular markers, have been used to scrutinize DNA sequence variation(s) in and among the animal species and create new sources of genetic variation by introducing new and favorable traits from landraces and related animal species.

What can you expect to know

Improvements in marker detection systems and in the techniques used to identify markers linked to useful traits have enabled great advances to be made in recent years. While restriction fragment length polymorphism (RFLP) markers have been the basis for most works in laboratory animals, valuable markers have been generated from random amplified polymorphic DNA (RAPDs) and amplified fragment length polymorphisms (AFLPs). Simple sequence repeats (SSR) or microsatellite markers have been developed more recently for laboratory animals, and this marker system is predicted to lead to even more rapid advances in both marker development and implementation in breeding programs. Ethical issues related to laboratory animals in research and experimentation have been debated, defended, and protested by both individuals and organizations at various levels. Responses range from personal lifestyle decisions and fervent philosophical treatises to strident arguments, violent demonstrations, and direct action. The continuum of attitudes about animals and the human relationship with animals spans the range between those who support no regulation of the human use of animals and those who advocate absolute animal liberation from all human use.

Introduction

The various genotypic classes are indistinguishable at the phenotypic level because of the dominance effect of the marker and low genome coverage. The first work on the detection of genome variation in animal livestock was based on morphological, chromosomal, and biochemical markers. Most morphological markers are sex-limited, age-dependent, and are significantly influenced by the environment. Biochemical markers show a low degree of polymorphism (Duran et al., 2009). Recent advances in molecular biology provide novel tools for addressing evolutionary, ecological, and taxonomic research questions, and the application of methods based on population genetics and statistics allows the development of animals with a high productive efficiency. Important advances to some of the economically important characteristics in several species of livestock have been achieved based on phenotypic performance; however, several limitations of these methods of improvement (based on population genetics alone) have become evident with time. Their efficiency decreases when the characteristics are difficult to measure or have low heritability (Beuzen et al., 2000). Additionally, selection has been generally limited to those characteristics that can properly be measured in large numbers of animals.

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Some characteristics, such as the rate of survival, are expressed too late in life to serve as useful criteria for selection. Also, traditional selection within populations has not been very efficient when the selection objective involves several characteristics with unfavorable genetic correlation, for example, milk production and the protein content of milk.

Molecular techniques allow the detection of variations or polymorphisms that exist among individuals in the population for specific regions of DNA. These polymorphisms can be used to build up genetic maps and to evaluate differences between markers in the expression of particular traits in a family that might indicate a direct effect of these differences (in terms of genetic determination) on the trait. Everybody in the field of biological science has started using molecular marker technology for the purpose of the so-called gene discovery. This has resulted in the development of new types of molecular markers and various technological simplifications to resolve the problems associated with molecular marker technology (Teneva, 2009). The development of molecular biology over the past three decades has created new means for studying livestock genetics and animal breeding. Selection according to the genotype has become an important tool in the breeding of farm animals. Molecular markers capable of detecting genetic variation at the DNA sequence level have removed the above-mentioned limitations of morphological, chromosomal, and protein markers, and they possess unique genetic properties that make them more useful than other genetic markers. They are numerous and are distributed ubiquitously throughout the genome. DNA-based markers have many advantages over phenotypic markers in that they are highly heritable, relatively easy to assay, and are not affected by the environment (Montaldo and Meza-Herrera, 1998).

Information collected by the Food and Agriculture Organization (FAO) of the United Nations indicates that approximately 30% of the world's farm animal breeds are at risk of extinction. Conservation policies of native breeds will depend to a large extent on our knowledge of historic and genetic relationships between breeds, as well as of economic and cultural factors. In the Secondary Guidelines for Development of National Farm Animal Genetic Resources Management Plans, the FAO proposed an integrated program for global management of livestock genetic resources using reference microsatellites (Oldenbroek, 2007). In India, The National Bureau of Animal Genetic Resources (NBAGR), Karnal, is entrusted with the characterization of important indigenous livestock breeds. India has 27 breeds of cattle, eight of buffalo, 42 of sheep, 20 of goats, six of horses, and 17 of poultry. Genetic variation of the animal is the basic material, which is utilized for changing the genetic makeup or genetic potentiality of domestic species to suit our needs. Mechanization, unplanned and indiscriminate breeding among native stocks, and human bias in favor of certain breeds are directly or indirectly responsible for the dilution of Indian livestock germplasm. Hence, the characterization of indigenous germplasm is essential for their conservation. The increasing availability of molecular markers in laboratory animals allows the detailed analyses and evaluation of genetic diversity, and also the detection of genes influencing economically important traits (Erhardt and Weimann, 2007).

Molecular markers have threefold applications in gene mapping: (1) a marker allows the direct identification of the gene of interest instead of the gene product, and consequently, it serves as a useful tool for screening somatic cell hybrids; (2) the use of several DNA probes and easy-to-screen techniques, a marker also helps in physical mapping of the genes using in situ hybridization; and (3) molecular markers provide sufficient markers for the construction of genetic maps using the linkage analysis. Genetic maps are constructed on the basis of two classes of molecular markers. Type I markers that represent the evolutionary conserved coding sequences (e.g., classical RFLPs and simple sequence length polymorphisms (SSLPs)) are useful in comparative mapping strategies where polymorphism is not an essential prerequisite. However, these are mostly single locus and di-allelic (SLDA) and thus are not useful for the linkage analysis. On the other hand, type II markers (like microsatellites markers) have higher polymorphism information content than conventional RFLPs and can be generated very easily and rapidly. Therefore, major efforts are being made to produce gene maps based on the type II markers. Further utilization of molecular markers developed from DNA sequence information, namely allele-specific oligonucleotide (ASO) and sequence-tagged microsatellites (STMS) polymorphic markers, is also helpful in the rapid progress of gene mapping (Table 18.1).

From the late 1980s, the number of publications generated has been at an unexpectedly high volume, projecting that genetic analysis in animals would be a "cake walk." Everybody in the field of biological science started using molecular marker technology for the purpose of the so-called gene discovery. The hype of this technology was so prominent and started to divide scientists working in "animal biology" into various groups. This kind of situation is not new in science. At any point in time, the typical scientific discipline tends to be seized by a particular methodology or enthusiasm and other approaches get dumped. This has resulted in the development of new types of

TABLE 18.1 Various molecular markers used for genetic analysis of animals.

Year	Acronym	Nomenclature
1974	RFLP	Restriction fragment length polymorphism
1986	ASO	Allele-specific oligonucleotides
1988	AS-PCR	Allele-specific polymerase chain reaction
1989	SSCP	Single-stranded conformational polymorphism
1989	STS	Sequence-tagged site
1990	RAPD	Randomly amplified polymorphic DNA
1991	RLGS	Restriction landmark genome scanning
1992	SSR	Simple sequence repeats
1994	ISSR	Inter simple sequence repeats
1994	SNP	Single-nucleotide polymorphisms
1995	AFLP	Amplified fragment length polymorphism
1999	MSAP	Methylation-sensitive amplification polymorphism
2000	MITE	Miniature inverted-repeat transposable element
2002	SSLP	Simple sequence length polymorphisms

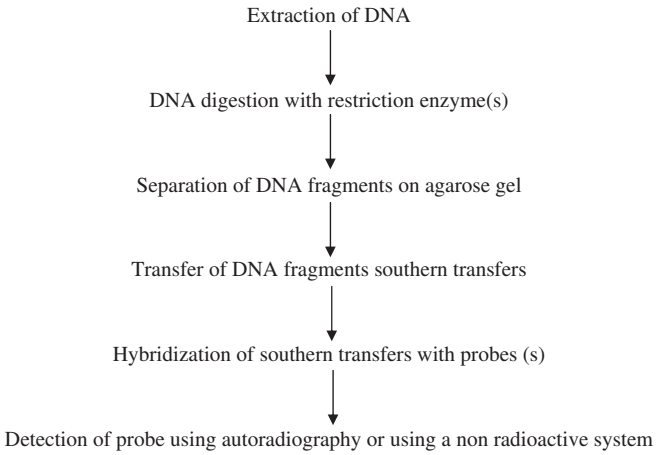
molecular markers and various technological simplifications to resolve the problems associated with the molecular marker technology. The recent PCR-based approach, gel-free visualization of PCR products, and automation at various steps are boons to the molecular marker approaches adopted for genome mapping and genetic diversity analysis in the animal kingdom.

Methodology

Molecular markers are also the prerequisite for the identification of positional and functional candidate genes responsible for quantitative traits. In this presentation, the detailed use of molecular markers for the evaluation of genetic diversity and identification of economically important traits in animal production is explained using different examples.

Restriction fragment length polymorphism

Restriction fragment length polymorphism (RFLP) analysis is a method used to indirectly collect information on mutations occurring at specific short regions (restriction sites) (Botstein et al., 1980). RFLP enabled the detection of polymorphisms at the DNA sequence level. RFLP are first-class genetic markers, allowing the construction of highly saturated linkage maps. RFLP is a technique in which organisms may be differentiated by the analysis of patterns derived from the cleavage of their DNA (Flow Chart 18.1). If two organisms differ in the distance between sites of cleavage of a particular restriction endonuclease, the length of the fragments produced will differ when the DNA is digested with a restriction enzyme. The similarity of the patterns generated can be used to differentiate species (and even strains) from one another.



FLOW CHART 18.1 Steps involved in the RFLP Analysis.

Steps involved in restriction fragment length polymorphism analysis

1. The first step in DNA typing is extraction of the DNA from the sample, be it blood, saliva, semen, or some other biological sample.
2. The purified DNA is then cut into fragments using restriction enzymes. For example, take the pattern GCGC and imagine that it occurs more than once in the DNA. The number of times it occurs is unique to the individual. The restriction enzyme chops the DNA into two at every place where the GCGC pattern occurs.
3. The restriction fragments have negative charge and can be separated by a technique called gel electrophoresis, which separates the pieces of DNA based on their size. The samples of DNA that have been treated with restriction enzymes are placed in separate lanes on a slab of electrophoretic gel across which is placed an electric field. The fragments migrate toward the positive electrode, the smaller fragments moving faster than the larger fragments, thus separating the DNA samples into distinct bands.
4. Put the nylon membrane onto the porous support, then the plastic mask (the mask must be slightly smaller than the gel), being very careful to slide the gel onto the mask. Close the apparatus and start the pump (you should be able to see if a vacuum is formed; if not, look for the problem).

5. If the membrane to be used is being employed for the first time, then an overnight pre-hybridization is needed; otherwise, 4–5 hours should be enough. Incubate in the rotating hybridization oven at 65°C. After prehybridization, start hybridization by adding the boiled, labeled probe to the pre-hybridization.
6. Incubate overnight. The bands can be visualized using luminescent dyes.

Applications of restriction fragment length polymorphism

The analysis of RFLP variation in genomes was a vital tool in genome mapping and genetic disease analysis. If a researcher were to try to initially determine the chromosomal location of a particular disease gene, he/she would analyze the DNA of members of a family afflicted by the disease and look for RFLP alleles that show a similar pattern of inheritance as that of the disease. Once a disease gene was localized, the RFLP analysis of other families would reveal who was at risk of the disease, or who was likely to be a carrier of the mutant genes. RFLP analysis was also the basis for early methods of genetic fingerprinting, useful in the identification of samples retrieved from crime scenes, determination of paternity, and characterization of genetic diversity or breeding patterns in animal populations. RFLP assay was carried out for the rapid identification of three target species (*Siganus canaliculatus*, *S. corallines*, and *S. javus*) using mitochondrial gene regions to facilitate studies on species-specific spatio-temporal patterns of larval dispersal and population connectivity to aid fishery management (Ravago-Gotanco et al., 2010).

The CD4 molecule is a cell-surface glycoprotein receptor expressed by helper T cells, monocytes, macrophages, and dendritic cells. Its structure consists of four immunoglobulin-like domains (D 1–D 4). In pigs, it was identified that amino-acid substitutions of domain 1 in CD4.B gave rise to the failure of some CD4 expressing cells to react with particular anti-pig CD4 monoclonal antibodies. In addition, a PCR-RFLP method was developed that enabled simply identification of the CD4 sequence variant to anti-pig CD4 monoclonal antibodies without the need to use the flow cytometric analysis. Similarly, this tool provides a simple and cost-effective approach to determine the identity and distribution of Onchocerca species, and will be valuable for future genetic studies that focus on parasites infecting blackflies. The Malayan box turtle (MBT, *Cuora amboinensis*) is a vulnerable and protected turtle species, but it is a lucrative item in the illegal wildlife trade because of its great appeal as an exotic food item and in traditional medicine. RFLP assay was validated for the screening of raw and processed commercial meatballs, burgers, and frankfurters, which are very popular in most countries. The optimized RFLP assay was further used to screen MBT materials in 153 traditional Chinese medicines of 17 different brands and 62 of them were found MBT positive, wherein the ingredients were not declared in product labels (Asing et al., 2016).

Allele-specific oligonucleotide

An ASO is typically an oligonucleotide of 15–21 nucleotide bases in length. It is designed (and used) in a way that makes it specific for only one version, or allele, of the DNA being tested. An ASO is a short piece of synthetic DNA complementary to the sequence of a variable target DNA. It acts as a probe for the presence of the target in a Southern blot assay or, more commonly, in the simpler dot blot assay.

Applications of allele-specific oligonucleotide

It is a common tool used in genetic testing, forensics, and molecular biology research, and is important in genotype analysis and the Human Genome Project. With an ASO test, it was shown that the mutation co-segregated with the recessively inherited yellow coat color in the Labrador retriever. Golden retrievers also appeared to be homozygous for the mutation (Everts et al., 2000).

Allele-specific polymerase chain reaction

Polymerase chain reaction primers are chosen from an invariant part of the genome and might be used to amplify a polymorphic area between them. In allele-specific PCR, the opposite is done. At least one of the primers is chosen from a polymorphic area, with the mutations located at (or near) its 3′-end. Under stringent conditions, a mismatched primer will not initiate replication, whereas a matched primer will. The appearance of an amplification product therefore indicates the genotype (Wangkumhang et al., 2007).

Applications of allele-specific polymerase chain reaction

It is a convenient and inexpensive method for genotyping single nucleotide polymorphisms (SNPs) and mutations. It is applied in many recent studies, including population genetics, molecular genetics, and pharmacogenomics. Two genetic variants of the bovine β-casein gene (A1 and B) encode a histidine residue at codon 67, resulting in potential liberation of a bioactive peptide (β-casomorphin) upon digestion. An allele-specific PCR (AS-PCR) was evaluated to distinguish between the β-casomorphin-releasing variants (A1 and

B) and the non-releasing variants. AS-PCR successfully distinguished β-casein variants in 41 of 42 animals as confirmed by sequence analysis (Keating et al., 2008).

Single-strand conformation polymorphism

Single-strand conformation polymorphism (SSCP) method is one of the simplest and perhaps one of the most sensitive PCR-based methods for detecting multiple mutations and polymorphisms in genes and family analysis ("fingerprinting") (Huby-Chilton et al., 2001).

Applications of single-strand conformation polymorphism

It is used as a way to discover new DNA polymorphisms apart from DNA sequencing, but is now being supplanted by sequencing techniques because of efficiency and accuracy. These days, SSCP is most applicable as a diagnostic tool in molecular biology. It can be used in genotyping to detect homozygous individuals of different allelic states, as well as heterozygous individuals who should demonstrate distinct patterns in an electrophoresis experiment. SSCP is also widely used in virology to detect variations in different strains of a virus, the idea being that a particular virus particle present in both strains will have undergone changes due to mutation and that these changes will cause the two particles to assume different conformations, and thus be differentiable on an SSCP gel (Kubo et al., 2009). The genetic diversity of Jamunapari goats (*Capra hircus*) was investigated using an optimized non-radioactive polymerase chain reaction single-strand conformation polymorphism (PCR-SSCP) method to detect α-lactalbumin polymorphism, indicating that Jamunapari goats have high genetic variability at loci exon I of the α-lactalbumin gene (Kumar et al., 2006).

The adrenergic receptor β3 (ADRβ3) is the major regulator of lipolysis and homeostasis and predominantly expressed in brown and white adipose tissues. The ADRβ3 gene which encodes for this receptor was studied as a candidate gene associated with growth traits, body dimensional measurements, and conformation indices of Barki sheep. SSCP was used to identify the variation in the intron region of ADRβ3 gene. Genetic diversity of Sri Lankan goats (*Capra hircus*) was evaluated using PCR-SSCP method to detect polymorphism in four candidate genes. The studies on genetic diversity allow not only the genetic characterization of desired breeds but also the documentation of correlations between genotypic variations and related phenotypic traits. Growth hormone (GH) gene has been described as a candidate gene for marker-assisted selection in different farm animals. SSCP was used to identify the polymorphism in GH gene and its association with variation of wool traits in Egyptian sheep breeds. This detected mutation and was found to have some effects on wool traits. SSCP was used to analyze the polymorphism of growth hormone receptor (GHR) gene in French, Iranian, and Danish strains of *Oncorhynchus mykiss*. A monomorphic SSCP pattern of AA genotype in the French and Iranian strains and a dimorphic AA and AB genotype in the Danish strain were observed in 3′ non-coding regions of GHR gene (Gorji et al., 2016).

Sequence-tagged site

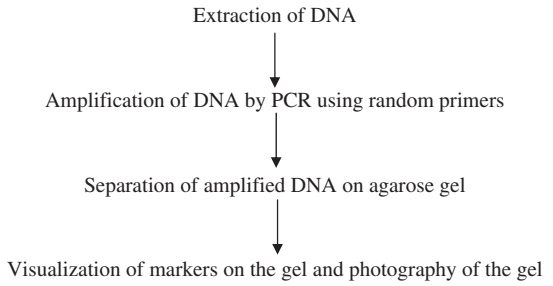
A sequence-tagged site (STS) is a short region along the genome (200–300 bases long) whose exact sequence is found nowhereelse in the genome. The uniqueness of the sequence is established by demonstrating that it can be uniquely amplified by the PCR. The DNA sequence of an STS may contain repetitive elements, sequences that appear elsewhere in the genome, but as long as the sequences at both ends of the site are unique, unique DNA primers complementary to those ends can be synthesized, the region amplified using PCR, and the specificity of the reaction demonstrated by gel electrophoresis of the amplified product.

Applications of sequence-tagged site

Sequence-tagged sites are very helpful for detecting microdeletions in some genes. For example, some STSs can be used in screening by PCR to detect microdeletions in Azoospermia (AZF) genes in infertile men. Identification of genes in elephants could provide additional information for evolutionary studies and for evaluating genetic diversity in existing elephant populations. Sequence tagged sites (STSs) were identified in the Asian and the African elephant for the following genes: melatonin receptor 1a (MTNR1A), retinoic acid receptor beta (RARB), and leptin receptor (LEPR) (Burk et al., 1989). Studies suggest that prolactin receptor (Prlr) is a potential causative gene for chicken early-feathering (EF) and late-feathering (LF) phenotypes. Using the published primers for STS junction (P1 and P2), the fusion gene was amplified with chicken genomic DNA. The research on the function and regulation of the candidate genes will help elucidate the molecular basis of the chicken-feathering trait.

Random amplified polymorphic DNA

Random amplified polymorphic DNA (RAPD) analysis is a PCR-based molecular marker technique that



FLOW CHART 18.2 Steps involved in the RAPD Analysis.

shows that the difference in the pattern of bands amplified from genetically distinct individuals behave as Mendelian genetic markers. It employs a single primer (a 10-mer) for a random amplification under specific PCR conditions. The number of amplified fragments depends on the distribution and number of annealing sites throughout the genome. In fact, the amplification takes place only when primers anneal on each strand at sites not more distant than 3–4 kb. PCR random products are then detected easily on an agarose gel, and the resulting banding pattern represents the DNA fingerprint. In comparison with other genetic markers, RAPD provides a more arbitrary sample of the genome and can detect an unlimited number of loci, simply by changing the base combination in the oligomer used. The most limiting property of RAPD markers is probably the dominant expression of alleles, making difficult the interpretation of multi-locus patterns. Even problems of amplification reproducibility were raised in the past. RAPDs have been widely employed for studies on taxon identification, hybridization, reproductive behavior, and population genetic structure. This technique has been employed for targeting genes of economic value (Flow Chart 18.2). RAPD markers are amplification products of anonymous DNA sequences using single, short, and arbitrary oligonucleotide primers and thus do not require prior knowledge of a DNA sequence (Bardakci, 2011).

Steps involved in random amplified polymorphic DNA analysis

1. The first step is the extraction of the DNA from the sample, be it blood, saliva, semen, or some other biological sample.
2. The yield of DNA per gram of tissue isolated is measured using a UV spectrophotometer at 260 nm. DNA purity is determined by estimating the ratio of absorbance at 260 nm to absorbance at 280 nm.
3. Oligonucleotide primers are used for amplification to standardize the PCR conditions. The reactions are carried out in a DNA thermocycler (Bio Rad).
4. The restriction fragments have negative charge and can be separated by a technique called gel electrophoresis, which separates the pieces of DNA based on their size. The samples of DNA that have been treated with restriction enzymes are placed in separate lanes on a slab of electrophoretic gel across which is placed an electric field. The fragments migrate toward the positive electrode, the smaller fragments moving faster than the larger fragments, thus separating the DNA samples into distinct bands.
5. Gels with amplification fragments are visualized and photographed under UV light. A medium-range DNA ruler is used as a molecular marker for the size of the fragments.

Applications of random amplified polymeric DNA analysis

Inbreeding indicates the degree of homozygosity at a locus within a population. Normally, inbreeding is estimated in terms of a coefficient calculated from the pedigree of an individual. If no history is available, however, there is no way to estimate the inbreeding coefficient. Sometimes, data on individuals are missing, and that too can prevent the estimation of the inbreeding coefficient, which is essential for the formulation of a breeding program at the farm level and for the breed development. The RAPD analysis was carried out on 20 randomly selected animals of three Indian cattle breeds (namely, Red Sindhi, Hariana, and Tharparkar) maintained at three farms: Central Cattle Breeding Farm, Chiplima, Orissa, India; Shree gaushala, Jind, Hariana, India; Central Cattle Breeding Farm, Lakhimpur-Kheri, Uttar Pradesh, India (Bhattacharya et al., 2003).

Serra da Estrela Protected Designation of Origin (PDO) cheese is the most famous Portuguese cheese and has a high commercial value. However, the adulteration of production with cheaper/lower-quality milks from non-autochthones ovine breeds compromises the quality of the final product and undervalues the original PDO cheese. A RAPD method was developed for the efficient detection of adulterant breeds in milk mixtures used for the fraudulent production of this cheese. Fish is a relatively inexpensive source of protein and an important source of foreign exchange in many regions of the world. Genetic study of bacterial isolates found in the guts, gills, and skin samples of catfish was carried out using RAPD markers to identify potentially harmful bacteria. Similarly, the patterns of morphometric and genetic variation were carried out using RAPD-PCR techniques for the first time on two species of Cyprinus and single species of *Oncorhynchus mykiss* from Srinagar, Kashmir, India (Ganaie and Ali, 2016).

Restriction landmark genome scanning

Restriction landmark genomic scanning using methylation sensitive endonucleases (RLGS-M) is a powerful method for the systematic detection of the DNA methylation. This approach is based on the assumption that CpG methylation, particularly of CpG islands, might be associated with the gene transcriptional regulation.

Applications of landmark genome scanning

It is a method that was used to study a mouse brain that was scanned for genomic DNA from various developmental stages to detect the transcriptionally active regions (Kawai et al., 1993).

Single nucleotide polymorphisms

Single nucleotide polymorphism means a polymorphism corresponding to a difference at a single nucleotide position (substitution, deletion, or insertion). It includes the detection of a single nucleotide change by a direct sequence protocol. SNPs occur in both coding and noncoding regions of the genome. SNP detection technologies are used to scan for new polymorphisms and to determine the allele(s) of a known polymorphism in target sequences. SNP detection technologies have evolved from labor-intensive, time-consuming, and expensive processes to some of the most highly automated, efficient, and relatively inexpensive methods (Kwok and Chen, 2003).

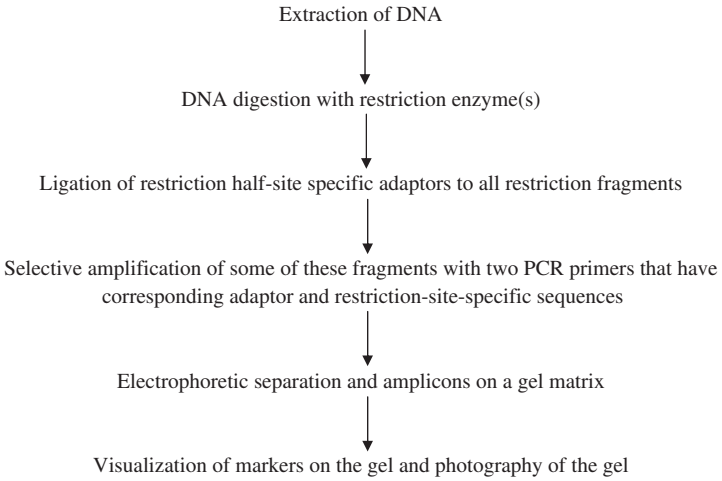
Applications of single-nucleotide polymorphisms

The Atlantic cod (*Gadus morhua*) is a groundfish of great economic value in fisheries and an emerging species in aquaculture. Genetic markers are needed to identify wild stocks in order to ensure sustainable management, and for marker-assisted selection and pedigree determination in aquaculture. The SNPs were tested on Atlantic cod from four different sites, comprising both North-East Arctic cod (NEAC) and Norwegian coastal cod (NCC). The average heterozygosity of the SNPs was 0.25 and the average minor allele frequency was 0.18. The SNP markers presented here are powerful tools for future genetic work related to management and aquaculture. In particular, some SNPs exhibiting high levels of population divergence have the potential to significantly enhance studies on the population structure of Atlantic cod (Moen et al., 2008).

GeneSeek (Neogen Corp., Lexington, KY) designed a new version of the GeneSeek Genomic Profiler HD BeadChip for Dairy Cattle, which originally had >77,000 SNPs. Australian fishes Murray Cod (*Maccullochella peelii*) and Trout Cod (*Maccullochella macquariensis*) are widespread over the Murray Darling Basin. Genetic single nucleotide polymorphism (SNP) data were combined together with mitochondrial sequences to examine hybridization and introgression between Murray Cod and Trout Cod in the upper Murrumbidgee River. Fertility in cows is a complex trait that is regulated in part by genetics. The incorporation of genomic information through genome-wide single nucleotide polymorphism (SNP) arrays has improved the reliability of genetic estimates. Genotyping was performed for Holstein cows. A total of 39 SNPs associated with the three fertility traits were discovered. The hypothalamic decapeptide gonadotropin-releasing hormone (GnRH) plays a critical role in the reproductive development. *GnRHR* gene was analyzed as a SNP genetic marker candidate in 40 Iraqi goats, out of which SNP was detected in 27. Multiple tools have been developed to improve the accuracy of animal selection and the rate of improvement in economically important traits in beef cattle. Brahman cattle exhibit lower carcass quality characteristics. SNPs were detected in six candidate genes (Royer et al., 2016).

Amplified fragment length polymorphism

An amplified fragment length polymorphism is a highly sensitive method for detecting polymorphisms (Flow Chart 18.3). A different procedure combines enzymatic digestion with PCR. AFLPs are the result of digestion with restriction endonucleases, a ligation of specifically designed oligonucleotide adaptors to the ends of each fragment, and PCR amplification using primers complementary to the adaptor sequence to which an extended sequence of a few bases is added in order to reduce the number of amplified fragments.



FLOW CHART 18.3 Steps involved in the AFLP Analysis.

Banding patterns obtained in this way reveal useful information for a series of applications like population genetics and systematic and kinship analysis. It discriminates heterozygote from homozygote when a gel scanner is used.

Steps involved in amplified fragment length polymorphism analysis

1. To prepare an AFLP template, genomic DNA is isolated and digested with two restriction endonucleases simultaneously. This step generates the required substrate for ligation and subsequent amplification. The success of the AFLP technique is dependent upon complete restriction digestion; therefore, much care should be taken to isolate high-quality genomic DNA intact without contaminating nucleases or inhibitors.
2. Following heat inactivation of the restriction endonucleases, the genomic DNA fragments are ligated to *EcoR I* and *Mse I* adapters to generate template DNA for amplification. These common adapter sequences flanking variable genomic DNA sequences serve as primer binding sites on these restriction fragments. Using this strategy, it is possible to amplify many DNA fragments without having prior sequence knowledge.
3. PCR is performed in two consecutive reactions. In the first reaction, called pre-amplification, genomic DNAs are amplified with AFLP primers, each having one selective nucleotide. The PCR products of the preamplification reaction are diluted and used as a template for the selective amplification using two AFLP primers, each containing three selective nucleotides.
4. Products from the selective amplification are separated on a 5% or 6% denaturing polyacrylamide (sequencing) gel. The resultant banding pattern ("fingerprint") can be analyzed for polymorphisms either manually or using analytical software.

Applications of amplified fragment length polymorphism

Amplified fragment length polymorphism is a multiplex PCR-based method in which a subset of restriction fragments are selectively amplified using oligonucleotide primers complementary to sequences that have been ligated to each end. AFLP analysis allows the reliable identification of over 50 loci in a single reaction. This technique combines the reliability of the RFLP and ease of the PCR; thus, AFLP is a new typing method for amplifying DNA of any origin or complexity. AFLP was tested to assess the frequency of extra-pair parentage in a bluethroat (*Luscinia svecica namnetum*) population. Thirty-six families totaling 162 nestlings were analyzed. Using a combination of three primer pairs, an exclusion probability of 93% for the population was reached. This probability can reach 99% when considering families independently. It was revealed that extra-pair fertilizations are very common: 63.8% of all broods contain at least one extra-pair young, totaling 41.9% of all young analyzed. However, with the technique and the three primer pairs used, it was not possible to attribute the parentage exclusions to extra-pair paternity, maternity, or both. As brood parasitism has never been reported in this species, it seems likely that the exclusions are due to extra-pair males. This study shows that dominant AFLP markers can be useful for studying the mating system of taxa for which no microsatellite primers are available. This technique allows the approximate estimation of parentage exclusions despite the fact that it is not possible to know which parent has to be excluded (Questiau et al., 1999). Psittacidae are frequentely bred as pets worldwide, but little is known about the zoonotic risks of these animals. Shiga toxin-producing *Escherichia coli* (STEC) in the feces of psittacine birds was detected using AFLP, indicating the zoonotic risk of breeding psittacidae in home environments.

Methylation-sensitive amplification polymorphism

Methylation-sensitive amplification polymorphism is a modification of the amplified fragment length polymorphism (AFLP) method that makes use of the differential sensitivity of a pair of iso-schizomers to cytosine methylation.

Applications of methylation-sensitive amplification polymorphism

The methylation levels of genomes were compared in swine, cattle, sheep, rat, chicken, and duck, using the methylation-sensitive amplification polymorphism technique (MSAP). The results showed that the methylation levels in genomes of the species investigated were mostly 40−50% (except cattle). The methylation level varied in different species. The methylation pattern in various tissues of each species was specific; for the same species, the methylation level of the tissue genome was mostly higher than that of the blood genome. The difference in the methylation level between birds and mammals was not significant; however, mammals appeared to have a lower hemimethylation frequency and higher full methylation frequency than birds (Shao-Qing et al., 2007).

New studies are revealing a molecular link between environment, phenotype, and genotype in the form of heritable methylation of DNA. MSAP was used across multiple stages of evolutionary divergence in natural

populations of North American stream fishes. It was found that epigenetic differentiation between methylomes is greater than genetic divergence among closely related populations across river drainages. Similarly, natural polyploidy (diploid, triploid, and tetraploid) was studied in loach (*Misgurnus anguillicaudatus*) fishes. It was revealed that the total methylation and full methylation rates decreased on increased ploidy individuals. Moreover, MSAP analysis showed that gravid females of polychaete worm *Pygospio elegans* offspring differing in larval developmental mode have significantly different methylation profiles and that the females with benthic larvae and non-reproductive females from the same location also differ in their epigenetic profiles (Kesäniemi et al., 2016).

Miniature inverted-repeat transposable element

Miniature inverted-repeat transposable element assay involves transposon display (TD), which is a modification of the AFLP procedure where PCR products are derived from primers anchored in a restriction site and a transposable element rather than in two restriction sites. For this candidate, primers in transposable elements are designed based on a consensus sequence generated of transposable elements. Miniature inverted-repeat TEs (MITEs) are a special type of Class 2 non-autonomous element that is present in high copy numbers in many eukaryotic genomes (Yujun and Wessler, 2010).

Applications of miniature inverted-repeat transposable element

Amphioxus is consistently used as a model of genome evolution for understanding the invertebrate/vertebrate transition. The amphioxus genome has not undergone massive duplications like those in the vertebrates, or disruptive rearrangements like in the genome of Ciona (a urochordate), making it an ideal evolutionary model. Transposable elements have been linked to many genomic evolutionary changes, including increased genome size, modified gene expression, massive gene rearrangements, and possibly intron evolution. Five novel MITEs were identified using an analysis of an amphioxus DNA sequence: *LanceleTn-1, LanceleTn-2, LanceleTn-3a, LanceleTn-3b*, and *LanceleTn-4*. Several of the *LanceleTn* elements were identified in the amphioxus ParaHox cluster, and it was suggested that these had important implications for the evolution of this highly conserved gene cluster. The estimated high copy numbers of these elements implies that MITEs are probably the most abundant type of mobile element in amphioxus and are thus likely to have been of fundamental importance in shaping the evolution of the amphioxus genome (Osborne et al., 2006). Fourteen novel MITE families were found in the Florida carpenter ant genome, *Camponotus floridanus*, which constitute approximately 0.63 % of the entire genome. The analysis of their insertion time showed that most members of these MITEs were inserted into their host genome in less than eight million years ago.

Microsatellites

DNA microsatellite sequences are valuable genetic markers due to their dense distribution in the genome, high variability, co-dominant inheritance, and relative ease of detection. As hyper-variability is highly significant for detecting differences in a population and between individuals, microsatellite typing can reveal the degrees of polymorphism that is easy to interpret. Another complementary approach to genetic analysis by microsatellites involves the use of gene markers (functional markers) for domestic species as an indication of the extent of variation (especially at loci of economic importance) and may provide information related to the functional differences between the breeds and may have relevance in selective breeding programs to establish new breeding stocks for the purpose of maintenance of useful genetic diversity.

This candidate gene approach involves a gene with a known expression of certain proteins and appears to provide genomic information that can be used for the genetic improvement of livestock. Microsatellites are also known as simple sequence repeats (SSRs), short tandem repeats (STRs), inter simple sequence repeats (ISSR), simple sequence tandem repeats (SSTR), variable number tandem repeats (VNTR), SSLP, and STMS.

Simple sequence repeats/Short tandem repeats/ Simple sequence tandem repeats

Simple sequence repeats or short tandem repeats, also known as microsatellites, are repeating sequences of 2–6 base pairs of DNA. STRs are typically codominant. They are used as molecular markers in genetics, and for kinship, population, and other studies. They can also be used to study gene duplication or deletion. STRs are also known to be causative agents in human disease, especially neurodegenerative disorders and cancer.

Applications of simple sequence repeats

To reduce the analysis cost and sample consumption and to meet the demands of higher sample throughputs, PCR amplification and detection of multiple markers (multiplex STR analysis) has become a standard technique in most forensic DNA laboratories.

STR multiplexing is most commonly performed using spectrally distinguishable fluorescent tags and/or non-overlapping PCR product sizes. Multiplex STR amplification in one or two PCR reactions with fluorescently labeled primers and measurement with gel or capillary electrophoresis separation and laser-induced fluorescence detection is becoming a standard method in forensic laboratories for analysis of the 13 CODIS STR loci.

Simple sequence repeat was used in five domestic animal species (namely, buffalo, cattle, goat, sheep, and yak) and has been used as a model to investigate the relative abundance of the type of repeat motif, their distribution in coding and non-coding regions, evaluation of mitochondrial SSR data for appropriateness of the phylogenetic tree, dynamism of length and repeat motif, and extent of conservation of flanking regions across loci with respect to time (Shakyawar et al., 2009). SSRs and guanine-cytosine (GC) distribution in intragenic and intergenic regions of ten primates was studied, suggesting that they have potential roles in transcriptional or translational regulation.

Inter simple sequence repeats

Inter simple sequence repeat (ISSR)-PCR is a technique that involves the use of microsatellite sequences as primers in a PDR to generate multilocus markers. It is a simple and quick method that combines most of the advantages of microsatellites (SSRs) and amplified fragment length polymorphism (AFLP) with the universality of RAPD. ISSR markers are highly polymorphic and are useful in studies on genetic diversity, phylogeny, gene tagging, genome mapping, and evolutionary biology (Reddy et al., 2002). Sequences amplified by ISSR-PCR can be used for DNA fingerprinting. Since an ISSR may be a conserved or non-conserved region, this technique is not useful for distinguishing individuals, but rather for phylogeographical analyses or maybe delimiting species; sequence diversity is lower than in SSRPCR, but still higher than that in actual gene sequences. In addition, microsatellite sequencing and ISSR sequencing are mutually assisting, as one produces primers for the other (Reddy et al., 2002).

Applications of inter simple sequence repeats

For the first time in spiders, the inter simple sequence repeat (ISSR) technique was used to study the genetic variability in Mexican populations of *Brachypelma vagans*. A non-lethal technique was used to collect samples from six populations in the Yucatan peninsula, and seven ISSR primers were tested. Four of these primers gave fragments (bands) that were sufficiently clear and reproducible to construct a binary matrix and to determine genetic variability parameters. It revealed a very high percentage of polymorphism (P 5 98.7%), the highest yet reported for tarantula spiders. The results show that the ISSR-PCR method is promising for intraspecific variation in tarantula spiders (Machkour-M'Rabet et al., 2009).

Inter-simple sequence repeats markers were used to determinate the genetic variability of *Fasciola hepatica* populations recovered from sheep and cattle from Spain. ISSR technique was used to assess genetic variation and phylogenetic relationships between *Lymnea natalensis* collected from different regions of Egypt. Moreover, eleven ISSR primers were identified while investigating the genetic structure of domestic duck populations from Turkey. Even the gene pools of beef cattle breeds bred in Russia were characterized on the basis of ISSR (Sulimova et al., 2016).

Variable number of tandem repeat markers

Variable number of tandem repeat markers are located in a genome where a short nucleotide is organized as a tandem repeat. These can be found on many chromosomes, and they often show variations in length. Each variant acts as an inherited allele that allows its use for identification. VNTRs are multiallelic loci that consist of repeated core sequences (> 6 nucleotides) known as mini-satellites, are tandem repeats, and are flanked by segments of non-repetitive sequences. This allows the VNTR blocks to be extracted with restriction enzymes and analyzed by restriction fragment length polymorphism, to be amplified by polymerase chain reaction (PCR) and to have their size determined by gel electrophoresis. The versatility and efficiency of these markers in genotyping and diversity studies were tested in the laboratory; their versatility was a great advantage. They are easy to score and can be run on agarose gels, which makes them an interesting tool, especially for use in less sophisticated laboratories.

Applications of variable number of tandem repeat markers

Variable number of tandem repeat markers are an important source of RFLP genetic markers used in linkage analysis (mapping) of genomes. They have become essential in forensic crime investigations. The technique may use PCR, size determined by gel electrophoresis, and Southern blotting to produce a pattern of bands unique to each individual. Therefore, VNTRs are being used to study genetic diversity (DNA fingerprinting) and breeding patterns in animals. VNTRs also have clinical applications like VNTR typing, the next gold standard in genotyping for early diagnosis of *M. tuberculosis* super-infection or mixed infection. *Mycobacterium avium paratuberculosis* (MAP) is a member of the *M. avium* complex (MAC)

and the causative agent of Johne's disease, one of the most economically important diseases in ruminants. VNTR is a frequently employed typing method of MAP isolates. *Anaplasma phagocytophilum* causes abortions in domestic ruminants, resulting in significant economic impact. The VNTR allele acts as a marker of *A. phagocytophilum* involved in abortions.

Sequence tagged microsatellite site

A sequence-tagged microsatellite site is a another form of microsatellite marker, which if cloned and sequenced, can be subjected to PCR amplification; such microsatellite loci can be recovered by PCR. Microsatellite markers in the STMS format can be completely described as information in databases and can serve as common reference points that will allow the incorporation of any type of physical mapping data into the evolving map. The advantage is that band profiles can be interpreted in terms of loci and alleles, and allele sizes can be determined with high accuracy.

Applications of sequence-tagged microsatellite site

One of the main applications of STMS is characterizing and understanding animal genetic variation. The use of STMS markers in genetic distancing of breeds is gaining momentum. The ever-increasing knowledge of mammalian genetic structure and the development of convenient ways of measuring that structure have opened up a range of new possibilities in the areas of animal and product identification.

Simple sequence length polymorphisms

Simple sequence length polymorphisms are used as genetic markers with PCR. An SSLP is a type of polymorphism: a difference in DNA sequence between individuals. SSLPs are repeated sequences over varying base lengths in intergenic regions of DNA. Variance in the length of SSLPs can be used to understand genetic variance between two individuals in a certain species (Rosenberg et al., 2002).

Applications of sequence length polymorphisms

Genetic monitoring is an essential component of colony management, and for the rat has been accomplished primarily by using immunological and biochemical markers. SSLPs are a faster and more economical way of monitoring inbred strains of rats. Sixty-one inbred strains of rats were characterized using primer pairs for 37 SSLPs. Each of these loci appeared to be highly polymorphic, with the number of alleles per locus ranging from 3 to 14; as a result, all 61 inbred strains tested in this study could be provided with a unique strain profile. These strain profiles were also used for estimating the degree of similarity between strains. This information may provide the rationale for selecting strains for genetic crosses or for other purposes (Otsen et al., 1995). Misidentification or cross-contamination of cell lines is a serious problem, leading to erroneous research. Mouse cell lines registered with the JCRB cell bank were examined by SSLP to identify their strains; 12 of the 80 cell lines (15%) were found to differ from registered information.

Example of microsatellites

A microsatellite is a stretch of DNA with mono-, di-, tri-, or tetranucleotide units repeated. Microsatellites are short sequences of nucleotides (typically 1–5 bp) that are tandemly repeated.

1. Repeat units
   AAAAAAAAAAA = (A) 11 = mononucleotide (11 bp)
   GTGTGTGTGTGT = (GT) 6 = dinucleotide (12 bp)
   CTGCTGCTGCTG = (CTG) 4 = trinucleotide (12 bp)
   ACTCACTCACTCACTC = (ACTC) 4 = tetranucleotide (16 bp)
2. Homozygous microsatellite
   …CGTAGCCTTGCATCCTT**CTCTCTCTCTCTCT**ATC GGTACTACGTGG… (46 bp)
   …CGTAGCCTTGCATCCTT**CTCTCTCTCTCTCT** ATCGGTACTACGTGG… (46 bp)
   5′ flanking region microsatellite locus 3′ flanking region
3. Heterozygous microsatellite
   …. CGTAGCCTTGCATCCTT**CTCTCTCTCTCT CT**ATCGGTACTACGTGG…(46 bp)
   CGTAGCCTTGCATCCTT**CTCTCTCTCTCTCTC TCT**ATCGGTACTACGTGG…(50 bp)
   5′ flanking region microsatellite locus 3′ flanking region

The most common way to detect microsatellites is to design PCR primers that are unique to one locus in the genome and the base pair on either side of the repeated portion (Fig. 18.1). Therefore, a single pair of PCR primers will work for every individual in the species and produce different sized products for each of the different length microsatellites.

The PCR products are then separated by either gel or capillary electrophoresis. Either way, the investigator can determine the size of the PCR product and thus how many times the dinucleotide "CA" was repeated for each allele (Fig. 18.2). It would be nice if microsatellite data produced only two bands, but often there are minor bands in addition to the major bands; these are called stutter bands and they usually differ from the major bands by two nucleotides.

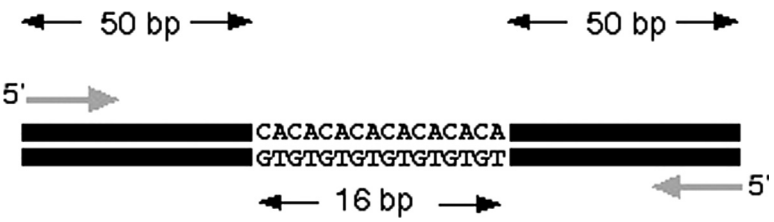


FIGURE 18.1 Detection of microsatellites from genomic DNA: Two PCR primers (forward and reverse gray arrows) are designed to flank the microsatellite region. If there were zero repeats, the PCR product would be 100 bp in length. Therefore, by determining the size of each PCR product (in this case, 116 bp), one can calculate how many CA repeats are present in each microsatellite (there are eight CA repeats in this example).

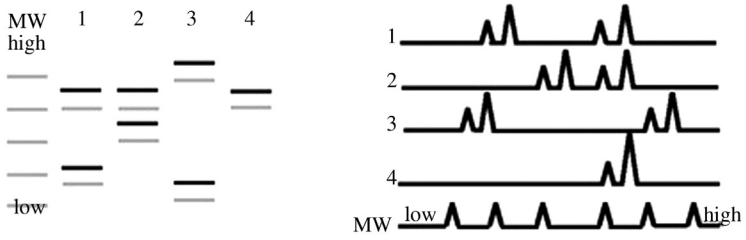


FIGURE 18.2 Stylized examples of microsatellite data: Left Half: Four sets of data were produced by gel electrophoresis, including major (black) and stutter (gray) bands (MW: molecular weight standards). Right Half: These data were produced by the analysis on an automated capillary electrophoresis-based DNA sequencer. The data are line graphs with the location of each peak on the X-axis representing a different sized PCR product; the height of each peak indicates the amount of PCR product. Major bands produce higher peaks than the stutter peaks.

Advantage of microsatellite marker

They require low quantities of template DNA (10–100 ng). They are found in large numbers and are relatively evenly spaced throughout the genome or are randomly distributed throughout the genome. They have a high level of polymorphism and follow a typical Mendelian inheritance, which usually expresses in a co-dominant fashion, and are often multi-allelic, giving mean heterozygosity of more than 70%. Microsatellites are unaffected by environmental factors and generally do not have pleiotropic effects on quantitative trait loci (QTL).

Disadvantages of microsatellite marker

They have high development costs, and heterozygotes may be misclassified as homozygotes when null-alleles occur due to mutations in the primer annealing sites. The underlying mutation model (infinite allele model or stepwise mutation model) is largely unknown. Sometimes, stutter bands may complicate accurate scoring of polymorphisms. Homoplasy due to different forward and backward mutations can underestimate genetic divergence. They are time-consuming and expensive to develop.

Some general applications of microsatellites

Microsatellites have several applications like parentage determination, genetic distance estimation of twin zygosity and freemartinism, sexing of pre-implantation embryos, and identification of disease carriers. Applications are briefly discussed in the following section.

Parentage determination

Since the breeding value of an animal is generally estimated using the information available from its relatives, the knowledge of correct parentage is therefore a prerequisite. Parentage testing using molecular markers yields much higher exclusion probability (> 90%) than testing with blood groups (70–90%) or other biochemical markers (40–60%). Highly polymorphic DNA fingerprinting markers are quite useful for this purpose. Recently, DNA fingerprinting with oligoprobes (OAT18 and ONS1) has been successfully used for determining the parentage of an in vitro fertilization buffalo calf. With the advent of PCR-based microsatellite assays, a large number of microsatellite panels have been reported that are useful for parentage testing in different livestock species.

Determination of twin zygosity and freemartinism

Correct knowledge of zygosity twins, particularly in monotocus animals, is important. Monozygotic twins provide a means for epidemiological and genetic studies, and also help in transplant matching. Individual-specific DNA fingerprinting techniques have the potential for the determination of twin zygosity and demonstration of spontaneous XX/XY chimerism.

Identification of disease carrier

Many of the most serious incurable diseases result not from infections with bacteria or viruses, but from defects in genomes of the host. Certain allelic variations in the host genome lead to susceptibility or resistance to a particular disease. DNA polymorphisms

occurring within a gene help to understand the molecular mechanism and genetic control of several genetic and metabolic disorders, and allow the identification of heterozygous carrier animals which are otherwise phenotypically indistinguishable from normal individuals.

Ethical issues

The use of genetic biomarkers in epidemiological studies raises specific social and ethical issues related to the selection of molecular markers and methods of analysis, obtaining participants, the storage of biological samples and their linkage with individual data, the disclosure of information, and the publication of results. Several of these issues are similar to those associated with the use of any type of biomarker in epidemiology. Other problems are specifically related to the use of genetic material and the perception that genetic information raises special concerns regarding privacy, risk of abuse, and psychosocial impact (Hainaut and Vähäkangas, 1999). Cloning raises concerns from both ethical and practical points of view. Whether it is acceptable to clone humans is a difficult issue to deal with. Besides the low success rates seen in animals, the chance of abnormalities suggests that more information is required concerning the consequences of such practices before they would become routine in humans and animals. Advantages of animal breeding programs derived from cloning with no use of transgenesis are likely to be small (Van Vleck, 1999).

Most countries involved in biomedical research now have in place regulations governing the use of experimental or other donor animals as providers of tissues. These apply to higher animals assumed to have sufficient brain capacity and organization to feel pain and distress, and generally do not apply to lower vertebrates such as fish or invertebrates. Usually a more highly evolved animal is assumed to be sentient after halfway through the embryonic development; restrictions apply to the method by which the animal is killed or operated upon (if it is to remain alive) such that the animal suffers minimal pain or discomfort. Restrictions also apply to the way the animal is housed and maintained, whether in an animal house during husbandry, under experimental conditions, or in a veterinary hospital under clinical conditions. In each case, control is usually exercised locally by an animal ethics committee and nationally by a governmental or professionally appointed body.

The current status of opinion and debate regarding ethical issues can be divided into three broad categories of relevance to animal biotechnology. The first is scientific integrity, where the focus has been on scientific fraud and the integrity of the research process. The second concerns possible harms or risks to parties affected either directly by research (including animals themselves) or through the eventual commercialization or development of products from animal biotechnology. The final category concerns a responsibility to serve as a guardian of the public interest with respect to application and development of technologies derived from new genetic sciences. It is plausible to see the scientific community as a whole having such a fiduciary obligation to the broader public with regard to the technical complexity of the issues and owing to public funding and institutional support for scientific research. The overall conclusion is that in the latter two categories especially, there is an urgent need for new participation in the deliberative consideration of ethical issues by working scientists (Hojjati et al., 2011).

The European Group on Ethics (EGE) is an independent, pluralist, and multidisciplinary body that advises the European Commission on ethical aspects of science and new technologies in connection with the preparation and implementation of community legislation or policies. There are two ways in which farm animals in society are viewed: (1) There is the 18th-century view of animals as living creatures with functional value (Duran et al., 2009); (2) they are seen in terms of their purpose, which is determined by society. Farm animals can also be viewed as sentient beings with inherent, intrinsic value (European Group of Ethics in Science and New Technologies (EGE), http://ec.europa.eu/bepa/expert-groups/ege/index_en.htm; Beuzen et al. (2000)). The functional view of farm animal cloning includes commercial production of large numbers of high-value, elite animals, or using cloning as a method to disseminate genetically engineered animals. Using cloning for greater productivity will inevitably perpetuate many of the serious welfare problems already widespread in high-tech farming (e.g., lameness in broiler chickens, pigs, and dairy cows). Cloning and genetic engineering of farm animals is taking us in the wrong direction, toward perpetuating factory farming when all other societal trends point toward sustainable farming and respect for animals as sentient beings. The very aims of cloning are therefore an ethical issue. The practice of cloning raises ethical and welfare concerns, detailed as follows.

Invasive Medical Interventions are performed on donor animals (for oocyte extraction) and on surrogate mothers. Oocyte extraction for pigs and sheep is usually surgical, with all the accompanying stresses of recovery.

Suffering Caused to Surrogate Mothers. Pregnancy is typically prolonged, and cloned calves and lambs may be 25% heavier than normal. Higher birth weights lead to painful births and often the need for cesarean section.

Abnormal Fetal Development and Late Pregnancy Mortality lead to frequent deaths at various stages of development. Death in the second-half of gestation is common.

Postnatal Mortality. The viability of cloned offspring at delivery and up to weaning is reduced compared with that at normal births. Surviving newborn clones have altered neonatal metabolism and physiology, and a high proportion of them die before weaning. Typical complications include gastroenteritis, umbilical infections, defects in the cardiovascular, musculoskeletal, and neurological systems, and susceptibility to lung infections and digestive disorders. These animals have short lives of suffering.

Health Problems During Life. Clones may have a greater propensity in later life for respiratory problems and immune system deficiencies compared with normal animals. Many clones have died or have had to be put down at a young age. Underlying weaknesses in cloned animals may not be fully revealed until the animals are stressed in some manner. A study undertaken at the US Department of Agriculture (published in October 2005) suggested that clones may be born with crippled immune systems.

Inefficiency and Wastage of Life. This includes embryos, fetuses, and mature animals that are killed as part of the procedures. A recent paper from New Zealand refers to the process as still inefficient and highly prone to epigenetic errors.

The Legal Situation in the EU. There are two important pieces of European Union law that should be considered with reference to cloned farm animals. The Protocol on Improved Protection and Respect for the Welfare of Animals in the Amsterdam Treaty of 1997 recognizes that animals are "sentient beings" and calls on the EU to "pay full respect to the welfare requirements of animals." The EU directive on the protection of animals kept for farming purposes (Directive 98/58/EC) says "Natural or artificial breeding or breeding procedures which cause, or are likely to cause, suffering or injury to any of the animals concerned must not be practiced." The directive also says "No animal shall be kept for farming purposes unless it can reasonably be expected, on the basis of its genotype or phenotype, that it can be kept without detrimental effect on its health or welfare." In other words, if the animal (in this case the cloned animal) is likely to suffer because of its inbuilt genetic or physiological weaknesses, then it should not be on a farm. In view of the widespread suffering caused to cloned animals themselves, to the females from whose eggs they are extracted, and to the surrogate females who have the cloned embryos inserted and who usually face cesareans, Compassion in World Farming calls on the EGE to recommend a ban on the cloning of animals for food.

Translational significance

A wide range of molecular marker technologies are now available for genetic studies that give us information about the kinships between breeds and the domestication process. On the other hand, molecular markers already provide new opportunities to speed up the selection of routinely measured traits, to select for new traits that are costly and/or difficult to record in farm animals and to improve animal production and productivity. Among these, the favorite markers for these studies are mainly microsatellites; RAPD, AFLP, ISSR, and SSR marker systems are also emerging as lead technologies. The RAPD marker system is not considered as convenient because of its inconsistency. However, RAPD assays are still used for DNA fingerprinting, along with other dominant markers such as AFLP and ISSR markers. SSR markers remain the markers of choice for genome mapping and genetic diversity analysis.

Several variations of the above-mentioned marker systems are also available. These new generations of markers (namely, MITE) are in the early phase of usage and are not routinely employed in molecular marker technology laboratories. Despite an array of molecular markers available to researchers, it is still important to choose the right marker for the right problem. There is clearly potential to enhance the rates of genetic improvement by using molecular information. The full realization of this potential will require an investment in infrastructure and knowledge, and will therefore be limited in the first step mainly due to nationally and internationally important species and breeds within species. Moreover, the technology must be carefully targeted to provide optimal returns to breeding organizations and farmers.

Clinical correlations

LncRNAs are linked to many diseases including diabetics, cardiovascular diseases, central nervous system disease, and cancer. The difficulty in knocking down nuclear-retained lncRNA may be overcome by ASOs, another nucleic acid-based technology which enables specific targeting of any gene. Thalassemia is a genetic disease in which there is a relative or complete lack of α- or β-globin chains. Allele-specific oligonucleotide polymerase chain reaction (ASO-PCR) and haemagglutination methods were used to determine the presence of Rhesus (Rh) C, c, E, and e antigens. The advantage of this new ASO-PCR method compared with a RFLP-PCR method is that with the former all four genes can be amplified at the same time by PCR (Hojjati et al., 2011). APOE gene encodes the apolipoprotein E, which

is involved in the transportation of cholesterol and lipids in blood circulation and central nervous system. Genetic polymorphisms of apolipoprotein E (APOE) are associated with various health conditions and diseases, such as Alzheimer's disease, cardiovascular diseases, and type 2 diabetes. Allele-specific PCR method was developed and applied to a genetic association analysis of APOE and schizophrenia. This convenient method shall meet the different needs of various research and clinical.

A number of males are having infertility problem and spermatogenic failure. Non-obstructive azoospermia (NOA) is a rigorous condition of spermatogenic failure (SSF) influencing infertile men. Y chromosomal microdeletions (YCMs), sex chromosome abnormalities, translocations, cryptorchidism, testicular torsion, radiation, and toxins are predicted to responsible for NOA genetic disorders in males. Many Y chromosome microdeletions have been identified which results from non-allelic homologous recombination (NAHR). In clinical practice, the European Academy of Anthrology (EAA) and the European Molecular Genetics Quality Network (EMQN) have published a guideline that adopts the use of six sequence-tagged sites (STSs) to detect complete deletions. Twenty to 30 STSs have been suggested to be sufficient for providing good coverage of the important regions of the Y chromosome. The emerging technique of next-generation sequencing (NGS) provides a unique opportunity to depict the whole portrait of Y chromosome deletions. Numerous STSs within the palindrome rather than the entire sequences provide unique landmarks that can be used to track deletions. This set of STSs in combination with the NGS technique is perfectly suited for the identification of deletions across the Y chromosome. Y-chromosomal microdeletion (YCM) serves as an important genetic factor in non-obstructive azoospermia (NOA). The NOA patients tended to carry more and rarer deletions that were enriched in nearby intragenic regions. Multiplex polymerase chain reaction (PCR) is routinely used to detect YCMs by tracing sequence-tagged sites (STSs) in the Y chromosome.

Turning point

For genome editing, CRISPR/Cas9 technology has come into sight as an influential and simple tool. This is very helpful as many model organisms more genetically tractable. It has been found that CRISPR/Cas9 gene targeting technology can successfully interrupt with genes in zygotes or ES cells, in the view of generating animal models and even manipulating them genetically. CRISPR/Cas9 gene targeting technology has many advantages such as, it is rapidly increasing, necessitating cost-effective, rapid, and sensitive genotyping strategies. With respect to the conventional method of gene targeting in many experimental organisms involves the incorporation of foreign DNA and/or allele-specific sequence necessitating for genotyping strategies. Such as conventional polymerase chain reaction (PCR) followed by agarose gel electrophoresis lacks the sensitivity to detect products that differ by only a few nucleotides, based on binary readout of PCR product amplification and size selection. Molecular markers such as RFLP and other techniques are relying on generating or elimination of a definite restriction site within the targeted locus. These assays are expensive, time-consuming, lack sequence specificity, and require costly capital equipment. Moreover, several of these techniques fail to distinguish biallelic combinations for all modes of inheritance: wild type, heterozygous, compound heterozygous and homozygous mutations. In contrast, alleles created by non-homologous end-joining (NHEJ) repair of double-stranded DNA breaks generated by Cas9 are much less amenable to such strategies. This novel genotyping strategy is cost-effective, sequence−specific, and allows for accurate and efficient multiplexing of small insertion−deletions and single-nucleotide variants characteristic of CRISPR/Cas9 edited alleles.

World wide web resources

The issue of animal cloning has received a great deal of attention in public discourse. Bioethicists, policy-makers, and the media have been quick to identify the key ethical issues involved and to argue (almost unanimously) on such attempts. Meanwhile, scientists have proceeded with extensive research agendas for the cloning of animals. Despite this research, there has been little public discussion of the ethical issues raised by animal cloning projects. Polling data show that the public is decidedly against the cloning of animals. To understand the public's reaction and fill the void of reasoned debate about the issue, it is necessary to review the possible objections to animal cloning and assess the merits of the anti-animal cloning stance. Some objections to animal cloning (e.g., the impact of cloning on the population of unwanted animals) can be easily addressed, while others (e.g., the health of cloned animals) require more serious attention by policy makers and the public. For more than 40 years, Congress has entrusted the Animal and Plant Health Inspection Service (APHIS) with the stewardship of animals covered under the Animal Welfare and Horse Protection Acts. APHIS continues to uphold that trust, giving protection to millions of animals each

year, nationwide. APHIS provides leadership for determining the standards of humane care and treatment of animals. APHIS implements those standards and achieves compliance through inspection, education, cooperative efforts, and enforcement.

Similarly, The Bioethics Research Library at Georgetown University is an interdisciplinary and multi-format collection on ethical issues related to health care, biomedical research, biotechnology, and the environment. There are a number of organizations worldwide that are actively working for the humane treatment of animals. The Universities Federation for Animal Welfare (UFAW) is an independent registered charity that works to develop and promote improvements in the welfare of all animals through scientific and educational activity worldwide. The Great Ape Project (GAP) is an international movement that aims to defend the rights of non-human great primates (e.g., chimpanzees, gorillas, orangutans, and bonobos), our closest relatives in the animal kingdom. The mission of the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) is to "enhance the quality of research, teaching, and testing by promoting humane, responsible animal care and use." The organization awards accreditation to institutions that "meet or exceed AAALAC standards" regarding animal care.

Moreover, the Canadian Council on Animal Care (CCAC) is an autonomous and independent body created in 1968 to oversee the ethical use of animals in science in Canada. The CCAC is registered as a nonprofit organization and is financed primarily by the Canadian Institutes of Health Research (CIHR) and the Natural Sciences and Engineering Research Council of Canada (NSERC), with additional contributions from federal science-based departments and agencies, and private institutions participating in its programs. It is governed by a council of representatives from 22 national organizations that are permanent member organizations and up to three limited-term member organizations.

The CCAC acts as a quasi-regulatory body and sets standards (its guidelines documents and policy statements) on animal care and use in science that apply across Canada. It is accountable to the general public and is responsible for the dissemination of information on the use of animals in science to Canadians. In addition to guidelines, documents, and policy statements, the CCAC develops comprehensive annual statistics on the number of animals used in science and produces an annual report to disseminate information to its constituents and the general public.

The various web resources related to animal welfare and ethical issues are given below:

http://repository.upenn.edu
http://www.aphis.usda.gov
http://bioethics.georgetown.edu.
http://www.ufaw.org.uk
http://www.greatapeproject.org/newsletters/
http://www.ccac.ca
http://www.awionline.Org
http://www.vetmed.ucdavis.edu/whatsnew/article.cfm?id = 2528 http://www.labanimal.com/laban/index.html
http://ec.europa.eu/bepa/european-group-ethics/publications/proceedings-ege-roundtables

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Glossary

Biochemical Markers Genes that encode proteins that can be extracted and observed (e.g., isozymes and storage proteins).

Freemartinism The normal outcome of mixed-sex twins in all cattle species that have been studied; it also occasionally occurs in other mammals, including sheep, goats, and pigs. A freemartin or freemartin (sometimes martin heifer) is an infertile female mammal that has masculinized behavior and non-functioning ovaries. Genetically, the animal is chimeric (karyotyping of a sample of cells shows XX/XY chromosomes). Externally, the animal appears female, but various aspects of female reproductive development are altered due to the acquisition of anti-Müllerian hormones from the male twin.

Homoplasy This occurs when characters are similar, but are not derived from a common ancestor. Homoplasy often results from convergent evolution.

Inbreeding Reproduction from the mating of parents that are close genetic relatives. It results in increased homozygosity, which can increase the chances of offspring being affected by recessive or deleterious traits.

Isoschizomers Pairs of restriction enzymes specific to the same recognition sequence. For example, *Sph I* (CGTAC/G) and *Bbu I* (CGTAC/G) are isoschizomers of each other. The first enzyme discovered that recognizes a given sequence is known as the prototype; all subsequently identified enzymes that recognize that sequence are isoschizomers.

Molecular markers They allow the detection of variations or polymorphisms that exist among individuals in the population for specific regions of DNA (e.g., RFLP, AFLP, and SNP).

Morphological markers The first marker loci available had an obvious impact on the morphology of plants. Genes that affect form, coloration, male sterility, or resistance (among others) have been analyzed in many plant species. Examples of this type of marker include the presence or absence of awn, leaf sheath coloration, height, grain color, aroma (rice), etc. In well-characterized crops like maize, tomato, pea, barley, or wheat, tens or even hundreds of such genes have been assigned to different chromosomes.

Pharmacogenomics The branch of pharmacology that deals with the influence of genetic variation on drug response in patients by correlating gene expression or single-nucleotide polymorphisms with a drug's efficacy or toxicity. Pharmacogenomics aims to develop rational means to optimize drug therapy with respect to patients' genotypes to ensure maximum efficacy with minimal adverse effects.

Pleiotropy It occurs when one gene influences multiple phenotypic traits. Consequently, a mutation in a pleiotropic gene can have an effect on some or all traits simultaneously. This can become a problem when selection of one trait favors one specific version of the gene (allele), while selection of the other trait favors another allele.

Polymorphism It occurs when two or more clearly different phenotypes exist in the same population of a species (i.e., more than one form or morph). The term is also used somewhat differently by molecular biologists to describe certain point mutations in the genotype, such as SNPs.

Taxon A population or group of populations of organisms that are usually inferred to be phylogenetically related and which have characteristics in common that differentiate the unit (e.g. geographic population, genus, family, and order) from other such units. A taxon encompasses all included taxa of lower rank and individual organisms.

Traits A distinct variant of an organism's phenotypic character that may be inherited, environmentally determined, or a combination of the two. For example, eye color is a character or abstraction of an attribute, while blue, brown, and hazel are traits.

Transposon display A strategy that allows simultaneous detection of individual elements. For example, sequences flanking dTph1 elements are amplified by means of a ligation-mediated PCR. The resulting fragments are locus-specific and can be analyzed by polyacrylamide gel electrophoresis.

Abbreviations

AAALAC	Association for Assessment and Accreditation of Laboratory Animal Care International
AFLP	Amplified Fragment Length Polymorphism
APHIS	Animal and Plant Health Inspection Service
ASO	Allele-Specific Oligonucleotide
AS-PCR	Allele-Specific PCR
AZF	Azoospermia
CCAC	Canadian Council on Animal Care
CIHR	Canadian Institutes of Health Research
EGE	European Group on Ethics
EU	European Union
FAO	Food and Agriculture Organization
ISSR	Inter Simple Sequence Repeats
LEPR	Leptin Receptor
MITE	Miniature Inverted-repeat Transposable Element
MSAP	Methylation Sensitive Amplification Polymorphism
MTNR1A	Melatonin Receptor 1A
NBAGR	National Bureau of Animal Genetic Resources
NCC	Norwegian Coastal Cod
NEAC	North-East Arctic Cod
NSERC	Natural Sciences and Engineering Research Council of Canada
QTL	Quantitative Trait Loci
RAPD	Random Amplified Polymorphic DNA
RARB	Retinoic Acid Receptor Beta
RFLP	Restriction Fragment Length Polymorphism
RLGS	Restriction Landmark Genome Scanning
SLDA	Single Locus and Di-Allelic
SNP	Single Nucleotide Polymorphism
SSCP	Single-Strand Conformation Polymorphism
SSLP	Simple Sequence Length Polymorphisms
SSR	Simple Sequence Repeats
SSTR	Simple Sequence Tandem Repeats
STMS	Sequence Tagged Microsatellites
STR	Short Tandem Repeats
STS	Sequence Tagged Site
TD	Transposon Display
UFAW	Universities Federation for Animal Welfare
VNTR	Variable Number Tandem Repeats

Long-answer questions

1. What is a molecular marker? Explain the different types of molecular markers.
2. What ethical issues are raised when molecular markers are used for the study of animals, and how can they be resolved?
3. What are microsatellite markers and why are they more useful than any other marker for diversity analysis?
4. What are the general applications of molecular markers?
5. How can animal research be studied using various world wide web resources?

Short-answer questions

1. What are the ideal features of molecular markers?
2. Describe the brief history of the evolution of molecular markers.
3. What is an ethical issue?
4. Briefly explain the advantages and disadvantages of molecular markers.
5. How are molecular markers important in animal breeding?

Answers to short questions

1. Molecular markers allow the detection of variations or polymorphisms that exist among individuals in the population for specific regions of DNA (e.g., RFLP, AFLP, and SNP). Molecular markers have threefold applications in gene mapping: (1) A marker allows the direct identification of the gene of interest instead of the gene product, and consequently, it serves as a useful tool for screening somatic cell hybrids; (2) use in several DNA probes and easy-to-screen techniques, a marker also helps in the physical mapping of the genes using in situ hybridization. (3) Molecular markers provide sufficient markers for the construction of genetic maps using linkage analysis.
2. Genetic maps are constructed on the basis of two classes of molecular markers. Type I markers that represent the evolutionary conserved coding sequences (e.g., classical RFLPs and SSLPs) are useful in comparative mapping strategies where a polymorphism is not an essential prerequisite. However, these are mostly single locus and di-allelic (SLDA), and thus are not useful for linkage analysis. On the other hand, type II markers (like microsatellite markers) have higher polymorphism information content than conventional RFLPs and can be generated easily and rapidly. Therefore, major efforts are being made to produce gene maps based on type II markers. Further utilization of molecular markers developed from DNA sequence information (namely, ASO and STMS polymorphic markers) is also helpful in the rapid progress of gene mapping (Table 18.1).
3. Ethics, also known as moral philosophy, is a branch of philosophy that involves systematizing, defending, and recommending concepts of right and wrong behavior (e.g., ethical issues related to animal research). The use of genetic biomarkers in epidemiological studies raises specific social and ethical issues related to the selection of molecular markers and methods of analysis, obtaining participants, the storage of biological samples and their linkage with individual data, the disclosure of information, and the publication of results. Several of these issues are similar to those associated with the use of any type of biomarker in epidemiology. Other problems are specifically related to the use of genetic material and the perception that genetic information raises special concerns regarding privacy, risk of abuse, and psychosocial impact. Cloning raises concerns both from ethical and practical points of view.
4. The development of molecular biology during the past three decades has created new means for studying livestock genetics and animal breeding. Selection according to genotype has become an important tool in the breeding of farm animals. Molecular markers capable of detecting genetic variations at the DNA sequence level have removed the limitations of morphological, chromosome, and protein markers, and they also possess unique genetic properties that make them more useful than other genetic markers.

   They are numerous and distributed ubiquitously throughout the genome. DNA-based markers have many advantages over phenotypic markers in that they are highly heritable, relatively easy to assay, and are not affected by the environment. The disadvantage is that they have high development costs and heterozygotes may be misclassified as homozygotes when null-alleles occur (due to mutation in the primer annealing sites).
5. Information collected by the Food and Agriculture Organization (FAO) of the United Nations indicates that approximately 30% of the world's farm animal breeds are at risk of extinction. Conservation policies for native breeds will depend to a large extent on our knowledge of historic and genetic relationships among breeds, as well as on economic and cultural factors. Genetic variation of an animal is the basic material, which is utilized for changing the genetic makeup or genetic potential of domestic species to suit our needs.

Mechanization, unplanned and indiscriminate breeding among native stocks, and human bias in favor of certain breeds are directly or indirectly responsible for the dilution of Indian livestock germplasm. Hence, characterization of indigenous germplasm is essential for their conservation. The increasing availability of molecular markers in laboratory animals allows the detailed analyses and evaluation of genetic diversity, and also the detection of genes influencing economically important traits.

Yes/no-type questions

1. CRISPR/Cas9 is a gene targeting technology?
2. APHIS stands for Animal and Plant Health Inspection Service?
3. Non-obstructive azoospermia (NOA) is only a natural disorder?
4. There are no ethical issues related to genetic biomarkers?

5. Microsatellite markers requires very large quantities of DNA?
6. MITE assay involves transposon display (TD)?
7. SNP itself is sufficient to examine hybridization between Murray Cod and Trout Cod?
8. SNP corresponds to a difference at a single nucleotide position?
9. Can polymorphisms build up genetic maps?
10. SSCP is able to identify multiple mutations and polymorphisms in genes?

Answers to yes/No-type questions

1. Yes—It has been found that CRISPR/Cas9 can successfully interrupt with genes in zygotes or ES cells.
2. Yes—Congress has entrusted the Animal and Plant Health Inspection Service (APHIS) with the stewardship of animals covered under the Animal Welfare and Horse Protection Acts.
3. No—Testicular torsion, radiation, and toxins are also responsible for NOA genetic disorders in males.
4. No—The use of genetic biomarkers in epidemiological studies raises specific social and ethical issues related to the selection of molecular markers and the publication of results.
5. No—Microsatellite marker requires low quantities of template DNA (10−100 ng). They are found in large numbers and are relatively evenly spaced throughout the genome.
6. Yes—MITE assay involves TD, which is a modification of the AFLP procedure where PCR products are derived from primers anchored in transposable element.
7. No—Genetic single-nucleotide polymorphism (SNP) data were combined together with mitochondrial sequences to examine hybridization and introgression between Murray Cod and Trout Cod in the upper Murrumbidgee River.
8. Yes—Single-nucleotide polymorphism means a polymorphism corresponding to a difference at a single nucleotide position (substitution, deletion, or insertion).
9. Yes—Polymorphisms can be used to build up genetic maps and to evaluate differences between markers in the expression of particular traits in a family.
10. Yes—SSCP is one of the simplest and perhaps one of the most sensitive PCR-based methods for detecting multiple mutations and polymorphisms in genes and family analysis.

CHAPTER

19

Ribotyping: a tool for molecular taxonomy

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Summary

Ribotyping is the identification and classification of bacteria based on polymorphisms in taxon-specific "signature labels" of universal and highly conserved ribosomal RNA (rRNA) molecules or their genes. It began in 1980s and evolved into various forms. It is considered a relatively stable and dependable system for molecular taxonomy and is widely accepted by international agencies.

What you can expect to know

Microbial taxonomy is a dynamic and continuously evolving system, probably because of the fact that microorganisms multiply asexually and with very short generation time. They also have far greater adaptability and capability to undergo variation in response to their environment as compared with biological systems of higher order (i.e., eukaryotes). Before the advent of molecular techniques, microbial systematics was largely based on studying their phenotypic traits, such as morphological, biochemical, antigenic properties, and bacteriophage susceptibility. Some of the methods based on these properties of microorganisms are still used by many laboratories for their preliminary identification but need to be supplemented with molecular techniques for deriving definitive taxonomic conclusions. Although various molecular biological techniques are being practiced for this purpose, ribotyping (i.e., identification and classification based on rRNA or their genes) evolved in the 1980s and is still considered to be a relatively stable and dependable system that is widely accepted by international agencies. Ribosomal RNAs are the core molecule of ubiquitous ribosomes. Their genes have been under the least evolutionary selection pressure, providing highly conserved regions and some less conserved sequences, which provide taxon-specific "signature labels," the basis of taxonomy. Conventional ribotyping was based on the determination of homology in 16S rRNA sequences by analyzing the restriction polymorphism and hybridization on membranous support, which is now being supplemented with more convenient PCR ribotyping (i.e., determination of polymorphism in 16S and 23S rRNA intergenic space) and sequencing of whole ribosomal operons. Fluorescent in situ hybridization (FISH) for the detection of rRNA of nonviral organisms in their natural habitat has also become fairly popular owing to its simplicity, cost-effectiveness, and lack of need for culturing. The advent of the next-generation sequencing (NGS) techniques has made possible to rapidly analyze microbial genomes, transcriptomes, and interactomes in a high-throughput manner, creating newer possibilities in microbial systematics.

History and methods

Introduction

Taxonomy is a field of science that involves description, identification, and nomenclature of an organism, which can be used in its classification. The purpose of taxonomy is to give proper signatures to an organism so that relatives of a particular taxon can be identified in a systematic manner. The information thus gathered can be used for systematics (i.e., studying their evolutionary and adaptability aspects). One of the approaches involved in systematics is the cladistic approach, where clades are formed, each consisting of an ancestor organism with all its descendants. The

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basic assumption behind the cladistic approach is that members of a group share a common evolutionary history and are thus more "closely related" to each other than to other groups of organisms. Another approach is evolutionary or synthetic systematics, which emphasizes evolutionary relationships. This approach suggests that the degree of genetic differences between lineages should also be used in addition to their evolutionary similarities when developing taxonomic classifications. The third approach is phenetics, or numerical taxonomy, where importance is given to multiple traits instead of relying on only a few traits. Similarities or differences between the characteristics of organisms are calculated, and clusters are formed. The clusters so formed may not necessarily reflect evolutionary relatedness. In other words, systematics is either studying the relationships between the organisms or characterizing and discriminating between different levels of taxa down to the species level and even beyond.

Microbiology and its systematics took a long time to evolve due to its limitations, as it could not have an organismal approach where phenotypic traits played an important role. From the initial approaches of studying biochemistry or physiology for systematics and taxonomy, it advanced with time to incorporate most of the modern techniques for its taxonomy. It is strongly felt that microbiological systematics is useful not only in studying microbial properties and phylogeny, but also in diagnostics where an unknown organism can be identified up to the species and subspecies levels, and also to infer some practical information to explore its medical, industrial, or environmental use. The methods and markers used for the characterization of a biological unit need to have universality (i.e., applicability to all members), reliability, and feasibility, and should also be able to reveal substitutions taking place in it over the time. Of the various approaches used for this purpose, studying rRNA and its genes (rDNA) have gained wide popularity. Ribosomal RNA is universally present, large, has a high degree of sequence conservation, has an important role in the translation of genomic messages, and has stable rDNA toward horizontal gene displacement. For these reasons, this operon is an important semantophoretic molecule for systematics and developing phylogenies. In addition to taxonomic applications, this technique has opened up many new fields, such as metagenomic studies in a particular environment. These include unicellular eukaryotes, variability in their distribution under a given condition, and discrimination of microbial strains collected from different sources. The information can be further used in devising strategies to plan eradication or control of diseases, or in industries or environmental conservation, etc., depending upon the type of organism. The aim of this chapter is to provide a brief overview of microbiological systematics, its history, and how ribotyping as a tool justifies all the approaches of systematics with its practical utilities.

Historical developments in bacterial taxonomy

Origination of taxonomy was one of the major events in the biological sciences in the 18th century. It was then that Carl von Linné (Carolus Linnaeus) described binomial nomenclature (taxonomy), which was accepted worldwide. The science of scientific naming started in the late 16th century as plants and animals were assigned long, polynomial, descriptive names based on particular characteristics. Latin, being a scholarly language, was used for this naming. As more and more species were discovered, the polynomials got longer and increasingly unwieldy. Linnaeus simplified the naming system by designating one Latin name to indicate genus and the other name for the special epithet. These binomial names were easy to remember, and people soon started using them. Eventually these binomials replaced the polynomial names completely. Linnaeus' plant taxonomy was based on the number and arrangement of stamens and stigma. Later on, the system of classification became more organized, and John Ray's practice of using morphological evidence from all parts of the plant in all stages of its development was followed. One of the important facts was that the complex morphologies and variations were used systematically to build phylogenies.

This advancement in taxonomy provided an organizational structure to biological science, and it became the most fundamental building block for information sharing on biological resources. It was an impelling force for the development of zoology and botany, as construction of phylogenies and evolutionary studies could be performed. Studies on structural and functional relationships as well as diversities and their correlation with ecologies and evolution became feasible. Later, taxonomists devised rules for nomenclature, and subsequently, botanical and zoological codes for nomenclature were developed. Even Darwin's theory of evolution did not change the Linnaean classification; it merely provided a theoretical justification.

Microbiology as a separate field evolved in the late 19th century with the first description of a bacterial species. As early microbiologists were botanists, they adopted its binomial nomenclature. During the late 19th century and early 20th century, the scientific community realized the importance of microorganisms. These minute creatures could be found in all the ecological niches. Many new microorganisms were

identified, and the scientists working on them assigned novel names. However, there was no systematic and universal approach of nomenclature, which made exchange of knowledge difficult. This peculiar need in bacteriology led to the formation of a nomenclature committee at the first international microbiological congress of the International Society for Microbiology held in Paris in 1930. Later, in subsequent meetings, the rules for bacteriological codes were established and refined. This committee developed criteria for classifying microbial species, and prepared a list of species types. During this period, repositories for type and reference of culture collections were also developed. These events led to the significant efforts by bacteriologists to develop systematics.

Since microbial morphologies did not have enough structural variability to have much phylogenetic significance (as seen in two other organismal sciences, botany and zoology), more focus was placed on the biochemical aspects of the organisms. However, comparative biochemistry cannot answer the evolutionary purists as efficiently as comparative anatomy can, which might be one of the reasons for the slow development of microbial taxonomy. Also, for taxonomy it is imperative to select specimens for comparison to other individuals. The species types of higher plants, animals, or insects could be kept in satisfactory shape and size by proper preservation, but in bacteria, pure cultures had to be maintained indefinitely without a significant change in characteristics. Furthermore, versatility in terms of adapting to the environment (which is more pronounced in bacteria as compared to higher forms) was found to pose more complexity. Still, efforts by those early microbiologists started giving structure to microbial taxonomies. Microbial systematics was open to the development of biological sciences and other newly developed technologies that had relevance in taxonomy, and natural relatedness schemes were adopted. During the 1960s, one of the major events that gave a boost to microbial taxonomy was the work of Sokal and Sneath (1963). They proposed a taxonomic system using numeric algorithms, such as cluster analysis, rather than using subjective evaluation of their properties; they called their new system numerical taxonomy. This historical work is still followed, and has helped significantly in developing phylogenies. With the introduction of numerical phenetic analysis and molecular techniques, the inter- and intra-relatedness of species could be determined objectively. The methods being used ranged from phenotypic characterization, biochemical characterization, and DNA–DNA re-association, to base composition analysis. Another important event that occurred in the late 1970s was when Carl Woese devised a system of classification above the species level; he compared ribosomal RNA (rRNA) molecules (Fox et al., 1977). This newer view showed prokaryotes to comprise not one, but two unrelated major groups, archaea and eubacteria. The emergence of molecular approaches laid the foundation for different approaches in bacterial systematics. This led to differences among the bacterial systematists, and two schools were formed, one whose members held to traditional ideas of taxonomy and the other who embraced molecular approaches to taxonomy. Later, in 1987, an ad hoc committee on the reconciliation of approaches to bacterial systematics concluded that "bacterial taxonomy, which began as a largely intuitive process, has become increasingly objective with the advent of numerical taxonomy and techniques for the measurement of evolutionary divergence in the structure of semantides, i.e. large, information-bearing molecules such as nucleic acids and proteins ..." and "an ideal taxonomy would involve one system" (Wayne et al., 1987).

Typing methods used for bacterial systematics

Taxonomy requires two sources of information to be investigated as extensively as possible: genomic information and phenotypes. Genomic information is obtained from nucleic acids, either through sequencing or through DNA–DNA similarity, G + C mol.%, and ribotypes. Phenotype is the visible physical and biochemical expression of genotypes that result from the interaction of genotype and environment. Although many different types of techniques have been devised for analyzing microorganisms, each has its advantages and limitations as to the extent to which it can be used for taxonomy. An ideal technique would be cost effective, less time consuming, automatable, and would have good taxonomic resolution power. On the other hand, the ultimate purpose of identification needs to be defined. It may be limited to identification and to assignment of a definite taxon, or to understanding disease/infection, where the focus is on devising strategy for treatment and control (as for clinical isolates).

Phenotypic typing methods

Classification was initially based on morphology, biochemical characteristics, and growth conditions of the organisms. These investigations required the use of pure cultures and the characteristics studied were compared with the properties of reference strains. The similarities or differences between the reference strains and the isolate under question were the basis of bacterial identification and nomenclature (for a review, see Rosselló-Mora and Amann, 2001).

- *Morphology of bacteria*. The morphology of a bacterium includes the cellular and colony characteristics of the organism on cultivation. The cellular characteristics such as shape, Gram reaction, type and location of flagella and its motility, help in preliminary identifications. The colony characteristics include color, dimensions, and form. Bacteria show characteristic types of growth on solid media under appropriate cultural conditions, and the colony morphology can be used in presumptive identification. These colonies may vary in size and nature (circular, wavy, rhizoid, etc.), elevation (flat, raised, convex, etc.), and opacity (transparent, opaque, translucent). The color of the colony or the changes that they bring about in their surroundings is also an indicator used for the identification of bacteria. Some bacteria also produce pigments; this feature is also used as an aid in identifying the organism.
- *Biochemical characteristics of bacteria*. The biochemical features include both primary and secondary biochemical tests for identification of microorganisms, such as growth on selective or differential media, growth in the presence of various substances such as antimicrobial agents, carbohydrate utilization tests, or presence or absence of certain enzymes. These tests are commonly used, and characteristics based on these tests are widely described in *Bergey's Manual of Determinative Bacteriology* (Garrity, 2001). The limitation with these tests is that laboratory to laboratory variability may be seen. Furthermore, there is no rule of thumb in defining the range of diversity among the strains to be examined. The set of strains should be as large as possible, and should include cultures of historical, pathological, or environmental importance. It is important to include reference strains (type strains) whose identity has been established for comparative purposes. Moreover, routine tests should represent a broad spectrum of the biological activities of the organisms.
- *Serotyping of bacteria*. Bacterial species and types can be identified by specific antigen–antibody reactions. Antigens are substances that induce the production of antibodies in a foreign species. Bacteria and bacterial components serve as excellent antigens. The test includes production of antibodies in an animal host and testing of the antiserum by either the agglutination or precipitation test. These conventional tests are now supplemented with monoclonal antibody-based, highly sensitive ELISA, and a wide range of other serological tests.
- *Phage typing of bacteria*. This method is used for typing bacteria by testing the susceptibility of the culture to lysis by each of a set of type-specific lytic bacteriophages. The phage type of the culture is identified based on its pattern of susceptibility to the different phage strains. Phage typing allows subdivision of a serological entity, as in *Salmonella typhi*, which is divisible into more than 100 different phage types.

In addition to these conventional phenotypic typing methods, there are currently many spectrophotometric methods used in the characterization of bacteria that require specialized instrumentation. The most popular methods are the following:

- *Fourier transform infrared spectroscopy (FTIR)*. This technique is based on the observation of vibrational properties of chemical bonds when excited by an infrared beam. It enables one to assess the overall molecular composition of microbial cells in a nondestructive manner, reflected in the specific spectral fingerprints for different microorganisms. This vibrational spectroscopy technique is useful in providing rapid, relevant information of intact microbial cells and allows rapid discrimination, classification, and identification down to the strain level, as well as monitoring of metabolite production. This method generates little waste and thus has lower run costs.
- *Pyrolysis mass spectrophotometry*. This is a rapid and high-resolution method for the analysis of otherwise nonvolatile material and has been widely applied for discriminating between closely related microbial strains. This technique involves thermal decomposition of materials at very high temperatures. Large molecules are cleaved at their weakest points and produce smaller, more volatile fragments. These fragments can be separated by gas chromatography. This technique has the ability to analyze small amounts of biological material with minimum sample preparation to obtain fingerprint data that can be used for the identification and typing of the microorganism (Goodacre and Kell, 1996).
- *Fatty acid methylester (FAME) analysis by gas chromatography*. Fatty acids in microbial cells are primarily found in cell membranes as phospholipid and also in the cell wall of Gram-negative bacteria as lipid A component of LPS and lipoteichoic acid in Gram-positive bacteria. The length of fatty acid chains in most of the bacteria varies from 10 to 19 carbon atoms, with 16 or 18 carbon atoms long fatty acids as the most common. The 16-carbon saturated hexadecanoic acid, in particular, is highly conserved among prokaryotes. The cellular fatty acid composition of bacteria varies in terms of its quantities and also in the form of presence of some

unique fatty acids. The qualitative and quantitative fatty acid profile of microbial cells is genetically stable and specific to generic and species level. More than 300 fatty acids and related compounds are found in bacteria. The fatty acids found in Gram-positive bacteria are mostly branched where as those found in Gram-negative bacteria are straight chain fatty acids. These differences in fatty acids are exploited in FAME for identification of bacteria. The process involves extraction of cellular fatty acids through saponification and then conversion to fatty acid methyl esters through treatment with methanol. These fatty acid methyl esters are volatile and used to create unique microbial fatty acid profile for a given organism using gas chromatography. The chromatography data are then used for typing of bacteria. The only commercially available systems, that is, "MIDI Sherlock Microbial Identification System" has its software where the gas chromatographic profile of the bacteria under question is matched with that in its stored library for the identification of the bacteria (Sasser, 2001).

- *Matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS)*. This is a very sensitive fingerprinting method used for the classification and identification of microorganisms, with its applications in clinical diagnostics. MALDI-TOF is a soft ionization technique in which a biomolecule is irradiated on a UV-absorbing matrix by laser pulse. The ionized biomolecules are accelerated in an electric field and enter the flight tube where different molecules are separated according to their mass-to-charge ratio and reach the detector at different times, thus generating spectra. A database of known organisms is used to match the isolate under investigation, providing a matching score based on identified masses and their intensity. MALDI-TOF MS has the ability to measure peptides and other compounds in the presence of salts and to analyze complex peptide mixtures; this makes it an ideal method for measuring nonpurified extracts and intact bacterial cells. Different experimental factors, including sample preparation, the cell lysis method, matrix solutions, and organic solvents, affect the quality and reproducibility of bacterial MALDI-TOF MS fingerprints (De Bruyne et al., 2011).

Genotypic typing methods

During early 1960s, increasing knowledge of properties of DNA and development of molecular biological techniques supported the idea that bacteria might best be classified by comparing their genomes. Initially, overall base compositions of DNAs (mol.% G + C values) were used. Bacteria whose mol.% G + C values differed markedly were obviously not of the same species. However, single values obtained by the analysis of DNA base compositions allowed only superficial comparisons, and a much more precise method was needed. Thus, DNA–DNA hybridization techniques were developed. A great practical advantage of this method was that it often produced more sharply defined clusters of strains than those solely circumscribed by phenotypic traits. The phylogenetic definition of a species generally included strains with approximately 70% or greater DNA–DNA relatedness and with 5°C or less ΔT_m. Here, organisms tended to be either closely related or not at all. This method is still important and valid as far as bacterial systematics is concerned, with the limitation being use of only two strains at one time, one of which is essentially the reference strain.

In addition to these methods, and with advancements in the science of genomics, many newer genomic marker techniques, such as amplified fragment length polymorphism (AFLP), randomly amplified polymorphic DNA (RAPD), and restriction fragment length polymorphism (RFLP), are also being used to collect information on microorganisms.

Though such techniques have been encouraged by the ad hoc committee on species definition in bacteriology, which met in Belgium in 2002, they still consider DNA–DNA re-association and 16S rDNA sequence analysis as the major methods for the definition of bacterial species (Stackebrandt et al., 2002).

Basis of using rRNA and rRNA genes as taxonomic tools

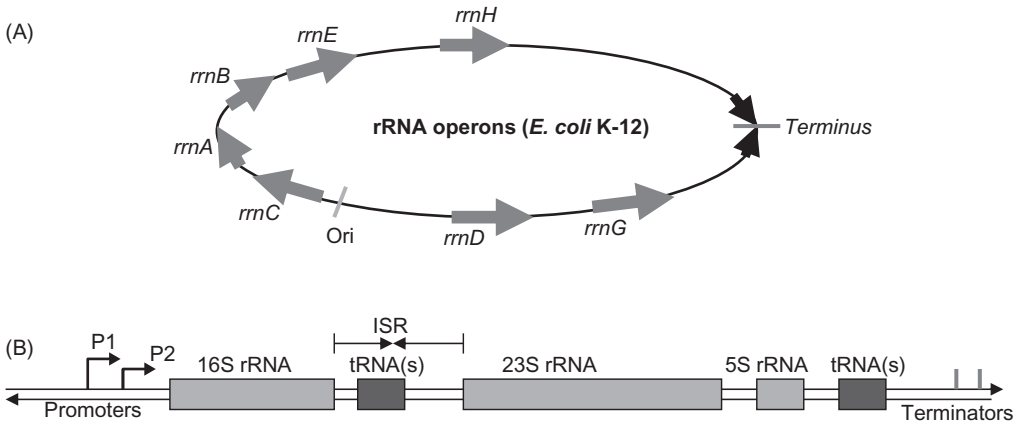
The ribosome is a structure of ancient origin and is necessarily ubiquitous. Each cell requires ribosomes for translation; their number in a given cell may range from a few hundred to 100,000 and is believed to be fixed within a particular species. Since they perform the central function of translation in a cell, the primary structures of ribosomes are sufficiently constrained. They are formed from proteins and ribosomal RNA. Ribonucleic acid is the core molecule for the translation process, forming the framework of the ribosome from a structural point of view; thus, it has much more stringent requirements. Even minor changes in a nucleotide sequence in rRNA can produce a deleterious effect on translation. Thus, during evolution rRNA had to be conserved in its basic sequences to maintain the translation apparatus with great efficiency. This may be the reason that rRNA gene sequences have

been found less prone to spread horizontally between the members of similar species. The differences between rRNAs of different cells represent discontinuities that occurred among the early ancestors of today's cells, making them suitable for phylogenetic study, where the number and distribution of different rRNA genes are taken as a measure of diversity.

Organization of the ribosomal operon

In prokaryotes, the smaller subunit of the ribosome (30S) has 16S rRNA (~1600 nucleotides long), and the larger subunit (50S) has 23S rRNA (~3000 nucleotides long) and 5S rRNA (~120 nucleotides long) molecules. The genes of these three rRNAs are typically linked together into an operon, called a ribosomal operon (Fig. 19.1A and B). This operon contains genes for 16S, 23S, and 5S rRNAs in order from the 5′ to 3′ direction. These genes are separated by a short segment of nucleotides called the intergenic spacer region (ISR), which may consist of genes for some tRNA molecules. The genes for rRNA are about 1.5 kb in size in all bacteria and have clusters of highly conserved regions, variable regions, and hypervariable regions containing species, genus, and family-specific signature sequences. This facilitates the determination of evolutionary interrelatedness, the generation of probes for hybridization and primers for PCR, and the formation of reference databases. Of the three rRNA genes, 16S rRNA gene sequences are considered to be the most conserved, and the species having 70% or greater DNA similarities usually have more than 97% sequence identity within this gene. The remaining 3% or 45 nucleotide differences are not evenly scattered along the primary structure of the molecule but are concentrated mainly in certain hypervariable regions, the positions of which are taxon specific. It is in these regions that species/genus-specific sequences may be clustered. The ribosomal RNA operon is transcribed into a single preRNA molecule consisting of regions of 16S rRNA, spacer, tRNA, spacer, 23S rRNA, spacer, 5S rRNA, spacer, and tRNA sequentially, from the 5′ to 3′ direction; this is then cleaved into rRNA and tRNA molecules.

Within the chromosome of an organism there may be multiple copies of the rRNA operon. The number of copies of ribosomal RNA operons containing the genes coding for rRNA genes (16S, 23S, and 5S) and their associated ISRs in bacterial species have been found to range from 1−15 (e.g., 7 in *Escherichia coli* and *Salmonella* and 10 in *Bacillus subtilis*). In *E. coli*, these copies are named as *rrnA*, *rrnB*, *rrnC*, *rrnD*, *rrnE*, *rrnH*, and *rrnG* (Fig. 19.1A). There is some degree of variation in the length of the spacer region between 16S and 23S rRNA genes in different organisms due to the



(C)

rrn Operons	ISR tRNA(S)	distal tRNA(S)
B, E, G	Glu2	–
C	Glu2	Asp1, Trp
A	Ile1, Ala1B	–
D	Ile1, Ala1B	Thr1
H	Ile1, Ala1B	Asp1

FIGURE 19.1 (A) Organization of seven different operons (*rrnA*, *rrnB*, *rrnC*, *rrnD*, *rrnE*, *rrnG*, and *rrnH*) present in *E. coli* K-12. (B) Organization of rRNA (rRNA and tRNA) genes and internal spacer region in a typical rRNA operon (*E. coli* K-12). (C) Type of tRNAs present in different rRNA operons of *E. coli* K-12. P1 and P2: promoters.

number and type of tRNA genes that may be present in this region (Fig. 19.1C); for example, in most of the Gram-negative bacteria, genes for $tRNA^{ala}$ and $tRNA^{ile}$ are present in this region, while in Gram-positive bacteria, no tRNA gene, or $tRNA^{ala}$ or $tRNA^{ile}$ (or both), may be present. The ISRs have been suggested to reflect intra-species phylogeny. Thus, rRNA or rRNA genes can be exploited to group or type and identify bacterial organisms using different molecular tools such as generating primers and probes; this process is called "ribotyping."

Different techniques of ribotyping

Grimont and Grimont (1986) first obtained several patterns of hybridized fragments from rRNA genes of different bacterial species and also observed that strains showing identical patterns were highly related. This laid the foundation for utilizing ribotyping as a tool for identifying and classifying bacteria based upon differences in ribosomal RNA molecules or the genes encoding them. Ribotyping generates a highly reproducible and precise fingerprint that can be used to identify and classify bacteria. The methodology involves the fingerprinting of genomic DNA restriction fragments that contain all or part of the genes coding for the 16S and 23S rRNA (initially phylogeny based on 5S rRNA homology was also attempted but was replaced by larger RNA molecules due to availability of more information, universality, and experimental ease). Currently, ~2,500,000 16S rRNA sequences of various species are available. This also includes a significant number of sequences retrieved by PCR from yet-uncultured microorganisms. This was the first technique used to differentiate Archea from Eubacteria. Advances in biotechnological tools have given rise to different techniques that are currently being used to resolve this operon and extrapolating the data obtained for the identification of bacteria from samples of different origins for further use as per requirements.

Conventional ribotyping

Conventional ribotyping, a restriction fragment length polymorphism technique, is based on the restriction endonuclease cleavage of total genomic DNA followed by electrophoretic separation, Southern blot transfer, and hybridization of transferred DNA fragments with a radiolabeled ribosomal operon probe (Fig. 19.2). After the availability of ribosomal gene sequences and DNA analytic software, it became possible to select an appropriate restriction enzyme that cuts once within the 16S rRNA genes and once within the 23S rRNA gene, which enables the detection of DNA sequence polymorphisms in ribosomal operons or immediately adjacent upstream and downstream genes flanking each ribosomal operon. The DNA is extracted from a single bacterial culture of the test organism derived from a single colony and is subjected to restriction digestion to get its discrete sized DNA fragments. These fragments are then resolved by electrophoretic separation and transferred to a membrane (Southern blotting) (Fig. 19.3A), and regions of rRNA operons are probed by hybridization with radioactively labeled probes (alternatively, other reporter systems such as fluorescent or chemiluminescent-labeled probes can be used). During species/genus-specific probe preparation, a whole 16S–23S–5S operon is first

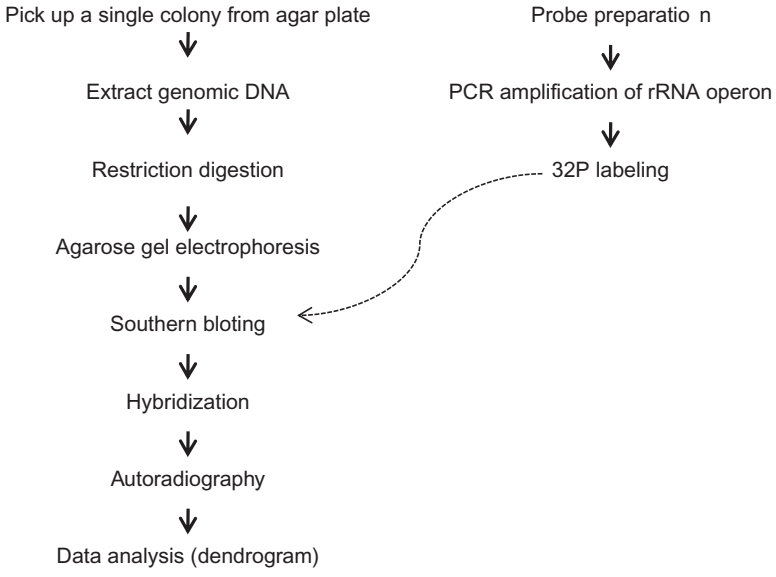


FIGURE 19.2 Flow diagram (ribotyping).

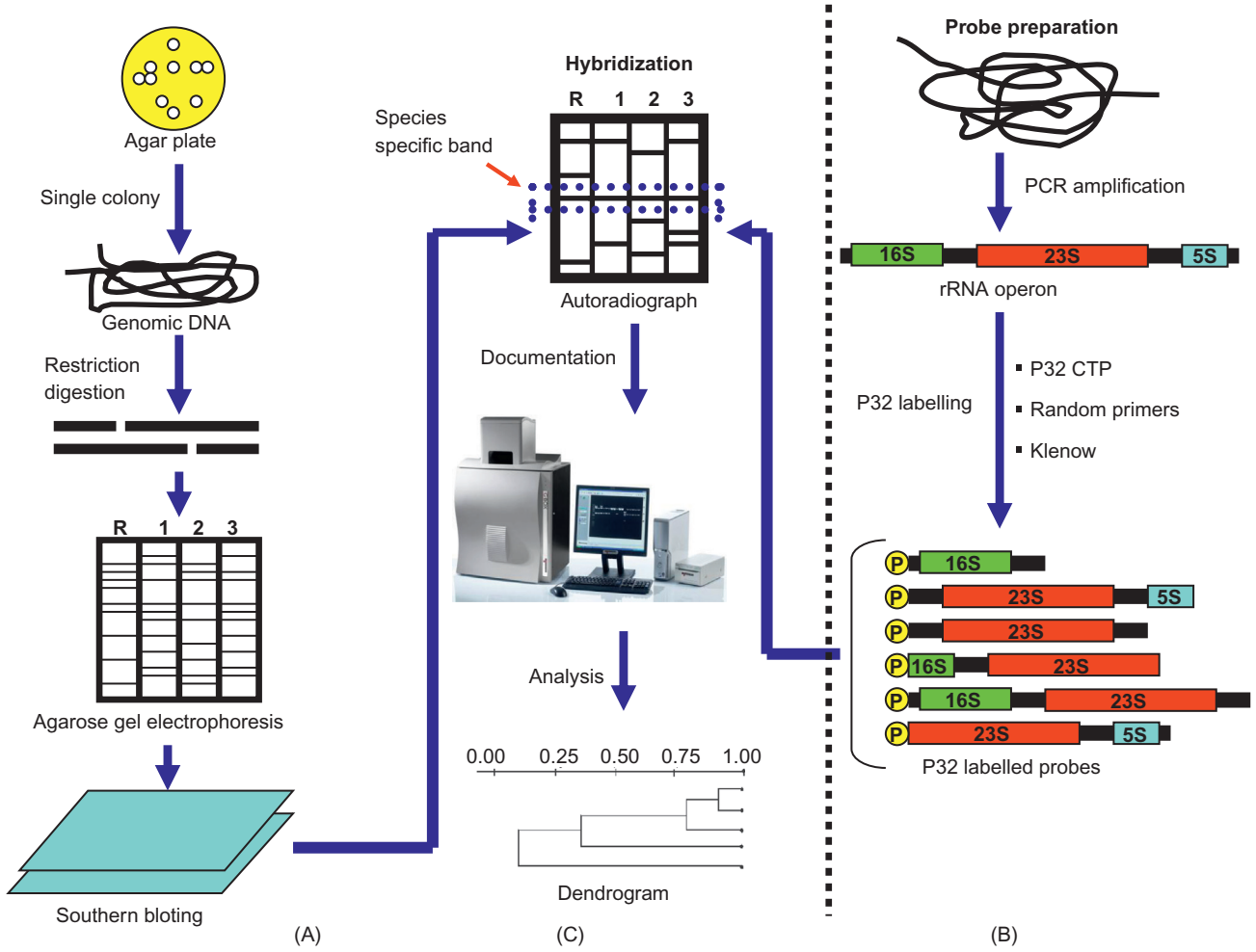


FIGURE 19.3 Ribotyping methodology.

amplified by PCR; then, this amplified fragment is used to generate radioactive or other labeled probes using random primers and a large DNA polymerase I (Klenow) fragment (Fig. 19.3B). There are other approaches to generate probes. For example, any segment of the operon (the 16S or 23S region, or the whole operon) can be cloned separately and used as a probe. Another method employs cDNA synthesized using reverse transcriptase and a 16S or 23S rRNA template as the probe sequence. Oligonucleotides that are chemically synthesized with a "signature sequence" of the specific level of the taxon can also be labeled and used as probes. Following electrophoresis, the pattern of the rRNA gene (i.e., ribotypic bands) is then recorded, digitized, analyzed manually or with appropriate software, and stored in the database (Fig. 19.3C). The variations in ribotypes among bacteria may occur due to both the position and intensity of bands complementary to rRNA genes and are used to identify and classify the bacteria. If the entire *rrn* operon is used as the probe sequence, then the banding pattern is known as a ribotype, and if only the 16S rRNA sequence is used as the probe, the banding pattern is called a 16S ribotype. The polymorphism in ribotyping is the reflection of the conservation of rRNA operon sequences and the variability of chromosomal flank sequences. In certain situations, for the discrimination of strains, one, two, or more restriction endonucleases can be used simultaneously to identify a greater degree of restriction fragment length polymorphism.

Selection of restriction endonuclease for ribotyping by sequence analysis (in silico)

As of now, more than 250,000 ribosomal gene sequences are available in public databases, and with the use of DNA analytic software, it has become possible to select an appropriate restriction enzyme that cuts once within the 16S rRNA genes and once within the 23S rRNA gene, which enables the detection of DNA sequence polymorphisms in ribosomal operons

or immediately adjacent upstream and downstream genes flanking each ribosomal operon. *Haemophilus influenzae* strain Rd was the first available bacterial genomic sequence, and has been used as a protoype for ribotyping (Bouchet et al., 2008). In silico analysis of *H. influenzae* strain Rd revealed the presence of six ribosomal operons and associated flanking sequences responsible for ribotyping polymorphisms. The initial ribotype protocol for *H. influenzae* includes the following steps:

1. Search the conserved restriction endonuclease cleavage sites within the six ribosomal operons.
2. Choose the ideal restriction enzyme that would cut once within the 16S rRNA gene and once within the 23S rRNA gene to create an internal species-specific fragment. The length, GC content, and specificity of the recognition site also determine the sensitivity of the ribotyping.
3. Scan for all publicly available 16S and 23S *H. influenzae* rRNA gene sequences (in addition to closely related species) to confirm conservation of the restriction site.
4. After selection of restriction endonuclease, the genomic DNA of the isolate to be ribotyped is digested, separated by electrophoresis, and transferred onto a nylon membrane by Southern blotting; it is then hybridized to a labeled probe containing conserved ribosomal operon sequences that enable the generation of ribotype RFLP patterns.

In silico analysis of *H. influenzae* revealed that its rRNA operon has two cutting sites for *Eco*RI, one within the 16S rRNA gene and one within the 23S rRNA gene that would result in ribosomal operons with two bands comprising the ISR located between 16S and 23S rRNA of the six rRNA operons. Interpretation of ribotyping depends in part on the size variation of ISR bands containing tRNA-encoding DNA sequences. ISR size is for the most part related to the number of tRNAs found within the ISR region (e.g., one or two in the case of *H. influenzae*) and is therefore referred to as a species-specific signature band. The high degree of conservation among ribosomal operons suggests that the ribotype polymorphism is not due to the rRNA gene, but rather to flanking sequences of the rRNA genes. In silico analysis of 50,000 bp immediately upstream and downstream of the six ribosomal operons of *H. influenzae* strain Rd revealed that rDNA flanking sequences are primarily composed of neutral housekeeping genes (encoding proteins, but not subjected to diversifying selection) evolved through point mutations (Bouchet et al., 2008). The number of variant alleles of a housekeeping gene is vast and, therefore, provides the basis for ribotyping. During evolution, the bacterial genome undergoes random point mutations throughout its genome and, thus, the polymorphisms in ribotyping are dependent on the rate of these point mutations in the genes flanking ribosomal operons. Change in a single base pair in a 6-bp restriction recognition site (*Eco*RI) will result in loss of the recognition sequence, and a consequent change in the ribotype RFLP fingerprint profile.

The importance of this process lies in the fact that it allows the detection of point mutations and, thus, can be used for strain differentiation within a species (which is important from a taxonomic point of view). The basic in silico approach described for *H. influenzae* for design and interpretation of a conventional ribotyping scheme has been similarly implemented for several other bacterial species (e.g., *E. coli*, *Pseudomonas aeruginosa*, *Burkholderia cepacia*, *Salmonella pneumoniae*, *Neisseria meningitis*, *Bordotella pertussis*, *Bordotella parapertussis*, *Bordotella broncoseptica*).

Automated ribotyping

Automated ribotyping was first time introduced by DuPont Qualicon in 1995 for the identification and characterization of bacterial isolates by the generation of ribosomal RNA fingerprints (RiboPrint pattern) from bacteria. This system is reproducible, convenient, and fast. In automated ribotyping, a single colony is picked up from an agar plate, suspended in lysis buffer, transferred to a sample carrier, and loaded into the RiboPrinter. Later, inside the RiboPrinter (as with conventional ribotyping), restriction digestion of DNA (mostly *Eco*RI), separation of DNA fragments by electrophoresis on agarose minigel, transfer and capture to nylon membranes, and hybridization with a chemiluminescent-labeled DNA probe derived from *E. coli rrn* rRNA operon takes place. After washing to remove unhybridized probes, the chemiluminescent patterns are then electronically imaged, processed, and compared to other DNA patterns in the RiboPrinter database. A single batch run takes place in about 8 hours, and the generated data can be distributed into the network. This system allows virtually all bacterial species to be characterized according to their specific ribotype and to be identified as per their existing reference pattern. It is particularly useful in the epidemiological surveillance of bacterial diseases and for the rapid identification of the source of infection, which may help in formulating a strategy to prevent further spread of disease and reduce the number of victims. However, the system is expensive, the run costs are high, and a pure culture is needed, besides having

database from the well characterized isolates in the database.

The conventional ribotyping procedure involves Southern transfer and hybridization with radioactively labeled probes, making it cumbersome, besides requiring specialized laboratories with facilities for the physical containment of radiation. Following the advent of new genetic tools and rapid sequencing schemes, attempts were made to develop more rapid and less labor-intensive typing schemes for microorganisms related to ribosomal RNA operons. These alternative schemes are more practical than conventional ribotyping for the identification and classification of bacteria, even beyond the species level.

Polymerase chain reaction ribotyping

PCR ribotyping is based on the amplification of the ISR, along with some sequences of 16S and 23S rRNA genes; it also detects the length and number of polymorphisms in the bands after gel electrophoresis (Kostman et al., 1995). Conserved regions in 16S rRNA and 23S rRNA genes allow designing of primers that can anneal with the genes of a wide number of bacterial genera, enabling the amplification of ISRs through PCR. In this method, the primers are chosen in such a way that they are complementary to the 3′ end of the 16S rRNA gene and the 5′ end of the 23S rRNA gene (Fig. 19.4). Following PCR, the amplified product is resolved through agarose or polyacrylamide gel electrophoresis and the data of size and number of bands Fig. 19.4A and 19.4B are analyzed. The length of the spacer region can vary in different copies of the rRNA operon within the same chromosome. This is probably because of the fact that the ISR is under the least selection pressure and thus may vary in length and sequence within the organisms of the same genus and species. If only one or very few bands appear after electrophoresis, techniques such as denaturing gradient gel electrophoresis, temperature-gradient electrophoresis, or SSCP can be applied to further resolve the polymorphism. After electrophoresis, more than one band may be obtained, but in a particular strain it will be constant. This technique was found to be especially suitable in clinical microbiology laboratories in epidemiological studies for determining the relatedness and discrimination of strains isolated from different sources.

Polymerase chain reaction ribotyping and endonuclease subtyping

If PCR ribotyping does not result in resolving conclusive heterogeneity in the amplified bands of multiple strains of one or different species, subtyping of the strains by restriction analysis of the amplified product may generate additional data on heterogeneity in the genomic sequences, which in turn may help in discrimination of the bacterial strains of a single species. This heterogeneity results due to the high degree of sequence variability among multiple copies of the ISR, ranging from a point mutation to the deletion or addition of larger segments. Such variability is mainly due to the fact that ISRs in some organisms code for one or two tRNAs. They also possess multiple alleles of the rRNA gene cluster, with considerable variation in the lengths and sequences of the ISRs both at the level of operons in the same genome and between operons of different strains within a species.

Polymerase chain reaction ribotyping followed by sequencing of ISR

Mycoplasma and mycobacterium are the organisms that have only one or two copies of rRNA operons in a single organism and pose difficulty in the resolution of ISR variability in terms of length and number of operons. In such situations, direct nucleotide sequencing of

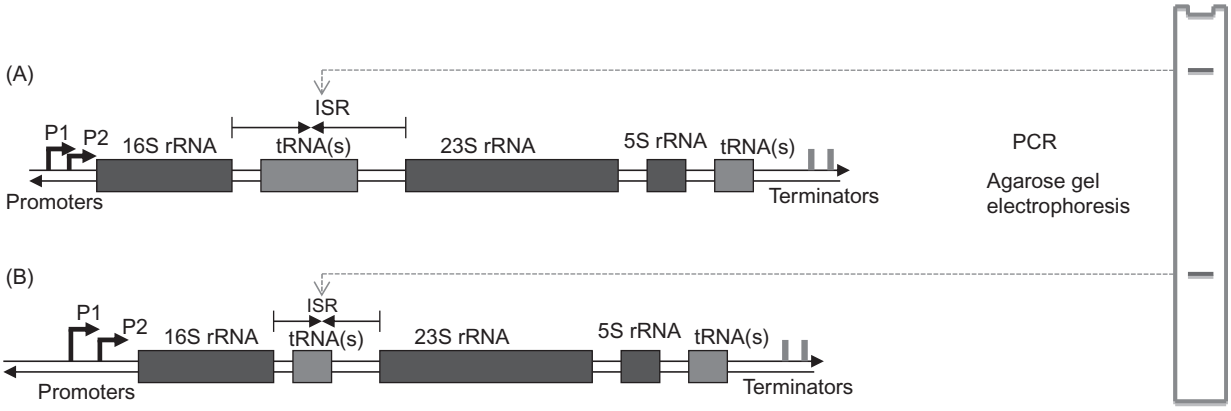


FIGURE 19.4 Length heterogeneity of ISR of rRNA operons (A and B) among bacterial species/strains results in polymorphisms that can be detected by PCR and agarose gel electrophoresis (PCR ribotyping).

amplified product following PCR ribotyping of the spacer region may result in the identification of variations in the sequences of genomes, thus helping in the discrimination of different strains of a species.

Since the sequences of 16S and 23S RNA genes are highly conserved, the primers first described by Kostman et al. can be used to amplify ISRs of a wide range of bacteria, making PCR ribotyping widely applicable. Moreover, this method obviates the need for the time-consuming and cumbersome Southern transfer technique used in conventional ribotyping. It is also widely used in the clinical microbiology laboratory where the focus is on expedited identification of microorganisms and determination of the source and spread of infection (to facilitate control measures).

PCR ribotyping carried out on various bacteria has resulted in the realization that the choice of PCR primer is important in the successful detection of heterogeneity in the intergenic space, especially of the 23S rRNA gene, which is relatively less conserved than that of the 16S rRNA gene. Primers derived from certain regions of the 23S rRNA gene have been found to not be able to bind to all strains of a species and, thus, fail to amplify the intergenic space through PCR.

Amplified ribosomal DNA restriction analysis

Amplified ribosomal DNA (rDNA) restriction analysis (ARDRA) is a modified method of PCR ribotyping. This simple and effective technique was first reported by Vaneechoutte et al.; they could distinguish nine of the 13 well-described taxa of the eubacterial family *Comamonadaceae* (Vaneechoutte et al., 1992). ARDRA is based on the PCR amplification of the 16S rRNA gene, followed by restriction digestion of the amplified products. The restriction fragment patterns obtained are further used to determine the diversity or similarity between the bacteria based on the proportion of the fragments that are shared between the microorganisms under question. The advantage of this technique is that it can be used either for the identification of the organism in pure cultures or in microbial communities.

Initially, DNA is extracted from the sample. This sample may have an unknown microorganism either in pure culture, where it can be cultivated, or a complex population of microorganisms. This DNA is subjected to PCR using the primers derived from the conserved regions present on both ends of the 16S rDNA gene. The entire amplicon of size of about 1500 bp can then be either directly subjected to restriction enzyme digestion or cloned into *E. coli* cells to segregate the PCR products into individual cultures. Cloning helps yield better resolution compared to direct restriction enzyme digestion of the amplicons, especially when a complex population of organisms is being handled. As the amplicon is about 1500 bp long, the restriction enzymes having a recognition site of more than four bases long will have less (or no) resolution. A single tetra cutter also may not give good resolution. Therefore, at least three tetra cutter restriction enzymes are used. Proper selection of the restriction enzymes is also important, as different restriction enzymes may give different resolutions of banding patterns. One study (Moyer et al., 1996) found *Hha*I, *Rsa*I, and *Bst*UI appropriate for the detection and differentiation of bacterial taxa based on criteria of RFLP size−frequency distribution patterns. These enzymes yielded the highest frequency of restriction fragment band size classes in the 250- to 550-bp range. The restriction pattern resolved after electrophoresis can further be analyzed, and phyolograms or cladograms prepared and compared with the relevant databases for identification.

This technique is useful if proper selection of restriction enzymes and electrophoresis has been done to resolve the restriction bands properly. ARDRA has proven useful only for genus differentiation and species differentiation but not for intra-species discrimination of different bacterial isolates.

Terminal restriction fragment length polymorphism of 16S rRNA gene

This technique was first used by Liu et al. (1997). This technique is useful in studying microbial communities in different environments. It is a PCR-based method in which the 16S rRNA gene is amplified with primers labeled at the 5′ end. The amplified products are subjected to restriction digestion using tetra cutters, and fluorescent-labeled terminal restriction fragments are then sequenced on an automated sequencer. The members of closely related phylogenetic groups often have the same terminal restriction fragment sizes.

Long polymerase chain reaction ribotyping

The long PCR ribotyping method involves PCR amplification of an entire 16S−23S−5S ribosomal operon of length of up to 6 kbp, followed by restriction endonuclease digestion to detect restriction length polymorphisms. The products can be visualized in a simple manner in an ethidium bromide-stained agarose gel electrophoretogram. This method was first developed to type extremely heterogeneous (nontypable) *H. influenzae* strains and has also been found useful in rapidly characterizing novel organisms with a high level of discrimination, reproducibility, and ease (Smith-Vaughan et al., 1995). The improved enzymatic systems for synthesis of longer DNA segments made it possible to amplify PCR products up to 15 kb. This is achieved by replacing *Taq* DNA polymerase with a combination of a high level of thermostable DNA

polymerase lacking 3′−5′ exonuclease activity and a low level of thermostable DNA polymerase exhibiting 3′−5′ exonuclease activity (e.g., *Pfu* DNA polymerase) with improved base pair fidelity. It is considered more discriminatory than 16S sequencing alone because it covers the more highly variable ISR and also possesses 23S rRNA genes.

Broad-range polymerase chain reaction ribotyping

Availability of ribosomal RNA sequences of a wide range of organisms in databases has resulted in the exploration of many new possibilities. In clinical microbiology laboratories, in certain situations when organisms are visible but cannot be cultivated (especially after antibiotic therapy or if they appear to be very slow-growing), broad-range PCR-based ribotyping has yielded good results. Sequence analysis of ribosomal RNA genes of different groups of organisms has emphasized the fact that 16S rRNA genes (and also 23S rRNA genes) show highly conserved regions of varying levels of variable sites. Primers can be designed that are specific to broad groups or higher level phylogenetic taxa (i.e., bacteria, archaea, eukarya, fungi). Within such groups, PCR-based progressive differentiation can be achieved using primers that are targeted to detect division, family, and genus. Usually primers are directed to amplify about 300−900 bp regions of the rRNA gene, depending upon the quality of the template DNA; a smaller target is preferable for damaged or lower quality DNA. Appropriate positive and negative controls must be observed, and special precautions need be taken to prevent DNA contamination during sampling, in a laboratory environment, and of all reagents. Real-time PCR, if applied in place of conventional PCR, is more suitable in analyzing background false amplification.

Limitations of polymerase chain reaction ribotyping

Use of the 16S−23S ISR in studies of phylogeny, molecular evolution, or population genetics is a potentially powerful tool. However, there are certain limitations imposed by the possibility of multiple nonidentical rRNA operons which are as follows.

1. ISRs are of identical length, but different sequence will produce identical ISR fragment patterns after gel electrophoresis.
2. Preferential amplification of some operons: the organism may contain both tRNA-containing and tRNA-lacking rRNA operons, and the operons without tRNAs may be preferentially amplified during PCR. This phenomenon may impact analyses employing restriction enzyme digests of ISR PCR products. If the ISR from one operon is amplified preferentially in one isolate, while the ISR from a different operon is preferentially amplified in another isolate, comparisons of digests of PCR products from those two isolates may be flawed. Experimental designs should therefore contain safeguards against complications imposed by the presence of multiple nonidentical rRNA operons.
3. Selection of an ideal restriction endonuclease described above is not always possible.
4. rRNA genes are transcribed from the ribosomal operon as 30S rRNA precursor molecules, which are then cleaved by RNase III into 16S, 23S, and 5S rRNA molecules. rRNA molecules may likewise be fragmented due to the presence of intervening sequences (IVS) within either the 16S rRNA or the 23S rRNA. The presence of IVSs within the 16S or 23S rRNA may be misleading in the interpretation of ribotyping RFLP patterns by (1) containing endonuclease recognition sequences of the selected restriction enzyme and (2) altering the size of the signature band. The appearance of an additional restriction site within the IVSs would result either in truncation of the species-specific signature band or in alteration of the flanking DNA band, depending on the position of the new cut site.
5. Split rRNA operon: after availability of whole genome sequences of some of the bacteria, split rRNA operons have been detected. Boer and Gray (1988) first reported an unusual organization of rRNA genes in the mitochondrial DNA of *Chlamydomonas reinhardtii*, where each gene was discontinuous and dispersed throughout the genome. Later, split rRNA operons were also identified in eubacteria and archaea. In split rRNA operons, in the most common pattern, the 23S and 5S genes form an operon and the 16S rRNA gene is separate; there may be one or more copies of each of the genes. For example, *Helicobacter pylori* 26695 carries two 23S−5S operons, two single 16S genes, and one separate 5S gene.

Ribosomal DNA sequence analysis

Development of rapid and high-throughput NGS techniques has of late resulted in the availability of rapid and unambiguous nucleotide sequence information for 16S and 23S rDNA of several bacterial species. This information is available in regularly updated public databases (GenBank: www.ncbi.nlm.nih.gov; or Ribosomal Database Project II: Cole et al., 2011) or private commercial databases for discrimination of even closely related species. The technology has enabled the determination of 16S rRNA sequences without even culturing the organisms from clinical and environmental sources; this is known as "taxonomic profiling," which consists of amplification of a complete or

selected region of the 16S rRNA region through PCR, and sequencing of the amplicon. The choice of primers determines amplification of conserved or less conserved regions of the ribosomal operon. Such "microbiome" and "metagenomic" studies earlier involved Sanger's automated capillary sequencing, which is being increasingly replaced by various next-generation sequencing platforms that enable more samples to be sequenced at a higher depth and lower cost.

In situ hybridization targeted to detect rRNA

The technique of hybridization with labeled probes (mostly fluorescent) has also been applied for the phylogenic detection of nonviral microorganisms based on using taxon-specific (kingdom, family, genus, species, or subspecies) probes designed to anneal with rRNA in situ in their habitat. The technique was first applied for bacterial identification in late the 1980s using fluorescent labels (known popularly as FISH) (De Long et al., 1989) and has been further refined (review: Amann et al., 1995). Apart from being rapid and cost effective, the main advantage of this method is that the organisms can be quantitatively detected in their natural habitat (i.e., aquatic, biofilms, soil, clinical samples) without the need for culturing. This technique can even be applied in mixed populations for simultaneous detection of more than one type of organism.

The FISH procedure (Figs. 19.5 and 19.6) consists of first designing an oligonucleotide probe targeting the hypervariable to less variable regions of 16S or 23S rRNAs specific to various phylogenetic levels of the target organism; this uses available sequences in RNA databases and appropriate software tools for sequence comparison and probe design. The oligonucleotide probe is usually kept short in length (15–25 bases), and secondary structures are avoided. The oligonucleotide is labeled at the 5′ end with any of the fluorochromes (DAPI, FLUOS, TRITC, Texas Red, Cy3, or Cy5 are commonly used) emitting wave lengths of different colors. Instead of labeling with fluorochromes, oligonucleotides can be tagged with other reporter molecules such as alkaline phosphatase, horseradish peroxidase, or digoxigenin, but they require a corresponding detection system. The selected probe must be thoroughly checked for its specificity against known standards and related nontarget organisms. The hybridization reaction is the most crucial step and is carried out on multi-well glass microscopic slides or membrane filters (having fixed the sample with heat, formaldehyde, or alcohol) under carefully chosen conditions of temperature (usually optimized around 46°C) and concentration of formamide. This step is followed by washing with an appropriate buffer at a specific temperature (usually 48°C). The fixation procedure of the sample varies for Gram-positive and Gram-negative organisms owing to differential complexity of the cell wall structure affecting permeability of the probe. Simultaneous detection of multiple organisms can also be attempted if a combination of probes is used and they are labeled with carefully chosen fluorochromes for better visualization. In such situations, hybridization conditions of differential stringency also need to be adequately selected to allow annealing of all the target nucleic acids. Multiple probes labeled with different fluorochromes can also be applied for the detection of a single organism to improve probability of detection. Alternatively, a single oligonucleotide can be doubly labeled at the 5′ and 3′ ends with different fluorochromes to enhance the intensity and resolution (Double-labeled Oligonucleotide ProbE, DOPE-FISH).

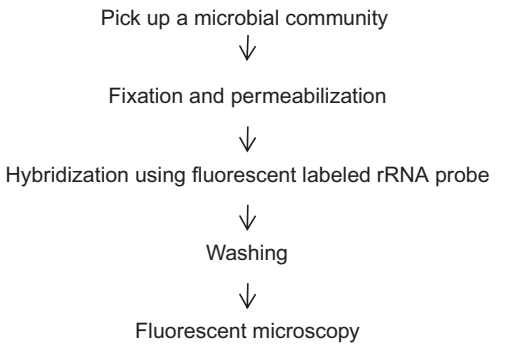


FIGURE 19.5 In situ hybridization.

Visualization of the fluorescent signals is conveniently achieved using an epifluorescence microscope and choosing an appropriate set of excitation and emission filters, depending upon the fluorochrome dye used. Inclusion of controls (positive and negative controls for autofluorescence of the cells) in the reaction greatly improves the reliability of the detection system. Confocal laser scanning microscopy (CLSM) can also be used for visualization of the fluorescence; this overcomes many of the problems encountered while using an epifluorescence microscope, especially if the specimen is thicker. Moreover, three-dimensional images can also be obtained with a CLSM, facilitating better resolution and quantitation of the target organisms. Another modification involves application of flow cytometry for the detection of fluorescence in the cells post-hybridization if the reaction has been attempted with cells in fixed and liquid suspension states. It also allows quantitation, specific sorting, and separation of desired cells in a mixed population, and increasing the concentration of target organism.

A modification of the in situ hybridization method aims to detect metabolically active cells in a community by growing the cells in the presence of a

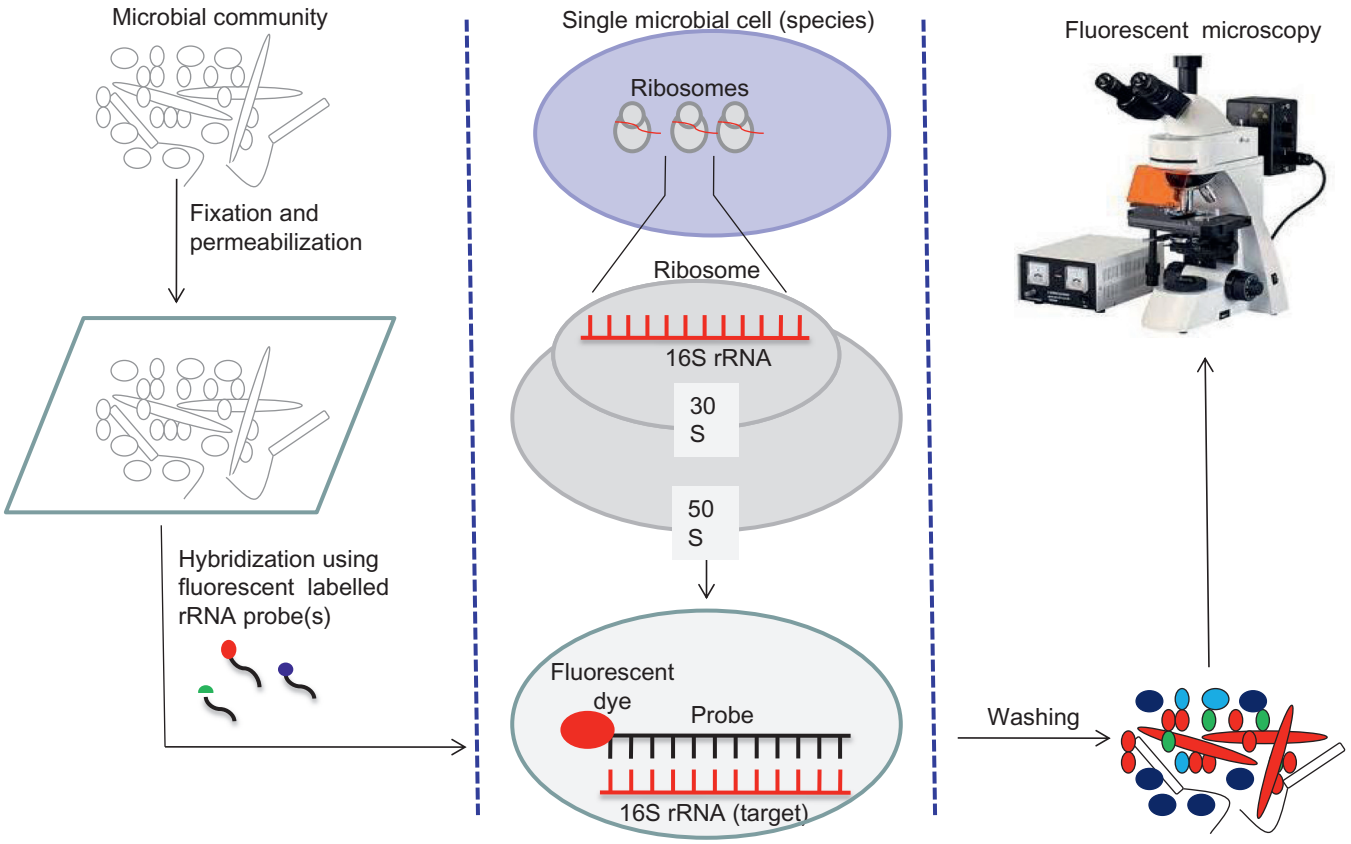


FIGURE 19.6 Steps in in situ hybridization.

radioactively labeled substrate (containing ^{14}C or ^{3}H). This results in accumulation of a radioactive label inside the cells that facilitates detection by microautoradiography (MAR) on a photographic emulsion. This is in turn followed by in situ hybridization to determine phylogeny. The technique is popularly known as MAR-FISH.

Clone-fluorescent in situ hybridization

The Clone-FISH technique is a modification of the FISH technique in which the hybridization reaction is not carried out in the target organism directly. In Clone-FISH, 16S rRNA genes or their fragments of the target organisms are cloned in suitable plasmid vectors and transcribed heterogeneously into rRNA in vivo within a suitable *E. coli* host. *E. coli* cells are then fixed and subsequently used as targets for in situ hybridization with the desired probe. Clone-FISH is a simple and fast technique, and is compatible with a wide variety of cloning vectors and hosts that have general utility for probe validation and screening of clone libraries. This technique is more applicable when the target organism is difficult to cultivate or is very slow growing, resulting in a very low rRNA-targeted hybridization signal and higher background noise.

Catalyzed reported deposition fluorescent in situ hybridization

The intensity of the signals in an in situ hybridization reaction depends upon the stage of growth of the cell and the ribosomal contents. To increase the sensitivity of the hybridization reaction, amplification of the signals can be achieved using horseradish peroxidase-labeled probes in combination with catalyzed reported deposition (CARD) of fluorescently labeled tyramides. Tyramide is a phenolic compound, which upon activation by horseradish peroxidase (HRP) enzyme, covalently binds to adjacent electron-rich tyrosine residues in proteins (even in fixed tissue or cell preparations). The hybridization reaction involves a single oligonucleotide that is covalently cross-linked to an HRP label. This enzymatic label is stable, and after the hybridization step, amplification of the signal is achieved by radicalization of the tyramide molecule derivatives linked to fluorophores by a single HRP molecule. Precautions need be taken to stop the activity of endogenous peroxidases with the blocking agents. Even multiple targets can be detected by sequential application of the labeled probes and tyramide labeled with different chromophores. This technique has been used for detection of pelagic marine (bacteria,

cynobacteria and acinobacteria) and sedimentary marine (archaea and bacteria) as well as marine algae to study the epiphytic bacterial community.

Recognition of individual gene fluorescent in situ hybridization

This is the method of detection of a single gene in bacteria. It involves a polynucleotide probe having only a few hundred nucleotides, but with multiple labels. With an extended hybridization time and high probe concentration, a network of probe molecules is formed that is typically characterized by halo-shaped fluorescence signals in the periphery of the cells (ring-shaped signals). The ring-shaped signals are believed to occur due to folding of the single-stranded RNA probe molecules into secondary structures, which results in the formation of a network of probes around the cell during whole-cell hybridization. This technique can be applied in combination with a conventional in situ hybridization reaction for identification of organisms based on 16S rRNA detection. The advantage RING-FISH is that the characteristic genes (e.g., virulence, antibiotic resistance) of the target organism can be detected along with the assignment of taxonomic level, especially in environmental samples.

Stable isotope-labeled probing of rRNA and rDNA

For the identification of functionally active microorganisms in environmental samples (also in biofilms), rRNA or their genes can be detected in addition to their metabolic genes using the technique of stable isotope probing (SIP). This method is based on growing the organisms in an environment containing stable isotope (nonradioactive)-labeled substrates in carefully chosen concentrations and duration of incubation, and subsequently analyzing isotope-labeled RNA (RNA-SIP) or DNA (DNA-SIP).

The commonly available isotopes for probing are compounds of ^{13}C (acetate, propionate, bicarbonate, phenol, etc.), ^{15}N (ammonium chloride), or ^{18}O (water). Labeled DNA or RNA is separated with density gradient centrifugation and subsequently 16S/23S rRNA genes are amplified by PCR or RT-PCR using the desired taxonomic level-specific primers. Isotope ratio mass spectrometry (IRMS) is used to distinguish labeled and unlabeled nucleic acids (isotope ratio). Another approach employs construction of DNA or cDNA libraries from the isolated "biomarked" nucleic acid and designing RNA probes for FISH. DNA-SIP requires a longer period of incubation and is less sensitive than RNA-SIP, as RNA is synthesized at a faster rate in growing cells than DNA, and more labels are incorporated during a shorter period of incubation.

Peptide nucleic acid probes

Although nucleic acid probes have been widely used for both solid- and liquid-phase hybridization, the use of peptide nucleic acid (PNA) probes is becoming more common of late owing to certain advantageous properties over nucleic acid probes. Peptide nucleic acids are nucleic acid analogs consisting of a neutral polyamide [i.e., poly-*N*-(2-aminoethyl)glycine] chain simulating a negatively charged poly-sugar phosphate chain of nucleic acids to which purine and pyrimidine bases are attached covalently (review: Corey, 1997). PNA probes hybridize with target nucleic acids both with DNA and RNA molecules in a similar fashion as the nucleic acid probes following conventional rules for base pairing. PNA probes are chemically and enzymatically more stable than nucleic acid probes, and the PNA-DNA/RNA hybrids have stronger binding affinity at low and medium salt concentrations. The specificity of PNA probes in terms of binding to target sequences with mismatches is also comparatively higher with lower background noise, and therefore are better suited for in situ hybridization reactions.

Limitations of ribotyping

The simplicity of performing, automation and availability of several large databases of reference sequences have made ribotyping as one of the most preferred techniques in diagnostic laboratories for identification and epidemiological studies. Its contribution to systematics has also been immense. For example, aerobic Gram-negative bacteria that used acetate as a sole carbon source were placed in a single group, the pseudomonads, which comprised several genera, including *Pseudomonas*. However, when 16S rRNA gene sequencing was introduced, it was discovered that these organisms arose by three different evolutionary routes that are now recognized as the alpha-, beta-, and gamma-proteobacteria. However, as all other techniques, it has its own limitations. The higher level of conserved sequences within rRNA operons results in lower discriminatory power. High similarity can be found in 16S rRNA gene sequences among some closely related microorganisms that lack resolution at the species level. It is well known that organisms can have identical 16S rRNA gene sequences and still belong to separate species that can be detected using other methods (Pontes et al., 2007). Another factor that leads to difficulties in interpretation is the different copy numbers of the gene in different bacterial species, which causes over or under representation of some species in rRNA target studies. Moreover, the process

TABLE 19.1 Comparative features of some commercially available microbial typing systems.

System	Manufacturer	Principle of detection	Database size	Accuracy (%)	Time of detection
Vitek t 2 Compact	Biomerieux, France	Biochemical	>450 taxons	42.5–94.0	2–24 h
BD Phoenic	Becton Dickinson, United States	Biochemical	>200 taxons	75.6–96.1	4–24 h
Biolog Microbial ID System	Biolog Inc., United States	Biochemical	>2500 species of bacteria, yeast and mold	47.5–97.5	4–26 h
API and ID32	Biomerieux, France	Biochemical	>600 species of bacteria and yeast		18–48 h
BBL Crystal	Becton Dickinson, United States	Biochemical	>400 taxons and 120 genera of clinically important organism		4–24 h
MicroSeq Identification System	Applied Biosystems, United States	Nucleic acid extraction, PCR amplification and rRNA base sequencing	>2300 bacteria species, 1100 fungi species	55.9–97.0	4–6 h
RiboPrinter System	DuPont Nutrition& Health, United States	Ribotyping	>5700 patterns covering more than 180 genera and 1200 species	81.0–93.8	8 h
MIDI Sherlock	MIDI, United States	Fatty acid methylester analysis	>2500 species including 700 environmental aerobic, 620 anaerobic microorganisms, and 200 yeast	75.0–77.8	Overnight
MALDI-Biotyper	Bruker Daltonik GmbH, Germany	MALDI-TOF mass spectrometry	>2500 species (5600 strains) of microorganism	79.0–98.7	Minutes
Vitek MS Plus	Biomerieux, France	MALDI-TOF mass spectrometry	>2000 clinically important microbes	79.0–98.7	<2 min
IFS-28B FT-IR spectrometer	Bruker Daltonik GmbH, Germany	Fourier transform infrared spectroscopy	730 bacterial strains covering 220 species out of 46 genera, 332 yeast strains covering 74 species out of 18 genera		Minutes

Note: Blank cell in the column accuracy indicate that information for this system could not be found.

is dependent on the efficiency of the DNA extraction method, and is also liable to PCR biases and artifacts, such as preferential amplification of certain sequence types, generation of chimeric sequences, and false positives due to experimental contaminants. Even following electrophoresis, the varying band patterns may exhibit only slight differences in size, which can further pose difficulties in evaluating the ribotypes.

Despite these limitations, ribotyping based on 16S rRNA gene sequencing has now been widely accepted as the primary technique for the identification of cellular microorganisms, particularly after establishment of boundary conditions (i.e., if a strain shows less than 97% of 16S rRNA gene homology with its highest match-described species, then it can be declared a novel species) (Stackebrandt and Goebel, 1994).

The comparative features of some commercially available microbial identification systems are summarized in Table 19.1.

Other genotyping methods

Multilocus sequence typing

Technological advancements and advent of many high-throughput sequencing platforms have opened new vistas in the field of microbial systematics and many new approaches are being tried, and multilocus sequence typing (MLST) is one of the earliest and most significant among them. This technique is an extension of the earlier used multilocus enzyme electrophoresis (MLEE) technique, which was based on detecting

variation in the electrophoretic mobility of some selected enzymes of microorganisms. In MLST, internal sequences (400−500 bp) of seven defined housekeeping genes coding for different enzymes are sequenced. The enzymes selected are those which participate in the basic metabolism of the cell and are least likely to be under selective pressure from the environment. Each gene is considered to be a single locus. The primers and regions for amplification are predefined and after PCR amplification the regions are sequenced. The variation in the sequence of each gene even in the form of a change in single base is considered a different allele and assigned a new number. A bacterial strain or type is identified as a "Sequence Type," having a definite allelic formula (Allelic Profile), consisting of a combination of components of all seven loci. The data generated are compared with allele sequences and sequence types available in central databases, that is, PubMLST, MLST databases at the ERI, UCC (Environmental Research Institute, University College Cork, Ireland) and the Institut Pasteur MLST databases. This scheme also has its limitations as it cannot discriminate between closely related pathogens (Enright and Spratt., 1999).

Whole genome sequencing

Ribotyping and other genotyping methods mentioned above are based on partial amplification/sequencing of the microbial genome. Ideally, to provide definitive typing, the method that sequences the entire genome of a microbial isolate would be of utmost importance. Sequencing entire genome has been made possible by the advent of NGS technologies, which are regularly being modified to become high throughput, inexpensive, routine and widespread to rapidly accelerate biomedical research. Along with the evolving NGS technologies, suitable bioinformatics tools for analyzing sequence data are also being developed. For targeted amplicon sequencing of 16S rDNA profiles, tools such as *QIIME*, *mothur*, and *VAMPS* were developed for the comparison and analysis of microbial communities. Since the high-throughput sequencing technologies introduce errors into sequence data, there are approaches to reduce these errors by clustering the flowgrams produced by the sequencer into a smaller number of sequences likely to be present in original samples using tools such as *Amplicon Noise* and *Denoiser*, which can be applied individually or used within the context of QIIME or mother tools (Kuczynski et al., 2011).

Although the approach of whole genome sequencing is becoming popular and wealth of data is being produced, the problem with whole genome sequencing is the difficulty of analyzing the huge amount of data generated and identifying and extracting the unique genetic information. To encounter this problem such that a valuable information can be extracted many systems have been proposed, central database such as Bacterial Isolate Genome Sequence Database (BIGSdb) is one of them. Such database can be used to device schemes for taxonomy in a manner that core genes contigs may be used for speciation and auxiliary gene may be used for strains identification and so on. The sequence databases also have the advantage that dependence on the maintenance of type cultures may be reduced as the sequences in the central databases may suffice for that requirement. The rapidly evolving discipline of Bioinformatics is also likely to provide the solution to analyze sequencing data and building interactive databases where data can be stored and accessed.

Single cell sequencing

1. Single cell sequencing analyses the sequence information from individual cells at optimized next-generation sequencing (NGS) platform, therefore providing a higher resolution of cellular differences and a better understanding of the function of an individual cell in the context of its microenvironment. It typically involves isolating cells and subsequent amplification of the single copy of the genome by multiple displacement amplification (MDA) which makes up to billions of copies of the DNA thereby allowing whole genome sequencing. It has revolutionized microbial genomics by assembling reference genomes for diverse, uncultivable bacterial species because great majority of bacterial species cannot be readily cultivated. Until recently, more than half of the 61 currently known phyla in Bacterial domains were identified only from their 16S rRNA gene sequence. The NGS is likely to fill remaining candidate phyla within the next few years. Micromanipulation under the microscope with the help of glass capillary to target specific bacteria by labeling with fluorescent DNA probes as well as Laser capture microdissection (LCM) are being used for single cell isolation. Several applications of single cell genomics are emerging such as targeted and untargeted pathogen-genome recovery, tracking pathogen persistence and transmission and the identification of novel bacteria that have pathogenic potential from the human microbiome (Gawad et al., 2016).

Metagenomics

Metagenomics or environmental and community genomics is the genomic analysis of unculturable microorganisms by directly extracting their DNA and comparing with established ribosomal sequences of

microorganisms so that the microbial population in the unseen world could be known. Uncultivable microorganisms are inevitably present in vast majority of all kinds of environment on earth. Although ribotyping revolutionized the microbial identification, it provided only a phylogenetic description of microbes, yielding little insight into the genetics, physiology, and biochemistry of the individual microbes. Metagenomics provides insights into the study of physiology and ecology of environmental microorganisms. Several novel genes and gene products have been discovered through metagenomics viz; new members of families of known proteins such as $Na^+(Li^+)/H^+$ antiporter, RecA, DNA polymerase, bacteriorhodopsin, novel antimicrobials and antibiotic resistance determinants. Assembly of metagenomes has provided functional insight of microbial communities such energy and nutrient cycling, genome structure and function and population genetics and microheterogeneity. Lateral gene transfer among microbial communities has resulted in analyses of genomic heterogeneity at much greater scale than has been viewed in the petri dish. The metagenomic sequence information is likely to facilitate culturing of as-yet uncultivable microorganisms (Arnold et al., 2016).

Taxonomy is a science of formal nomenclature of any biological agent that has to be universally accepted as universality is one of the most important needs. Although many newer methods based on whole genome sequencing have evolved with the passage of time, but universal rules for extrapolating the data generated have not yet been formulated. Until any rules for systematics are formed we consider ribotyping for taxonomy as the gold standard.

Future perspectives

Microbial taxonomy is periodically reviewed and subjected to modifications based on updating of information on newer isolates of all species across the world and is ever-developing due to new data-generating techniques. The present system of identification and classification, predominantly based on ribotyping, though widely accepted, appears to be somewhat skewed in favor of analyzing a single trait or mono-locus housekeeping gene. Moreover, the present taxonomic system merely provides information on characterization, identification, and probable model of evolutionary relationship among its constituents. The past two decades have witnessed great advancements in the assessment of overall homology and differences among microorganisms. Advancements have been seen in the areas of determination of molecular structure (phenotypic) details, complete genome sequencing, (faster) analytical software tools that facilitate incorporation of multiple characteristics and housekeeping genes, and determination of both phenotypic and genotypic characteristics. Also, there is great potential for automation based on microarrays or "biochip" techniques, which may have a far-reaching impact on the identification and classification of microbes. The taxonomic system, if based on holistic properties, may also provide practical and useful information on the medical, epidemiological, and environmental significance of microorganisms.

Ethical issues

So far, the technology for the classification of microorganisms has not raised any serious ethical concerns. However, it is possible that with further expansion of this technology, there may be ethical issues that develop.

Translational significance

Ribotyping of microbial isolates has helped in the taxonomic classification of organisms and building of phylogenies with a greater degree of authenticity, as discussed earlier (Grimont and Grimont, 1986). Its reproducibility and robustness have found utility in epidemiological tracking of infections, determination of clonality of strains, enabling of effective planning for prevention and disease management, and also tracking of sources of contamination in the food industry. Automated robots for ribotyping have been developed, reducing hands-on time for typing microorganisms. This technique has also been useful in studying the geographical distributions of microbiomes and in understanding the physiology of constituent uncultivable organisms.

Advancements in the biological sciences have led to the unraveling of many new phenomenon of the biosphere. Current scientific approaches have been directed toward systems biology (i.e., the study of complex interactions within biological systems). An important contribution of systems biology has been revelations about co-evolution and the close interaction between an animal and its microbiome. The role of the microbiome has been proven to be in host defense mechanisms, metabolism, reproduction, and all body functions. This intricate association between the vertebrate host and its microbiome led the National Institutes of Health to launch the Human Microbiome Project in 2008. Its goal is the identification and characterization of microorganisms associated with healthy and diseased individuals (http://commonfund.nih.gov/hmp/). Ribotyping, an important tool for giving

signatures to microbes, has been one of the useful methods in deciphering this microbiome.

Another area in which ribotyping has proved to be useful is in studies related to nutrigenomics, where deciphering nutritional health effects requires an understanding of the composition of gut microflora, effects of changing diet on changes in gut microflora, and also corroboration of these changes with the health of the host.

Clinical significance

The conventional and modern automated systems based on genomic (ribotyping) and phenotypic characters (biochemical, fatty acid composition or protein based) of microbial population have tremendous contributions in detection, characterization and identification of pathogenic microorganisms in clinical samples, that is, blood, urine, pus, secretions, discharges, tissues samples, with great degree of precision. The presence of organisms can be detected qualitatively as well as quantitatively directly from the clinical samples, without the need to culture them using DNA based method of hybridization with labeled probe or amplification by polymerase chain reaction. The microbial populations can even be characterized from complex and mixed populations in metagenomic studies. The methods based on phenotypic characterization however, require growing culture in pure form. The identification of causative organism of any disease helps in formulation of appropriate treatment and in selecting proper antibiotic regimen. The microbial strain typing, that is, discrimination of variations in different isolates of same species using PCR ribotyping and other methods, allows epidemiological studies in detection of the origin, pattern and mode of spread of the infection and to suggest control measures and strategies for designing of appropriate vaccine. The accumulation of data on origin and spread of infection in different geographical settings also helps in surveillance of antibiotic resistance, acquisition of newer virulence properties by microorganisms. Emergence of new microbial strains/species can be detected, characterized and defined to their taxonomic status.

World Wide Web resources

http://www.ncbi.nlm.nih.gov: The web site for the National Center for Biotechnology Information provides access to molecular biology, biomedical, and genomic information.

http://www.microbial-ecology.net/probebase: probeBase is an online resource for rRNA-targeted oligonucleotide probes.

http://www.psb.ugent.be/rRNA/: The European rRNA Database provides access to all complete, or nearly complete, ribosomal RNA sequences from both the small ribosomal subunit (SSU) and large ribosomal subunit (LSU).

http://ribosome.fandm.edu. This is a 16S and 23S ribosomal RNA mutation database that provides information on RNA of numerous organisms and is currently under revision to include mutations in ribosomal proteins and ribosomal factors of more organisms.

http://greengenes.lbl.gov: An online, full-length (ssu) rRNA gene database. The Greengenes web application provides access to current and comprehensive 16S rRNA gene sequence alignments for browsing, blasting, probing, and downloading. The data and tools presented by Greengenes can assist researchers in choosing phylogenetically specific probes, interpreting microarray results, and aligning/annotating novel sequences.

http://ncbi.nlm.nih.gov/taxonomy: This site provides classification and nomenclature for all the organisms in public sequence databases, representing about 10% of described species on the planet.

http://commonfund.nih.gov/hmp: This site elaborates the objectives of the Human Microbiome Project (HMP), and lists other sources for genomes of many reference strains in gene banks and strain repositories.

http://pubmlst.org: Public databases for molecular typing and microbial genome diversity.

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Abbreviations

AFLP	Amplified fragment length polymorphism
ARDRA	Amplified ribosomal DNA (rDNA) restriction analysis
CARD	Catalyzed reported deposition
cDNA	Complementary deoxyribonucleic acid
CLSM	Confocal laser scanning microscopy
DAPI	4′,6-Diamidino-2-phenylindol
DOPE	Double-labeled Oligonucleotide ProbE
ELISA	Enzyme-linked immunosorbent assay
ePCR	Emulsion polymerase chain reaction
FISH	Fluorescent in situ hybridization
FTIR	Fourier transform infrared spectroscopy
G + C	Guanine and cytosine ratio
HRP	Horseradish peroxidase
IRMS	Isotope ratio mass spectrometry
ISR	Intergenic spacer region
RFLP	Restriction fragment length polymorphism
MAR	Microautoradiography
MALDI-TOF MS	Matrix-assisted laser desorption/ionization-time of flight mass spectrometry
MLST	Multilocus sequence typing
NGS	Next-generation sequencing
PCR	Polymerase chain reaction
PNA	Peptide nucleic acid
RAPD	Random amplification of polymorphic DNA
rDNA	Ribosomal deoxyribonucleic acid
RING	Recognition of individual gene
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SIP	Stable isotope probing
T_m	Melting temperature
TRITC	Tetramethylrhodamine-5-(and-6)-isothiocyanate
tRNA	Transfer ribonucleic acid

Long answer questions

1. Define taxonomy. How are taxonomic principles different in microorganisms and eukaryotes?
2. Give an account of different typing methods used for bacteria.
3. What is ribotyping? What are the reasons for this technique to be widely used for taxonomic classification of bacteria?
4. Describe the principle and methodology of PCR ribotyping. In what way does it improve on conventional ribotyping?
5. What are different approaches of ribotyping? Mention their relative advantages and disadvantages.

Short answer questions

1. How does the concept of species in bacteria differ from that of eukaryotes?
2. What is the basis of ribotype polymorphisms?

3. PCR ribotyping is based on intergenic space heterogeneity. What are the applications of PCR ribotyping?
4. What would you infer from finding a single band during PCR ribotyping?
5. What are the reasons for conventional ribotyping becoming unpopular?
6. (a) How can the intensity of the signals in (F)ISH be enhanced? (b) How can a polynucleotide probe be enhanced to have better penetration and higher binding affinity?

Answers to short answer questions

1. Bacteria multiply asexually by binary fission, and thus limits to gene transfer up to the species level are difficult to ascertain.
2. Differences in the length and number of bands using the whole ribosomal operon as a hybridization probe.
3. PCR ribotyping is mainly used in the clinical microbiology laboratory for epidemiological studies and to discriminate bacterial organisms up to the species and strain level.
4. A single band in PCR ribotyping indicates a single ribosomal operon in the test organism.
5. It is a time-consuming and labor-intensive process of Southern transfer and hybridization with a labeled radioactive probe.
6. (a) By improving the penetration of probes through the cell wall via lysozyme treatment. (b) By using a double-labeled probe or more than one probe.

Yes/no type questions

Do you agree with the following statements?

1. The molecular approach in microbial systematics began with the observation of Carl Woese regarding ribosomal RNA of different microbial species.
2. The target of the microbial taxonomy is limited up to the level of species identification?
3. The biochemical properties of microorganisms are not the defining criteria for their taxonomic characterization.
4. The entire ribosomal operon is highly conserved and thus used for microbial species identification.
5. Conventional ribotyping is still widely used for bacterial identification.
6. Sequencing of ribosomal operon is more logical than sequencing of whole genome for taxonomic point of view.
7. Intergenic spaces between 16S and 23S ribosomal genes have little significance in bacterial identification.
8. MALDI-TOF spectroscopy is the molecular technique used for microbial identification based on proteomic study of organisms having great reliability and reproducibility.
9. The microbial identification systems based on genotypic properties are the only automated, reliable and practically used methods.
10. The in situ hybridization with labeled probe (FISH) is a very useful extension of ribotyping having many advantages over the methods based on amplification of genomic components.
11. Ribotyping does not require cultivation of the test organism while other methods based on phenotypic characterization essentially require pure cultures of organisms.

Answers to yes/no type questions

1. Yes. Carl Woese recognized two groups in prokaryotes as bacteria and archaea and eubacteria on the basis of the differences in their rRNA and thereafter began the era of ribotyping.
2. No. The microbial isolates are characterized even beyond the species level, that is, to discriminate the strains within a given species that has several applications.
3. Yes. At present, the accepted defining criteria for microbial taxonomy are the rRNA sequences.
4. No. The ribosomal operon consists of some highly conserved, less conserved variable, and hypervariable regions that are differently used for species defining and for strain typing.
5. No. Due to its procedural complexities, it has been almost completely replaced by PCR-based methods of ribotyping.
6. Yes. At present, the accepted basis of taxonomy are the ribosomal properties. Whole genome sequencing yields enormous data that are difficult to be analyzed by existing bioinformatics tools.
7. No. The intergenic spaces between 16S and 23S rRNA in terms of their size and sequence variation are used as the basis for PCR ribotyping largely for strain differentiation.
8. Yes. Since the technique is based on total protein composition and can be correlated to ribotypes with the precision to the extent of 98% it is fairly accurate.

9. No. There are many automated and reliable systems approved by international agencies that are based on phenotypic characters of microorganisms such as MALDI-TOF, FAME GS.
10. Yes. FISH techniques allow simultaneous detection of different microbial cells in their natural habitat even quantitatively in a cost-effective way.
11. Yes. Ribotyping requires separation of DNA that can be directly done from samples, cultures, etc., and can be used from mixed population of organisms. All other methods require pure cultures of organisms in growing state.

CHAPTER

20

Next generation sequencing and its applications

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Summary

Next generation sequencing (NGS) has revolutionized nearly every area of biotechnology and has become a core technology in the area of Genomics. With its unprecedented throughput, scalability, and speed of data generation, it enables researchers and clinicians to study biological systems at a level and resolution never before possible. It has been applied to various aspects of biological science including animal, human, and plant biotechnology. The enormous information produced by NGS assists in understanding genomic variations, disease mechanisms, and resistance helping development of better diagnostics, therapies, and better breeds.

What you can expect to know

The chapter describes the history and fundamentals of next generation sequencing (NGS) technologies. After reading this chapter, one should be able to distinguish between basic concepts of Sanger sequencing and NGS. One should know the available sequencing platforms, understand the advancements in sequencing technologies, chemistries, their advantages and limitations and novel applications, which were facilitated by NGS approaches. One may also expect to know how NGS technologies work and what their translational significance is.

Introduction

Before the Next Generation Sequencing (NGS) arrived, Sanger sequencing by dideoxy chain termination method had been the central approach for more than 30 years to determine DNA sequence and was considered as gold standard for discussing genomic variations. During the recent past decade, research on genomics has been inclined toward DNA sequencing as a primary molecular research tool as genome-wide sequencing is now facilitated with high throughput NGS. Virtually every area of biotechnology has been benefited by the advent of high throughput sequencing. The NGS platforms with different sequencing chemistries and instrumentation produce sequencing data several times higher than traditional ways of sequencing in terms of number of base pairs sequenced in a massively parallel way and hence sequencing of a complete genome with desired depth is now a matter of days or weeks in contrast to Sanger sequencing. Besides the massive increase in data output, NGS has transformed the way scientist think about genetic information. Massively parallel sequencing has revolutionized the ways to identify genomic variations, disease-associated markers, and facilitated molecular evolutionary analysis in animals and plants.

History of DNA sequencing

DNA sequencing was started with the work of Frederick Sanger at MRC Centre, the University of Cambridge, England, in 1972. Sanger developed and published a method for "DNA sequencing with chain-terminating inhibitors" in 1977 (Sanger et al., 1977a) with the use of chain-terminating dideoxynucleotide analogs that caused base-specific termination of primed DNA synthesis. The first full DNA genome to be sequenced was that of bacteriophage φX174 in 1977 (Sanger et al., 1977b). Simultaneously Maxam and Gilbert (1977) published "DNA sequencing by chemical degradation" in which terminally labeled DNA fragments were subjected to base-specific chemical cleavage and then the reaction

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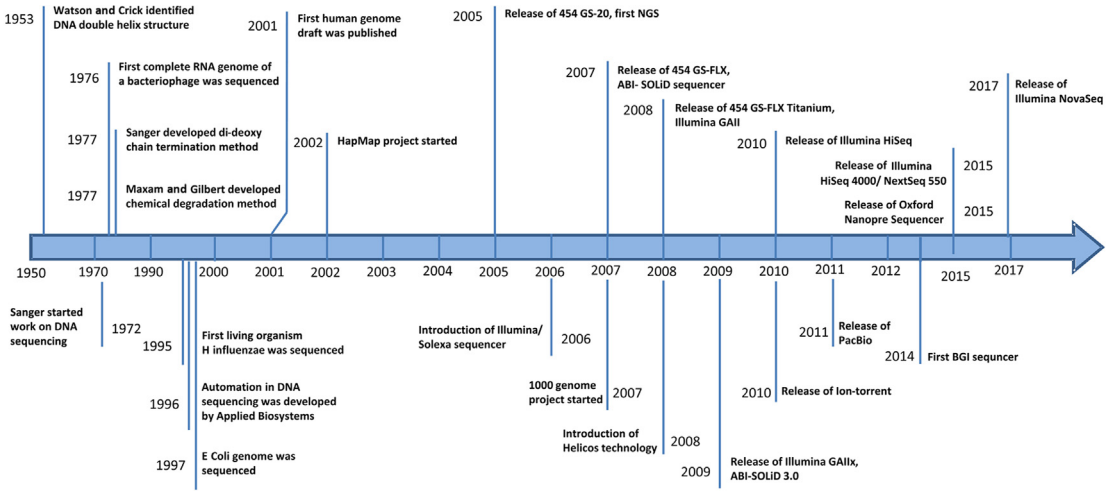

FIGURE 20.1 Timeline of introduction of DNA sequencing technologies and platforms.

products were separated by gel electrophoresis. But the method did not get much popularity due to its technical complexity and use of hazardous chemicals. The Sanger method, on the other hand, became more popular being more efficient and using fewer toxic chemicals. A number of sequencing trials then initiated, with the help of Sanger sequencing, including sequencing of Mycoplasma, *Escherichia coli*, *Caenorhabditis elegans*, and *Saccharomyces cerevisiae*. The Institute of Genomic Research (TIGR) was first to publish the full genome sequence of a free living bacterium *Haemophilus influenza* using shotgun approach (by random fragmentation without the need of mapping) in 1995. In 2001 one of the greatest discoveries of biological sciences, the sequence of human genome was published by two independent groups led by Craig Venter of Celera Genomics, who used shotgun sequencing approach and Francis Collins of National Human Genome Research Institute (NIH), who used bacterial artificial chromosome (BAC) mapping based method to sequence the human genome.

The Sanger sequencing has been used to sequence the DNA region in question and limited to few genes at a time. The complexity of emerging diseases and their association with genomic changes needed a much wider picture of the genome. The demand of sequencing a large number of human individuals and other organisms and limitation of Sanger sequencing to produce large amount of data very efficiently and rapidly drove the development of high-throughput NGS that can parallelize the sequencing process, producing thousands or millions of sequences at once.

The timeline where different NGS technologies and their platforms introduced is shown in Fig. 20.1.

Generation of sequencing technologies

The sequencing technologies have not observed rapid changes for the first 30 years in its development. It is only in the last decade, when sequencing changed with technologies, which can be defined as change of sequencing generations. Briefly "generation" refers to the chemistry and technology used by the sequencing process. The first-generation sequencing was obviously the Sanger and Maxam–Gilbert sequencing, which were able to sequence few hundred base pairs at a time, used for individual gene sequencing. Sequencing 3 billion base pairs of the human genome would take a very long time with Sanger sequencing, as it needs to sequence about 6 million fragments of DNA with 500 bp length.

After being served with Sanger sequencing for over 30 years, the scientific research was introduced to NGS technologies, which is also termed as second-generation sequencing. The major advancement from first generation was that the second-generation sequencers have been able to parallelize the sequencing reaction in a massive manner, thus generating huge amount of data very rapidly at a modest cost, thus revolutionized the field of genomics. It included Roche 454, Illumina and ABI-SOLiD technologies.

The term third-generation sequencing or next–NGS is given to technologies that interrogate single molecules of DNA, without amplifying them through PCR, thereby overcoming issues related to the biases introduced by PCR amplification and dephasing. However the third-generation sequencers were developed with a vision of making sequencing cheaper than second-

generation sequencing. Sequencers and sequencing technologies have been continuously upgraded to produce more data in less cost with the possibilities of wider range of applications. Companies are developing different models to cope up with cost per mega base (MB) sequenced, hence the demarcation of third-generation sequencers and beyond is difficult. Third-generation sequencers include Ion-torrent, PacBio, Helicos, etc. Complete Genomics, Oxford Nanopore, Plonator, MGI use different types of technologies and chemistries, not defined by third generation, and can be placed under other newer sequencing technologies.

Principle of Sanger sequencing versus NGS

Sanger sequencing works on the principle of chain termination, whereby the growing DNA strand is terminated by the incorporation of dideoxy nucleotides (ddNTPs), A, T, G and C in different reactions. The fragments of different lengths of the amplicon are then run on gel in different lanes to determine sequence. In newer dye terminator sequencing, the ddNTPs are labeled with fluorescent dyes to make the fragments readable though laser light source at unique wavelengths in capillary gels rather than on traditional slab gels. Sequencers based on Sanger sequencing produce a read length (the length of DNA fragment that can be sequenced at a time) of 800−1000 bp. Because only one read can be sequenced in one capillary of the sequencer at a time, the total output of the run is equal to the read length. However sequencers with multiple capillaries enable us to sequence multiple samples at a time, for example, 8, 16, 48, or 96.

On the other hand, next-generation sequencers work on the principle of sequencing millions of DNA fragments simultaneously in a massively parallel mode and produce sequence data in megabases, gigabases, and now terrabases. The whole genome or transcriptome of an organism is fragmented into millions of small pieces and sequenced independently in parallel. NGS technologies lowered down the cost and effort of DNA sequencing by producing huge amount of data in massively parallel mode. It can be compared by looking at the cost per MB of DNA sequence generated in September 2001 as >5000 USD with the same in October 2015 as close to 0.014 US Dollars. The first human genome, which was published in 2001, was a 13-year effort with sanger technology as a multinational multi-centric project. By comparison the full genome of James Watson was sequenced over a 4-month period in 2008 with the help of NGS at the cost of less than 1 million US Dollar, and the estimated cost of sequencing a full human draft genome in 2015 is around 1200 to 1300 US Dollars.

NGS technologies: Initial phase

Next Generation Sequencing employs massively parallel sequencing of millions of DNA fragments simultaneously. This can be achieved by different technologies in different NGS platforms. Depending on the technology used, they differ from each other in terms of read length, data produced, and data quality and therefore, bioinformatics required to process and analyze that data. In this section we will discuss important NGS technologies, their working principle, key features, and output data generated. A basic scheme of NGS experiment is illustrated in Fig. 20.2. The next generation technologies can be divided into those that were introduced in initial phase but out of much use or obsolete now due to inability to improve further (initial phase) and those that improved and in-use in today's NGS world (recent phase).

Pyrosequencing technology

Roche-454 GS FLX- Pyrosequencing was among the first NGS technology. But due to its inability over the time to increase the amount of data produced per run, increase read-length after a certain extent, and decrease the running cost, it has been phased out and no longer supported now. In 1993, Nyren and his group published a novel sequencing method based on chemiluminescent detection of pyrophosphate released during polymerase-mediated deoxynucleoside triphosphate (dNTP) incorporation, which was later commercialized by 454 Life Sciences with the technical refinement from Ronaghi et al. (1996) and the use of emulsion-based PCR. Later in 2007 Roche acquired 454. In pyrosequencing DNA to be sequenced is fragmented and subjected to its complimentary strand synthesis by DNA polymerase. As the polymerase incorporates a nucleotide in the growing chain, a pyrophosphate molecule is released. This pyrophosphate, through a series of enzymatic reactions, is converted into ATP. The ATP is used to enzymatically convert luciferin into oxyluciferin, which emits fluorescence recorded by the camera. By detecting this fluorescence the incorporation of a nucleotide is confirmed. The identity of the incorporated nucleotide is known, as four dNTPs (dATP, dTTP, dCTP, and dGTP) are introduced in the reaction separately in predefined cycles.

454 Sequencing used a massively parallel pyrosequencing system capable of sequencing upto 1000 bp of DNA in a 23-hour run on its newer Genome Sequencer FLX plus instrument. The technology worked by fragmenting the DNA into approximately 800−1000 bp in length called nebulization, ligating adaptors to DNA fragments making library and attaching the library to small DNA-capture beads. The beads were compartmentalized into

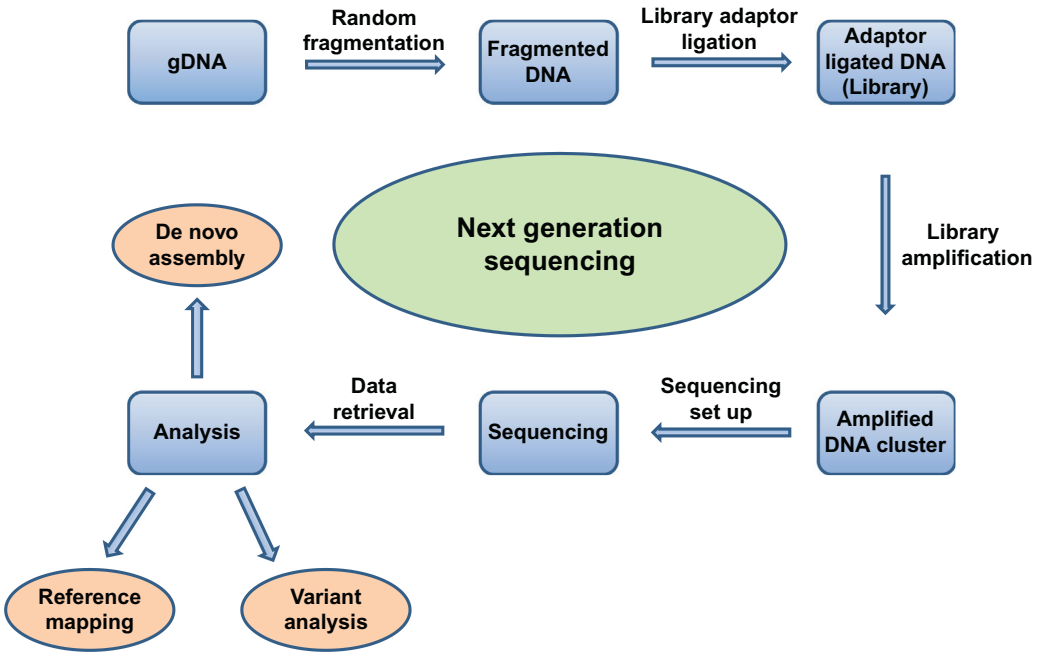

FIGURE 20.2 Basic scheme of a next generation sequencing experiment.

water-in-oil emulsion microvesicles, often called microreactors, where clonal multiplication of single DNA molecules bound to the beads occurred during emulsion PCR. After amplification, the emulsion was disrupted, and the beads containing clonally amplified template DNA were enriched. Each amplified DNA-bound bead was placed in tiny well on a PicoTiterPlate consisting of around 3.4 million wells. A mix of enzymes such as DNA polymerase, ATP sulfurylase, and luciferase were also packed into the well. The PicoTiterPlate was then placed into the sequencer machine for sequencing. The GS FLX platform could produce data of approximately 450 MB with 400–600 bp read length. The newer platform GS FLX plus was, however, able to generate approximately 700 MB data with a read length of 700–1000 bp.

Advantages: The advantage of 454 technology was within its ability to sequence reads in the read length of 700–1000 bp. The longer read length is advantageous in terms of downstream bioinformatics resulting in sequence assembly with longer contigs, higher N50 length, and less gaps specially in de novo sequencing projects. Longer paired-end reads produced by 454 platform also facilitated construction of better scaffolds.

Limitations: The 454 technology had certain limitations. The primary one was the difficulty to sequence homopolymer repeats due to simultaneous incorporation of same nucleotide producing light that cannot be discriminated after a certain length (>6 bp) with high accuracy (Mardis, 2008). Another disadvantage of this technology was the generation of relatively low bases or run, around 700 MB, as compared with other NGS technologies. This results in making it relatively expensive technology and not of priority, if resequencing is desired with a high $\times$ depth. This was one of the reason behind its discontinuation.

Sequencing by ligation technology

ABI SOLiD- The sequencing by ligation technology was marketed by Applied Biosystems, USA. This technology has also become obsolete due to its inability to produce large amount of data and being costlier than others. The name SOLiD stands for Small Oligonucleotide Ligation and Detection System. This technology was developed by George Church in 2005, which was further improved and distributed by Applied Biosystems in 2007 (Voelkerding et al., 2009). The principle of this sequencing relied on the ability of DNA ligase in detecting and incorporating bases in a very specific manner. In sequencing by ligation, DNA fragments attached to beads are clonally amplified by emulsion PCR. After PCR, specific primers hybridize to the adapter sequence of the amplified templates on the beads, which provides a free 5′ phosphate group for ligation to the fluorescently labeled probes, called interrogation probes, instead of providing a 3′ hydroxyl group as in normal polymerase-mediated extension. The interrogation probe is 8 bp in length, where first two bases are specific and rest of the six bases are degenerate. A set of four fluorescently labeled interrogation probes, consists of one of the 16 possible 2-base combinations at

the end (e.g., TT, GT, TC, CG, etc.) compete for ligation to the sequencing primer. Upon ligation, fluorescence is captured, which is corresponding to the probe ligated. For the second cycle, the fluorescence of the attached probe is removed and a 5′ phosphate group is regenerated. Multiple cycles of ligation, detection, and cleavage are performed with the number of cycles determining the eventual read length. Following a series of ligation cycles (usually seven), the extension product is removed and the template is reset with a primer complementary to the n-1 position for a second round of ligation cycles. This process is repeated each time with a new primer with a successive offset (n-1, n-2, n-3, etc.). Thus the sequencing was divided into library preparation, emulsion PCR, bead deposition, sequencing, and primer reset. A 6–7 days long instrument run in 5500 system claimed to generate sequence data at approximately 10–15 GB/day (total throughput 120–240 GB, 100 GB in case of SOLiD 4 system) with a read length of 75 bases (for mate-paired: 2 × 60 bp, for paired-end: 75 bp × 35 bp) with a sequence consensus accuracy of 99.99% (Voelkerding et al., 2009).

The SOLiD platforms have been discontinued with the development of other high throughput and cost-effective platforms.

Advantages: The advantage of this technology was generation of sequencing data of comparatively higher accuracy than other sequencing methods. One of the reasons behind high accuracy was the sequencing with successive offset primer less by one base pair, so that each nucleotide of the template was sequenced twice, therefore in order to miscall an SNP, two adjacent colors must be miscalled, which was usually not frequent.

Limitations: Among the limitations of this technology were less data output than Illumina and less read length requiring close genome sequencing for mapping. Even the time taken for a whole run was about 6–7 day to complete specially for bigger genomes.

True single molecule sequencing

Helicos Biosciences: Helicos sequencing technology was the first to introduce true single molecule sequencing (tSMS). However it files bankruptcy in 2012 due to low demand and has been revamped by SeqLL (http://omicsomics.blogspot.in/2016/10/seqll-helicos-van-winkle.html). It operates on the principle of single molecule sequencing. By directly sequencing single molecules of DNA or RNA on HeliScope platform, this technology termed as Helicos' tSMS™ and Direct RNA Sequencing (DRS™), increases the sequencing speed at a low cost. tSMS and DRS enables the simultaneous sequencing of large numbers of strands of single DNA or RNA molecules in which labeled bases are sequentially added to the nucleic acid templates captured on a flow cell.

Billions of single molecules of template DNA are captured on a specific proprietary surface. To this polymerase and one of the fluorescently labeled nucleotides (C, G, A, or T) is added, which is incorporated into growing complementary strands on all the templates in a sequence-specific manner. After a wash step, the incorporated nucleotides are imaged and their positions recorded. In the next steep the fluorescent group is removed in a highly efficient cleavage process, leaving behind the incorporated nucleotide. This process continues through each of the other three bases in multiple cycles providing a 25–55 bp read (average 35 bp) from each of those individual templates. From 600 million to 1 billion DNA strands, a total of 21–35 GB of sequence data are generated per run with 99.995% accuracy.

Advantages: Relying on single molecule sequencing principle and generating big data with low cost, makes helicos advantageous over other amplified sequencing techniques. Single molecules technology enables sequencing of RNA molecules from single cells directly without reverse transcription or amplification without cDNA synthesis and PCR biases or amplification-induced errors.

Limitations: Generation of small read length could be a major limitation of Helicos technology when compared with other NGS technologies. An important drawback to single molecule sequencing is that sometimes correct bases would not be seen due to the problem of "dark bases". So Helicos had a high single nucleotide deletion rate (http://omicsomics.blogspot.in/2016/10/seqll-helicos-van-winkle.html). This can be overcome with repetitive sequencing, but increases the cost per base for a given accuracy rate.

NGS technologies: recent phase

Reversible terminator technology

Illumina: This is one of the most prevalent technology of present time. The technology of Illumina sequencing was started with the concept of British Scientists Shankar Balasubramanian and David Klenerman, which involved sequencing of single DNA molecules attached to microspheres. They founded Solexa in 1998 keeping single molecule sequencing in mind, but because of certain limitations, they had to shift to sequencing of clonally amplified DNA, which was commercialized in 2006 as Solexa Genome Analyzer (Voelkerding et al., 2009).

The Illumina Genome Analyzers use flow cells consisting of optically transparent slides with multiple individual lanes. Small oligonucleotide anchors are immobilized on the surfaces of these lanes. The template DNA to be sequenced is fragmented, phosphorylated at 5′ end and adenylated to add a single A at 3′ end. Oligonucleotide adapters are ligated to the DNA

fragment and the ligation is facilitated by the presence of a single T overhang on the adapters. The adapter ligated oligonucleotides are complementary to the flow-cell anchors and hence attach to the anchors. These DNA templates attached to the anchors are used to generate clusters of the same DNA fragment by amplification. A DNA fragment bends and hybridizes with its distal end to an adjacent anchor complimentary to the distal end. On denaturation both the strand separate and again bend and hybridize with their distal ends to adjacent anchors complimentary to their distal ends. After multiple amplification cycles a single DNA template makes a clonally amplified cluster with thousands of clonal molecules. Millions of clusters of different template molecules can be generated per flow cell.

For sequencing, the technology uses four fluorescently labeled nucleotides to sequence the tens of millions of clusters on the flow cell surface in parallel. In each growing chain, a single labeled deoxynucleoside triphosphate (dNTP) is added in each cycle. Due to the incorporation of the labeled nucleotide, DNA polymerization terminates and the fluorescent dye is imaged to identify the incorporation. Then the label is enzymatically cleaved to allow incorporation of the next nucleotide (www.illumina.com).

Illumina has developed a wide range of platforms depending on the amount of data generated per run and thus offers instruments as per the required applications. According to product information available from Illumina, for most of the clinical applications, MiniSeq and MiSeq platforms are used, which produced 7.5 and 15 GB data respectively in 24 to 55 h of operation, producing 25 million reads per run. MiSeq is able to produce relatively longer read length of 2×300 bp, while nearly all other platforms can produce 2×150 bp read lengths. Their mid-range platform NextSeq produces maximum data of 120 GB in 30 hours run with 400 million reads. They have recently launched a smallest version of instrument called iSeq100, which produces nearly 1.2 GB data with 4 million reads, suitable for targeted sequencing with small panels. Illumina has also developed their high-end production scale instruments for research in large genome sequencing or population-based sequencing. While their HiSeq4000 instrument, a data of 1500 GB can be generated in 3.5 days with 5 billion reads, the highly parallel suits of HiSeqX instruments (HiSeqX Five and HiSeqX Ten) can produce a maximum data of 1800 GB in 3 days run with 6 billion of reads produced. Illumina has introduced NovaSeq, the highest throughput instrument with 6000 GB data with 20 billion reads produced in 13−44 hours. MiniSeq and MiSeq sequencers are used to sequence smaller regions but in large number of samples (e.g., 96) making them suitable for clinical use.

Advantages: Large data and low cost per base renders the technology a good choice for many sequencing applications where large read length and de novo construction of genome is not required, for example, clinical and population-based resequencing, ChIP sequencing, certain projects of RNA sequencing, etc.

Limitations: The major concern of Illumina technology has been that of dephasing, which means different fragments in a cluster are sequenced with different phases, that is, under- or over-incorporated nucleotides, because of block removal failure or else, result into fragments of varying lengths, which reduces precision in base calling at 3′ ends of the fragments. Dephasing increases with increased read length. It is more common at sequences of invert repeats or GGC (Nakamura et al., 2011). However they claim to have improved the technology to overcome the limitation upto certain extent. Illumina technology produces reads of short-length "micro-reads", hence assembly and downstream bioinformatics could be a challenge especially for certain de novo sequencing. Longer time required per run is also a limitation.

Ion semiconductor sequencing

Ion Torrent: This technology works on the principle of detection of hydrogen ion release during incorporation of new nucleotide in the growing DNA template. In nature when a nucleotide is incorporated into a strand of DNA by a polymerase, a hydrogen ion is released as a byproduct. Ion Torrent, with its Ion Personal Genome Machine (PGM™) sequencer, uses a high-density array of micromachined wells to perform nucleotide incorporation in a massively parallel manner. Each well holds a different DNA template. Beneath the wells is an ion-sensitive layer followed by a proprietary ion sensor. The ion changes the pH of the solution, which is detected by an ion sensor. If there are two identical bases on the DNA strand, the output voltage is doubled, and the chip records two identical bases called without scanning, camera and light. Instead of detecting light as in 454 pyrosequencing, Ion Torrent technology creates a direct connection between the chemical and the digital events. Hydrogen ions are detected on ion semiconductor sequencing chips. These ion Semiconductor Chips are designed and manufactured like any other semiconductor chips used in electronic devices. These are cut in the form of wafers from a silicon boule. The transistors and circuits are then pattern transferred and subsequently etched onto the wafers using photolithography. This process is repeated 20 times or more, creating a multilayer system of circuits.

Ion has come up with a variety of sequencers with small and large data output, which are to be used

according to applications and usage. Ion torrent PGM™ generates a total data output from 30 MB to 2 GB, depending on the type of ion semiconductor sequencing chip used. However in September 2012 Ion Torrent launched their bigger system, the Ion Proton. It uses larger chips with higher densities and thus can be suitable for transcriptome, exome, and bigger gene panels. Although Ion Proton is capable of generating much larger outputs, around 10 GB, it is substantially more expensive. Their newer higher throughput sequencers called Ion S5 and S5XL can generate data output of 2–130 million reads depending upon chip type used around 4 hours run depending on the Chip used. The read length obtained is 200 and 400 bp for IonTorrent and IonProton, while S5 and S5XL also have facility to sequence 600 bp reads.

Advantages: Ion torrent generates read length of around 200–600 bp, which is used to fill gaps in the assembly produced by other technologies. Due to low cost involved Ion platforms have gained recognition in clinical sector. The short run time of this technique also facilitate multiple runs for generation of more data in a given time.

Limitations: Ion platforms lie in between huge data technologies and long read length technologies. Whereas short read technologies are facilitated by huge data generated, Ion need to improve the total data output. The reported higher error rate and premature sequence truncation may pose difficulty for it to be a primary choice where very high quality data are required (PubMed Central ID: PMC4249215).

Single molecule real time sequencing

Pacific Biosciences: The sequencing technology of Pacific Biosciences works on the so-called Single Molecule Real Time (SMRT) technology, which enables the observation of DNA synthesis as it occurs in real time. This is possible with the use of Zero Mode Waveguide (ZMW) specially designed microholes used in the technology. Around 150,000 ZMWs are fabricated per SMRT cell enabling the potential detection of approximately 150,000 single-molecule sequencing reactions in parallel.

A ZMW is a nanohole made in a 100 nm metal film on a glass surface. Due to this small size of the ZMW laser light of wavelength approximately 600 nm cannot pass completely through the ZMW and exponentially decays as it enters into it. Therefore by applying a laser through the glass into the ZMW, only the bottom 30 nm of the ZMW becomes illuminated. A single DNA polymerase molecule is anchored to the bottom glass surface of the ZMW. Nucleotides, each labeled with a different colored fluorophore, are then flooded above the ZMWs. Labeled nucleotides travel down into the ZMW within microseconds, reaching the DNA polymerase, then diffuse back up and exit the hole. As laser light cannot penetrate up throughout the holes, it does not excite fluorescently labeled nucleotides present at upper side of the holes. Thus the labeled nucleotides above the ZMWs are dark. Only when they diffuse through the bottom 30 nm of the ZMW do they fluoresce. Upon correct incorporation of nucleotide into the growing strand, this results in higher signal intensity for incorporated versus unincorporated nucleotides, which is then detected. Thus single nucleotide incorporation can be detected inside the ZMW.

Earlier started with low throughput with its PacBio RS instrument, which generated read lengths of around 1000–3000 bp with an average of 1200 bp, PacBioRSII is able to generate maximum read lengths of 60 KB with half of the reads at 20 KB with the help of 150,000 ZMWs and 5–8 GB of data per SMRT and PacBioRSII can sequence 8 SMRT per day. The newer platform PacBio Sequel produces read numbers several times higher with the help of nearly 1,000,000 ZMWs.

Advantages: Pacific Bioscience sequencing technology, being a single molecule, real-time sequencing technology does not require the PCR amplification steps. This avoids usual amplification bias in the sequenced DNA fragments. It can produce read lengths upto 60 KB. Such a long read length try to overcome short read limitations of repeat handling and incomplete assembly and facilitates mapping and assembly of the sequenced reads in a much better way. The time taken from sample preparation to sequencing results is also less and can take less than 1 day. The technology has an ability to observe and capture kinetic information of the polymerase activity.

Limitations: PacBio technology generates around 500 MB to 5–8 GB data per SMRT cell (www.pacificbiosciences.com), which is very less when compared to the other existing technologies. However due to very less data output, at present, it stands as a struggling technology. However short run time can overcome the problem provided the cost remain limited with repeated runs for a desired depth. It is also complained with random insertion–deletion errors (Mardis, 2011).

Other newer sequencing technologies

Oxford Nanopore and Complete Genomics sequencing can be placed under "Other" sequencing technologies. These technologies use different types of sequencing chemistries, which are not satisfied by usual definitions of second or third generation. However Polonator chemistry resembles and in-fact

was a basis of development of a second-generation sequencing (ABI-SOLiD), its future idea of being modular, cheapest and open system makes it different from others and put it in the category of other/fourth-generation technologies, but it is still in development/initial phase. While complete genomics is so far optimized only for human sequencing, nanopore is growing its areas of applications being largest read technology.

Nanopore sequencing

Oxford nanopore- Nanopore sequencing is a method under development since 1995 for DNA sequencing. Recently in February 2012, Oxford Nanopore Technologies, UK, presented initial data of the technology. A nanopore is simply a small hole of the order of 1 nm internal diameter, which is made up of certain transmembrane cellular proteins. The nanopore sequencing works on the principle of minute changes in electric current across the nanopore, immersed in a conducting fluid with voltage applied, when a moving nucleotide (or DNA strand) passes through it. Every nucleotide on the DNA molecule, while passing through the nanopore, obstructs the nanopore to a different characteristic degree and the amount of change in current is characteristic for each different nucleotide. DNA may be forced to pass through the hole, one base at a time, like thread through the eye of a needle. The change in the current can be directly read and sequence of the passing DNA can be determined by detecting changes in the current generated specific to the base passed. Alternatively specific nanopore can be designed to produce current changes when a specific nucleotide passes through it.

Oxford Nanopore is known to produce ultralong reads. Long reads are considered as the most advantageous feature of SMS shared by PacBio and Oxford Nanopore to distinguish SMS from NGS (or the second-generation sequencing) and Sanger sequencing (the first-generation sequencing; Midha et al., 2019). Long reads overcome the limitations of NGS and drastically improves the quality of genome assembly. The technology has recently been scaled up to routine use in sequencing. Earlier they introduced their MinIon device, which is handy and can be applied in the field outside the laboratory. According to the product information (https://nanoporetech.com/products#comparison), the read length produced by the sequencer is equal to the DNA fragment length and longest reported read length has been >200 KB. This means that it can sequence the entire genome of the virus in one continuous read. This read length is much longer than the fragments when compared with other technologies. The initial system features to generate 30 GB with 7–12 million reads. Other high-end systems include GridIon producing 150 GB data and PromethIon claimed to generate 7–10 TB data expecting to reach 15 TB soon. The handheld MinION is already established for portable DNA sequencing. Oxford Nanopore earlier started developing an even smaller device, SmidgION (https://nanopore-tech.com/products#comparison).

Advantages: The potential advantages of the nanopore system are that it could deliver real-time sequencing of single molecules at low cost and is expected to read very long DNA molecules as a single read. Nanopore technology is expected to provide sequencing at a very low cost per gigabase of sequence. This is a cost of just a few thousand dollars to sequence a human genome to the standard 30-fold coverage. Being a very fast sequencing process it could be a future choice of sequencing for nearly all the applications. Nanopore sequencing is an attractive platform for clinical laboratories to adopt due to its low cost, rapid turnaround time, and user-friendly bioinformatics pipelines (Petersen et al., 2019).

Limitations: Nanopore sequencing has been seen to face the problem of base-calling accuracy compared to other platforms (Petersen et al., 2019). The technology may have concerns of increasing accuracy and throughput at this stage which have to be taken into account for its large-scale commercial use.

Polony-based sequencing technology

Polonator technology- This technology was developed by Dr. Church at Harvard Medical School. The vision behind the development of this technology is to keep costs down, must be affordable for the masses with the high-quality components used to develop a modular, easily upgradable *platform*, develop a system that can be used for a broad variety of applications, and develop an open system so as scientists can help with advances in hardware and software applications.

Polonator sequencing is basically a polony-based sequencing by ligation. DNA is randomly sheared, A-tailed, end repaired, and ligated. DNA molecules of 1 KB are selected, circularized, and amplified. Amplified circularized DNA is restriction digested and amplified in emulsion PCR resulting in 135 bp library molecules. Two flow cells are mounted within the instrument, one undergoes biochemistry while the other is imaged. Each flow cell has 18 individual wells, with a total of over 1 billion streptavidin-coated polystyrene beads. Reaction takes place with DNA on the surface of beads. Depending on library titration and technique, the typical output is 8 to 10 million mappable reads per lane, or about 150 million reads per dual flow cell run. With the

read length of 26 bases the run output is about 4 to 5 Gb. Alternatively library beads can be enriched by removing the unamplified beads increasing the output to 8−10 Gb.

Advantages: The Polonator is supposed to be the least expensive sequencing technology commercially available. All the software features are freely downloadable, which will facilitate its optimization for others to use.

Limitations: Polonator technology generates a read length of only 26 bp (13 + 13 bp paired end). The duration of a run is currently about 4 days. In general the best 92% of the mappable reads have a mean accuracy of above 98%.

DNA nanoball sequencing

Complete genomics/BGI/MGI- There is another sequencing technology developed and commercialized by Complete Genomics, specifically optimized for human resequencing applications. Complete Genomics was founded in 2006 and was acquired by BGI-Shenzhen, the world's largest genomics services company, in March 2013. The technology is based on a proprietary DNA arrays and ligation-based read technology and claimed to provide an accuracy of 99.999% facilitating human sequencing service to scientific community.

It claims to deliver very accurate and cheap human sequencing as it has brought together diverse technologies to create a comprehensive solution for large-scale sequencing of human genomes. There is a combination of technological advancements at each step of the workflow, that is, libraries, arrays, sequencing assay, instruments, and software. They use in-house developed high-throughput sequencing instruments.

Complete genomics technology has two primary components: DNA nanoball or DNB™ arrays and combinatorial probe-anchor ligation, or cPAL™, reads. DNA is packed efficiently on a silicon chip making the patterned DNB arrays. A highly accurate cPAL read technology, which helps to read the DNA fragments using small concentrations of low-cost reagents. It is claimed that this unique combination of proprietary DNB and cPAL technologies is superior in both quality and cost to other commercially available approaches of sequencing a whole human genome at a very low consumables cost and with a consensus error rate. The technology of DNA Nanoball sequencing involves shearing DNA that is to be sequenced into small fragments and circularizing the fragments using adapter sequence. The circular fragments are replicated by rolling circle mechanism resulting in many single-stranded copies of each fragment. The DNA copies concatenate head to tail in a long strand and forms the compacted DNA nanoball. The nanoballs are then adsorbed onto a microarray flow-cell in a highly ordered pattern, allowing a very high density of DNA nanoballs to be sequenced. A number of fluorescent probes are then ligated to the DNA at specific nucleotide locations in the nanoball, starting unchained sequencing reactions. The color of the fluorescence at each interrogated position is recorded to detect the fluorescence and a base call is determined. The maximum read length generated is 150 bp. But as they say, the high level and uniformity of base-call accuracy are achieved. The instruments can generate from 60 GB to 6 TB depending on the instruments.

Advantages: This technology is capable of packing very high density of DNA nanoballs to be sequenced in an orderly manner maximizing the number of reads per flow cell and a non-progressive cPAL, minimizing read error. The Complete Genomics technology gains special attention as it claims to provide unprecedented specificity and sensitivity of the sequencing. The data produced by MGIseq sequencers are comparable with Illumina Hiseq 4000 sequencers (Jeon et al., 2019). It is advantageous for those seeking human genome sequencing for a larger number of samples at a comparable cost.

Limitations: The limitation of Complete Genomics technology lies in two things. First it is specially optimized for human only and not open for other organisms. Second a 35 bp read length is supposed to have few, though not all, limitations of a short read technology such as problems in mapping for highly repetitive DNA. However claiming extraordinary accuracy, this limitation may somewhat be minimized. The use of multiple rounds of PCR can also introduce PCR bias in the sequences.

A comparison of important NGS platforms is summarized in Table 20.1.

Downstream bioinformatics

NGS-based bioinformatics analytics are designed to convert signals to data, data to interpretable information, and information into actionable knowledge. With the help of massively parallel sequencing, huge date is generated in the form of ATGC sequences of DNA. These sequences or reads have different lengths depending on the technology used and samples to be sequenced. These reads needs to be joined or analyzed to generate information with biological significance. The reads are primarily handled in downstream analysis, which can be divided into primary, secondary, and tertiary analyses.

TABLE 20.1 Comparison of Important Next Generation Sequencing platforms.

	Roche 454	Helicos	ABI SOLiD	Illumina	Ion Semiconductor	Pacific Bio	Oxford Nanopore	Complete Genomics / MGI
Sequencing methods	Pyrosequencing	Heliscope – Single molecule	Sequencing by ligation	Reversible Dye Terminators	H^+ Detection	ZMW – Single molecule	Nanopore with DNA transistor	DNA nanoballs
Max read Lengths[a]	700–1000 bp	25–55 (av 35 bp)	75 bp	2x300 bp	400 bp	60 Kb	> 200 kb (Longest determinedRead)	150 bp
Sequencing run time[a]	23 hrs	Less than a day	6–7 days	3–10 days	4 hrs	Less than a day	Not known, can be fast	12–37 hours
Max data generated[a]	700 MB / run	21–35 GB / run	320 GB/run	6 TB / run	15 GB (depends upon chip used)	5–8 GB / SMRT	10 TB (15 TB theoretical)	6 TB /run
Advantages	– Long read length – Small data files	– Big data among SMS	– High quality data	– Huge data	– Low cost – Very fast	– Very long read length – Fast	– Very long read length	Low error / High accuracy
Concerns	– Less data – Homopolymer – Obsolete	– Small reads – Higher raw error rate	– Short reads – Long run time	– Short reads – Dephasing	– Less data – Short read	– Random indel errors – Less data	– Only small device released so far	Short read

[a]*Max read lengths, sequencing time and max data output shown in the table are values among all the models of a technology. Different models are able to produce different combinations of read lengths, sequencing time and data output.*

Primary analysis

Primary analysis converts the raw signals generated by the NGS instruments into nucleotide bases with associated quality scores and demultiplexing, which ultimately resulted in producing short nucleotide sequences called reads. The programs and tools for primary analysis are usually provided by the NGS providers, but it can also be performed on other software tools for improved performance.

Secondary analysis

Secondary analysis generally consists of alignment and variant detection. Depending on the application, secondary analysis can occur at the level of the whole genome, exome, or enriched gene panels. Assembly is the process of using various computer programmes to align multiple sequencing reads that are overlapping with one another to reconstruct the long DNA fragment, may be the whole genome, as DNA sequencing cannot read whole genomes in one attempt, but reads small pieces of different length, depending on the technology used. De novo assembly refers to assembling reads to build a new sequence, where no existing sequence of same or related organism/species is available. More and longer reads allow better sequence overlapping for easy assembly (Fig. 20.5). Shorter sequences are faster to align but they complicate the assembly as shorter reads are more difficult to use with repeats or near identical repeats. A number of free and commercial programs are available that can be used to assemble sequence data depending on the type of platform, read length, quality scores, and abundance or total generated data.

Variant detection involves the comparison of the sequenced reads with the reference genome to determine regions that differ and aim to distinguish genuine genomic variations from errors. Mapping assembling reads against an already existing backbone sequence of same or related organism/species to build a sequence that is similar but not necessarily identical to the backbone sequence. Mapping is generally used to figure out genomic variation in the sequenced DNA compared with the reference sequence such as SNPs, insertions, deletions, and structural variations. Variant detection errors are common in NGS depending on the method and the platform used. The errors can be minimized by increasing sequencing depth to several magnifications. The erroneous variant detection can also be handled by filtering the reads based on the quality score and priority filters as per the application. Higher

depth of the sequencing data is helpful in detection of low-frequency variations.

Challenges in NGS bioinformatics are numerous and this area is a highly dynamic area of research with immense possibilities of improvements. Multiple open sources and commercial softwares exist for variant detection at any single step, each with their own strengths and weaknesses.

Tertiary analysis

Tertiary analysis pertains to annotation of the variants to determine their biological significance and enable functional prioritization and downstream interpretation. Frequency-based annotations are important component of tertiary analysis because variants that are common in the general population are unlikely to have biological relevance. The effect of variation is characterized on the basis of the type of mutation present such as non-sense mutations, deletions, and duplications. In RNA sequence the identification of fusion transcripts and their clinical relevance also lie in this category. Annotations are divided into structure based, prediction based, and evidence based. Tertiary analysis also includes analysis with consideration of sample type and each data set includes its own bias depending on the sample characterization. There are numerous commercial applications developed to facilitate NGS result annotation and data interpretation.

General principles of NGS methods in various applications

NGS can be used in a number of ways depending on the application. However in every type of sequencing the major changes occur in the sample processing and library preparation steps. Once the library is prepared, a particular sequencing platform uses the same chemistry to sequence the fragments. Major types of NGS applications and sequencing methods used are shown in Fig. 20.3 and an interconnected workflow for different protocols is shown in Fig. 20.4.

Whole genome de novo sequencing

Before the first completely sequenced genome of a free living organism, *Hemophilus influenzae* in 1995, DNA sequencing was used to sequence individual genes, clones, DNA fragment, and engineered products. The first draft of human genome was published in 2001 by two different Sanger sequencing approaches simultaneously. It took around 5 years to complete the sequencing and its assembly analysis. Since the arrival of massively parallel NGS, genomic research has been

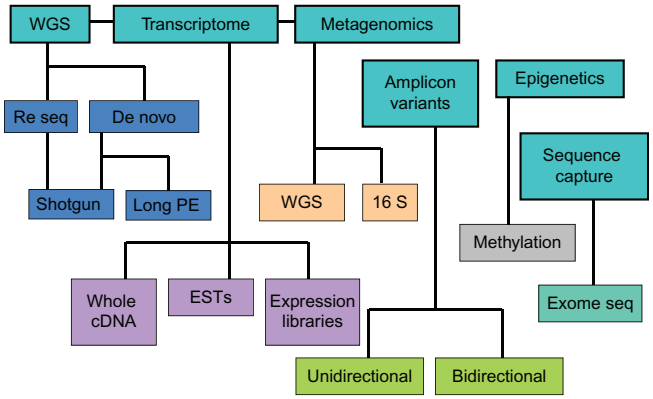

FIGURE 20.3 Schematic diagram of different NGS applications and sequencing methods.

benefited by success of sequencing the new genomes for the first time (no reference genome already available) with much less effort and time. Whole genome sequencing is now a preferred method of choice for genomic studies and a number of organisms, both eukaryotic and prokaryotic, have been sequenced at whole genome level.

Sequence assembly refers to aligning and merging fragments of a much longer DNA sequence in order to reconstruct the original sequence. Whole genome de novo sequencing is basically sequencing fragments of DNA and aligning sequence reads or assemble them to make a full-length genome without referring to any previous information of available sequence for the same species. De novo sequencing is a challenging task and needs more sequence data and relatively longer read lengths, so that reads can be aligned faster and a consensus sequence can easily be made. While with shorter read lengths, an assembly is difficult to make due to the presence of non-matching ends and repetitive sequences, longer reads have advantage to handle repetitive sequences more efficiently. However even long reads cannot construct a full genome without gaps. Initial assembly of reads in the form of contigs can be further extended with the addition of paired-end read data (Fig. 20.5). De novo sequencing assemblies face problems of handling terabytes of sequencing data which are a computer intensive process and need processing on computing clusters.

For de novo sequencing, the starting material is genomic DNA. DNA is fragmented, generally with a size in the range of read length capability of the sequencing platform. Platform-specific sequencing motifs/adaptors are then attached to the fragments, purified, and quantified. DNA fragments with appropriate adaptors are collectively termed as library, the quality of which is one of the key determining factors for the success of sequencing. The library fragments are separated individually, amplified to produce several

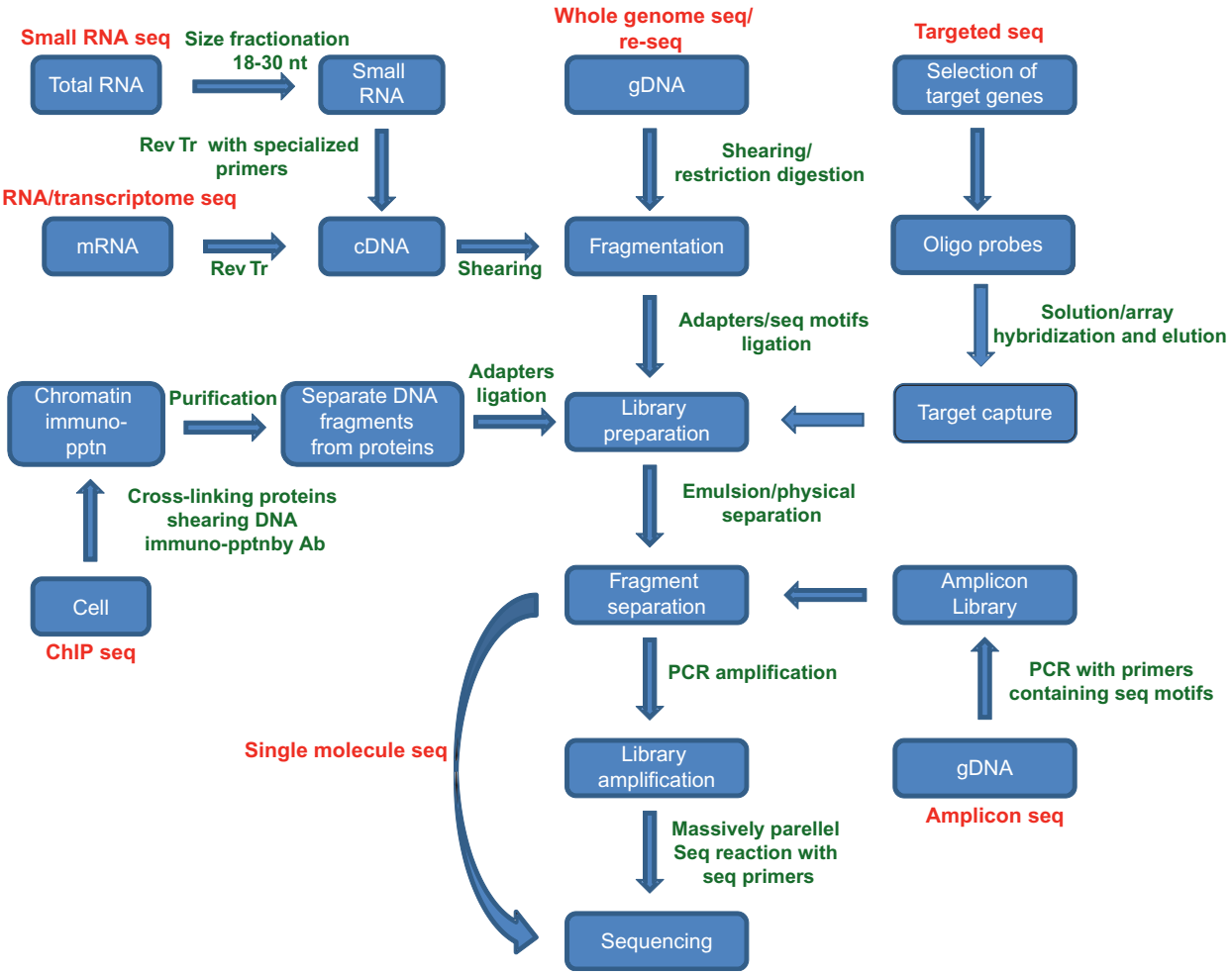

FIGURE 20.4 Flow chart of different protocols used for different sequencing applications and their interconnection.

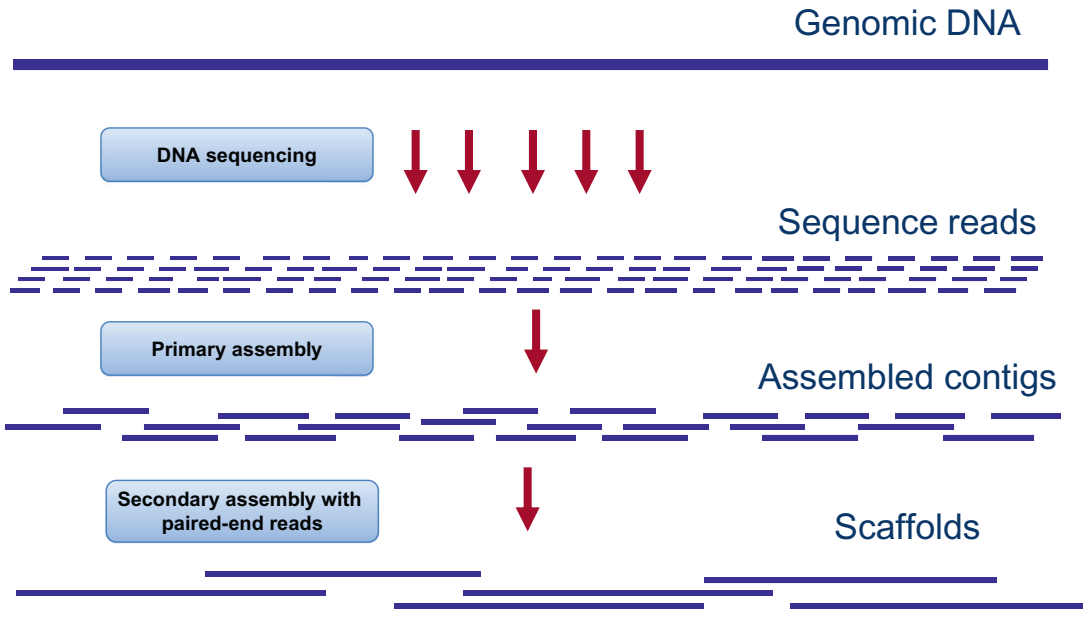

FIGURE 20.5 Schematic diagram of de novo sequence assembly using shotgun and paired-end read data.

clones of the same fragment, and sent to the sequencing reactions in massively parallel format. Single-molecule sequencing technologies are different in which library fragments are not amplified to avoid PCR bias and are sequenced as single molecules (Fig. 20.4).

Whole genome resequencing

With the help of NGS, reference genome sequences for many organisms are available in databases. Once the initial sequence for an organism's genome is available, it is possible to perform comparative sequencing or resequencing to identify polymorphisms, mutations, and structural variations between different or related organisms. Researchers are interested to identify and characterize sequence variation and understand their biological consequences. Resequencing of genes or other genomic regions of interest is a key step in detection of mutations associated with various phenotypes, strains, and diseases. Resequencing techniques can be used to test known mutations (genotyping) as well as to scan any mutation in a given region (variation analysis). Taking advantage of available complete genomes, researchers need to study genomic variations/alterations such as insertions, deletions, SNPs, CNVs, and so on in different strains/varieties, at different time points of growth, during drug stress, environmental stress, different phases of cell divisions and so on. Sequencing the whole genome of a species for which complete genome sequence information is already available, resequencing analysis is comparatively easy. The sequence reads generated can be easily mapped onto the sequence available in the database and the variations can be mapped. For resequencing platforms that can produce more data are more beneficial than those that generate longer reads with less data output. The protocol for resequencing experiments is similar to that of de novo sequencing.

Targeted resequencing

Targeted resequencing refers to sequencing regions of interest in the genome rather than sequencing whole genome. The most popular one is sequencing the exon part of the genome to get rid of unwanted introns. Targeted resequencing can be used to examine all of the exons in the genome, a part of the genome, specific gene families that are supposed to constitute known drug targets or regions and are thought to be involved in disease or pharmacogenetics effects through genome-wide association studies. But in the sequence enrichment step, there is a critical need for cost-effective solutions that can target specific genomic regions with high specificity and sensitivity, enabling the detection of both rare and common variants. Oligonucleotide microarrays and solution-based hybridization methods having probes specific to targeted regions/exons have been used to capture target regions from a pool of genomic fragments (e.g., Agilent, Nimblegen, Illumina, etc.). Capture probes can be immobilized on a solid surface in the form of microarray probes or can be used in solution. In microarray-based approaches, genomic DNA is fragmented and oligonucleotide linkers containing universal PCR priming sites are ligated to the fragments. The fragments with linkers are denatured, hybridized to an array, and eluted. The enriched DNA can be amplified by the PCR before sequencing by next generation sequencers.

Solution-based technologies use oligonucleotides up to 170 bases in length, containing PCR priming sequences with a restriction enzyme recognition site. The oligonucleotide library is amplified by the PCR, digested with restriction enzymes, and ligated to adapters containing the polymerase promoter site. In vitro transcription is performed to generate single-stranded biotinylated cRNA capture sequences. The system utilizes 120-mer biotinylated cRNA to capture regions of interest, enriching them out of an NGS genomic fragment library (e.g., Agilent Technologies). Other groups have captured specific genomic regions by using other solution-based hybridization methods, such as molecular inversion probes (MIPs) or Haloplex. In this technique the probes are single-stranded DNA containing sequences complementary to the target sequences in the genome. The probes hybridize to and capture the genomic targets. Probes are designed to have two genomic target complementary segments separated by a linker. When the probe hybridizes to the target, it undergoes an inversion and circularizes. Similar technology has been used extensively in the HapMap project for large-scale SNP genotyping, for detecting gene copy alterations and to identify biomarkers for different diseases such as cancer.

An alternative target enrichment approach developed by RainDance Technologies uses a novel microfluidics technology. In this technology, PCR primer pairs for the targeted regions, genomic DNA and PCR reagents are segregated individually in water in emulsion droplets. These droplets are merged and passed through an electrical impulse that causes them to coalesce. It generates PCR reactions at the rate of 10 million reactions per hour. The coalesced droplets are amplified by the PCR, and the amplicons are pooled and processed for NGS.

Transcriptome sequencing

Transcriptome pertains to RNA transcribed from a particular genome under investigation in a given

condition at a time. The RNA content of a cell provides direct knowledge of gene regulation and protein content information. Transcriptome sequencing, also called RNA-sequencing, refers to the sequencing of cDNA to get information about a sample's total RNA content at a given time in a given condition under study. This requires the conversion of mRNA to cDNA before the sequencing reaction. Different methods for in-depth characterization of transcriptomes and quantification of transcript levels have emerged as important tools for understanding cellular physiology and disease biology.

The technique has been benefited by NGS, which provide deep coverage at base-level resolution. It provides a comprehensive RNA sequencing with the information on differential expression of genes, differently spliced transcripts, gene alleles, post-transcriptional modifications, non-coding RNAs, alternative splicing, single-nucleotide polymorphism (SNP), and gene fusions. It is now possible to detect and identify nearly every transcribed molecule from short microRNA to the long 5′ and 3′ untranslated regions and even full-length mRNAs. Transcriptome sequencing has been used to discover novel gene sequences in the transcriptomes. Other uses include identification of cancer-related SNPs in transcribed sequences in human cancer transcriptomes, and confirmation of predicted genes in the bacteria (Mane et al., 2009, and references therein).

Transcriptome sequencing has begun to be utilized in various clinical diagnostic applications. Where current methods require RNA to be converted to cDNA by reverse transcription prior to sequencing and has been shown to introduce biases and artifacts that may interfere with proper characterization and quantification of transcripts, Helicos developed DRS™ that enables virtually unbiased view and quantification of transcriptomes. DRS claimed to sequence RNA molecules directly in a massively parallel manner without RNA conversion to cDNA or other biasing sample manipulations such as ligation and amplification.

Amplicon sequencing

The sequencing of PCR products is used to analyze genetic variations among different biological species and strains. NGS being a massively parallel sequencing has facilitated ultra-deep sequencing of amplicons making identification of rare variants present in very small frequency. Amplicons are generated by PCR with primers already containing appropriate sequencing motifs, and thus amplicons themselves are the sequencing library. As in amplicon sequencing, each amplified molecule within a mixture of amplicons can be sequenced individually, the technology is well able to detect rare variants with detection limits of 0.5% and even lower. Amplicon sequencing is suitable to study highly variant samples such as the hypervariable regions of antibodies as well as samples with low variance such as population samples for SNP detection. It is used in various projects such as population diversity screenings within different individuals, identification of rare mutations/SNP associated with various diseases, metagenome analysis (with 16S or 18S rDNA sequencing), study of methylation patterns, and so on.

Chromatin immunoprecipitation DNA sequencing

Chromatin immunoprecipitation (ChIP) sequencing is used to analyze DNA−protein interactions within the cell. It combines ChIP with massively parallel DNA sequencing to identify the binding sites of DNA-associated proteins. The protocol involves ChIP to isolate DNA bound to proteins followed by separation of DNA from proteins by purification. Purified DNA is then processed for sequencing similar to de novo sequencing but optimization for low-quantity DNA. It is used primarily to determine how transcription factors and other DNA-binding trans-acting proteins bind to DNA and how they influence the cell function. Determining how proteins interact with DNA to regulate gene expression, DNA structure and other cellular activities is essential for fully understanding many biological processes and disease states. ChIP-seq technology is currently seen primarily as an alternative to ChIP on chip technique, which requires a hybridization microarray. ChIP on chip has been the most commonly used technique so far, which has been utilized to study these protein−DNA relations. ChIP sequencing can be used to map global binding sites precisely for any protein of interest.

Small RNA sequencing

Small RNAs are typically 18−40 nucleotides in length and have been shown to play a critical role in regulating gene expression in many organisms, affecting developmental timing, cell fate, tumor progression, neurogenesis, and other key cellular processes. Animals, plants, and fungi contain several distinct classes of small RNA, including microRNA (miRNA), short interfering RNA (siRNA), piwi-interacting RNA (piRNA), and so on. The investigation and quantification of small RNAs in a high throughput manner may provide valuable insight into the mechanisms of gene regulation and involvement of non-coding RNA in important diseases such as cancer. With the enormous depth reached by NGS technologies, comprehensive information of the small RNAs of a cell

can be obtained in various clinically important samples. It allows researchers to examine tissue-specific gene expression patterns, disease associations, miRNAs isoforms, and to discover previously uncharacterized small RNAs having critical roles in the cell. Small RNA sequencing involves isolation of small RNA by size fractionation techniques followed by cDNA preparation with specialized primers capable of converting such a small molecule to cDNA. cDNA is then processed for NGS.

Animal biotechnology and the cattle genome

Animal biotechnology has a long history since traditional breeding techniques dating back to 5000 years before century. These techniques involve crossing different varieties of animals to produce greater genetic variety, and later selective breeding in the offspring to produce the maximum number of desirable traits. Today animal biotechnology is based on the concepts of genetic engineering and has been facilitated by high throughput technologies such as high-density DNA chips and NGS.

The sequencing of human genome was completed in 2001 by two independent research groups simultaneously. This was one of the remarkable achievements of biological sciences and molecular biology. Human genome sequencing opened the gates for genome sequencing of other mammalian species. The decreasing cost and time of genome sequencing at a rapid rate due to technological and methodological developments enabled researchers to plan sequencing of other important organisms at a genome wide level. In this continuation an assembled draft bovine genome sequence was released in 2007, and later in 2009 the complete sequence of the bovine genome was published (Elsik et al., 2009). The complete sequence of the cattle genome resulted in the discovery of large number of genetic variants in the form of SNPs and used as a powerful tool for identifying small genetic variations associated with the livestock. Following this low coverage sequencing of animals representing six other breeds of the cattle was also done to identify about 100,000 genetic polymorphisms (SNP) between individuals. A lot of sequencing data were generated over the next few years by the livestock research scientists for full-length cDNAs, Expressed Sequence Tags (ESTs), highly specific BAC end sequence markers, dense genetic linkage maps, physical maps, and cell-based resources (CSIRO).

The success story of large and complex genome sequencing added the chapter of draft sequence of the giant panda genome solely from paired-end next generation sequence reads, which proved suitability of using short sequence reads for the assembly of large and complex eukaryotic genomes (Li et al., 2010). The emergence of NGS also prompted the International Sheep Genomics Consortium to plan a Sheep Reference Genome Project in 2009. Till date more than 125 animals have been sequenced. NGS has facilitated scientific community to form various genome sequence consortium to sequence reference genomes of important animals useful for human being.

Applications of NGS in animal biotechnology

NGS has its potential in every subfield of animal biotechnology from revolutionizing traditional animal breeding applications to the identification of very small genomic variations. Recent molecular biological approaches have been benefited by the development of NGS technologies and supposed to help in animal research as well as their translational applications (Fig. 20.6).

Evolutionary research

Evolutionary biology is a field of biology concerned with the study of the evolutionary processes that have given rise to the diversity of life on Earth. Understanding the mechanisms how organisms adapt to changing environments is a central topic in modern evolutionary ecology. Many fundamental questions important to our understanding of adaptation genetics remain unanswered due to the lack of sufficient information about molecular events occurring during the process of evolution. Recently high throughput technologies facilitated the study of evolutionary biology with the development of NGS. NGS technologies make it easier and more possible to identify genetic loci responsible for adaptation. It revolutionized the way that biological and evolutionary processes can be studied at the molecular level. NGS offers the opportunity to perform genomics studies on many additional ecologically interesting species unlike earlier, where there is a requirement of a closely related genetic model organism to link evolutionary data with molecular events. NGS helps to find loci of small effect, because genotyping is becoming faster and cheaper, and it is possible to carry out improved mapping studies, with more individuals and markers (Stapley et al., 2010) such as high throughput genotyping in chicken at a low cost of around 50 USD per sample (Pertille et al., 2016). NGS provides a cost-effective way to perform preliminary genome-based analyses and allows examination of some fundamental developmental and evolutionary processes of a species in the absence of a closely related genome (Subramanian et al., 2010).

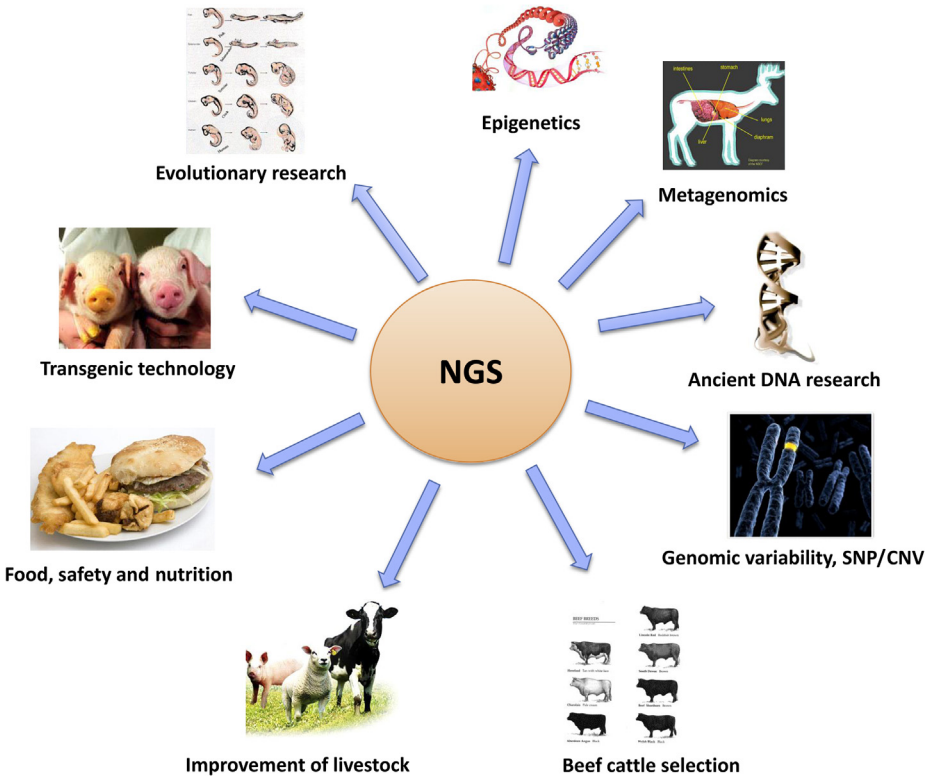

FIGURE 20.6 Different applications of next generation sequencing in animal biotechnology.

Epigenetics

Epigenetics is the study of heritable changes in gene expression or cellular phenotype caused by mechanisms other than changes in the underlying DNA sequence. In other terms it is a study of epigenetic factors that influence the molecular properties by binding to specific DNA sequences. Examples of such changes are DNA methylation and histone modification, both of which are known to regulate gene expression without altering the associated DNA sequence. Epigenetics is a field of rising importance in genetics and genomics. Some examples of these modifications include imprinting, X-inactivation, gene silencing, and embryonic reprogramming (Sellner et al., 2007). Epigenetic effects such as methylation involve the addition of methyl groups to certain cytosine residues and if this occurs in the promoters of genes, transcription machinery can be blocked from binding to the DNA. The highly methylated regions in DNA tend to be less transcriptionally active, which may have impact on the phenotypes of animals.

The study of epigenetics is important for animal breeding because it may help finding part of the missing causality and missing heritability of complex traits and diseases. It impacts many economically important traits from growth and development to more efficient reproduction and breeding strategies. Methylated DNA patterns are modified along the life of an individual by environmental forces and some environments are more likely to increase certain methylation patterns, and these patterns would contribute to the phenotypic variation between individuals. Further the environment may affect the methylation pattern of three generations cohabiting under the same specific circumstances at a given time during pregnancy: the productive female, the fetus, and the fetus' germ cells. Hence what happens to an animal during its lifetime may have consequences in future generations (González-Recio, 2011). Tissue-specific epigenetics studies in bovine genomes have potential to select or induce favorable effects and this information can be included into further breeding programs.

Recently the identification of methylated DNA is possible using some high throughput technologies based on NGS, which provide information on DNA methylation on a genome-wide basis. The NGS technologies can easily elucidate whether nucleotides are methylated, which allows rapid understanding of these effects and their influence on phenotypes in beef cattle. Epigenetic parameters resulting in transgenerational changes, heritable disorders, and areas of livestock immunity and stress have recently been discussed (Triantaphyllopoulos et al., 2016). The researchers highlighted the importance of nutrition and its linkage to epigenetic alteration. They also described how the information can be translated into directed efforts to improve animal health and welfare.

Metagenome sequencing

Metagenomics is the study of mixed genomes. It is the study of genetic material recovered directly from samples, where mixed population of various microorganism is expected to be present. Traditionally metagenome sequencing relies upon cultivating the microbial cultures from test sample and sequencing of cloned specific genes, often the 16S rRNA gene to identify the microorganism present in the natural sample and to produce a profile of diversity. In the culturing process, a number of micro-organism are usually missed out either due to their non-culturable nature or overgrowing co-cultures. Further amplification of 16S rRNA gene from natural sample also misses out representation of many organisms. It is now facilitated by massively parallel NGS technologies. Because of its ability to reveal the previously hidden diversity of microscopic life, metagenomics by these high-throughput techniques offers a powerful tool to see microbial world in a mixed microbial habitat.

A novel application of this technology that is becoming increasingly important and of much concern in the animals especially livestock is the sequencing of gut microbiomes. Most work in the past has been done in humans and mice and has focused on profiling the 16S ribosomal RNA (rRNA) gene to identify the microbes present in the gut using traditional Sanger sequencing. However, metagenome research has recently been accelerated due to NGS technologies.

Animals harbor several floras in their gut, which interact with the host and affect many biological processes by utilizing and converting a number of important nutrients. The study of gut microbiomes and their interactions with the genotype of the host is important because a substantial genetic diversity in the species present within the gut microbiome is expected. The gut microorganisms are supposed to have a profound impact on energy consumption/generation and utilization of important nutrients from food material.

Ancient DNA

In less than a year from its development, NGS increased the amount of DNA sequence data from extinct organisms by several orders of magnitude, which provided information to understand the origins of species. Although newer techniques aided in studying ancient DNA by increasing sensitivity and specificity, NGS has revolutionized ancient DNA research at a great extent.

Ancient DNA is usually highly fragmented with average fragment lengths ranging from 51.3 bp for some Neanderthal DNA to 142 and 164 bp, respectively, for DNA from permafrost mammoth hair. Early analyses of aDNA focused on mitochondrial and/or chloroplast DNA, which were abundantly available in the cell, making retrieval and reproducibility much easier. With the help of NGS, it has been possible to retrieve and study nuclear DNA on a more routine basis (Linderholm, 2015). More than 20 studies have already made use of NGS to obtain sequence data from ancient remains (Knapp and Hofreiter, 2010 and references therein). Ancient DNA isolated from fossils usually contains very different levels of contamination and are difficult to sequence. However large data generated by the various NGS technologies together with the short read length makes them ideal tool for ancient DNA research. This is evidenced by the massive increase of available sequence data from long-dead organisms since the invention of NGS (Knapp and Hofreiter, 2010). Recently researchers have been successful to sequence, align, and identify ancient DNA related to sheep, which was preserved in parchment (Teasdale et al., 2014). Due to the small amount of endogenous DNA and high background contamination, shotgun sequencing of ancient DNA is not of much uses, rather targeted sequencing with different capture methods can be very promising. NGS techniques helped ancient DNA research and thus helped understand evolution of the organism in due course of time.

Genomic variability, SNP/copy number variations discovery

Once the genome of a species is sequenced, it facilitates identification of genomic variation within different individuals by sequencing and comparing the data of individual genomes with already available reference genome. After successful alignment of the fragments of one or more individuals to a reference genome, different SNPs are identified and individuals are assigned with the genotype depending on the genomic variation they harbor. SNPs may be associated with diseases or susceptibility for diseases depending on their position in the essential genes. Massively parallel sequencing strategies generate large amount of sequencing data, producing very high depth of fragments sequenced and help finding out SNPs present even in very low frequencies.

Copy number variations (CNVs) are gains and losses of genomic DNA sequence, usually >50 bp between two individuals of a species (Mills et al., 2011). They are alterations of the DNA of a genome that results in the cell having an abnormal number of copies of one or more sections of the DNA. Since they cover a bigger portion of genomic sequence, they affect a wide range of phenotypic traits than SNPs, which are more frequent in the genome. CNVs thus have

potentially greater effects on phenotype of the organism than SNPs. CNVs can affect the gene structure and dosage that may result in altered gene regulation.

Several common CNVs have been identified and shown to play important roles in normal phenotypic variability and disease susceptibility in human and other higher organisms where they are known to be associated with diseases such as autism, schizophrenia, neuroblastoma, Crohn's disease, and severe obesity-related disorders. Recently interest in CNV detection has extended into domesticated animals (Bickhart et al., 2012 and references therein). CNVs have been discovered by cytogenetic techniques such as fluorescent in situ hybridization, comparative genomic hybridization, array comparative genomic hybridization, and by SNP arrays. The microarray-based methods limit their use as only a relative copy number (CN) increase or decrease can be reported with respect to the reference individual genome. Recent advances in NGS of DNA have further enabled the systematic identification of CNVs at a higher resolution and sensitivity.

CNVs can be passed to next generation of animals and have higher rates of accumulating mutations and may be associated with animal health under recent selection. *Bos taurus indicus* are better adapted to warm climates and demonstrate superior resistance to tick infestation than *Bos taurus taurus* breeds due to CNV in associated resistance genes in their genomes (Porto-Neto et al., 2011). Similarly milk production traits along with other important phenotypic traits show distinctive patterns due to CNVs in beef and dairy cattle breeds (Bickhart et al., 2012 and references therein).

Beef cattle selection

Beef cattle are raised for meat production (as distinguished from dairy cattle, used for milk production). Traditionally marker-assisted selection is used for the accurate selection of specific DNA variations that have been associated with a measurable difference or effect on complex traits. Recent advancements in sequencing and genotyping technologies have enabled a rapid evolution in methods for beef cattle selection from restriction fragment length polymorphism (RFLP) markers that were low throughput and time consuming to the new high-density SNP assays and NGS where marker genotypes are easily and inexpensively generated. With the rapid development of molecular technologies, new tools have become available for beef producers to efficiently produce high-quality beef for today's consumer. Technologies such as NGS help to shorten the generation interval to identify causal mutations, to provide information on gene expression, and thus strengthen our understanding of epigenetic changes and effect of gut microbiome on cattle phenotypes.

The ability to rapidly, accurately, and relatively low-cost sequencing of the genomes of individual animals has the potential to revolutionize selection in beef cattle. Massively parallel sequencing data provide information about novel as well as known polymorphisms within an individual. The discovery of mutations that actually cause variation within traits will become increasingly important and their knowledge will allow testing across breeds that will drastically reduce the number of loci that need to be tested to explain variation within a trait (Rolf et al., 2010). It is easier than earlier to identify signature sequences for diverse genomic selection during cattle domestication, breed formation, and recent genetic improvement (Xu et al., 2015).

Animal breeding and improvement of livestock productivity and health

Animal breeding is a branch of animal science that addresses the evaluation of the genetic value in terms of estimated breeding value, estimated breeding value (EBV) of domestic livestock. Animals have been selected for breeding with superior EBV in growth rate, egg, meat, milk, or wool production and other important desirable traits. This has revolutionized agricultural livestock production throughout the world. Traditional breeding has been done in the lack of molecular information of the genes, actually responsible for quantitative traits. The efficiency of these traditional methods remains limited in case of traits that have a low heritability and cannot be correctly measured in a large number of animals such as meat quality, internal parasite resistance, and slow genetic progress (Eggen, 2012). NGS is transforming animal breeding, due to low cost involved in whole genome sequencing studies facilitating identification of genetic markers at a genome wide level. NGS has revolutionized the planning and implementation of livestock breeding programs. For the livestock industry these high throughput technologies are expected to increase the efficiency and productivity of animal breeding, whereas for consumers it is supposed to enhance security and the quality of animal products (Eggen, 2012).

Food, safety and nutrition

The food safety produced through animals for human consumption remains a key question among the food and nutrition biologists. The food produced through animal biotechnology includes products with

genetic modifications and the concern of their effect on human health. The main areas of concern are allergens, bioactivity, and the toxicity of unintended expression products. As the food allergens introduced from expression of genetically modified proteins from genetically modified animals is a big concern, difficulty comes in how to anticipate these accurately before human consumption.

NGS strategies being more economically feasible with real-time results can be directly applied to improve poultry production and enhance food safety. Determination of the gut microbes, genes involved in metabolic pathways, or presence of plasmids and screening for function such as antibiotic resistance, or nutrient production can be carried out. These gut microbial flora of poultry and animals can be sequenced to determine the effect on human health and diseases (D'Souza and Hanning, 2012).

Transgenics

Transgenic animals are used as experimental models to perform phenotypic and for testing in biomedical research. They are important model systems for establishing the mutational fingerprint for various human diseases including cancer. Genetically modified animals are becoming more important to the discovery and development of treatments for many serious diseases. Transgenic mice are often used to study cellular and tissue-specific responses to disease. By modifying the DNA sequence or transferring DNA to an animal, certain proteins can be developed that can be used in medical treatment. Stable expressions systems of human proteins have been developed in many animals, including sheep, pigs, and rats for their commercial production. However the mutation detection assays by conventional DNA sequencing analysis used for these transgenic systems allow low-throughput detection of mutational fingerprint in phenotypically expressed individual mutants, which is costly, time consuming, and extremely laborious (Besaratinia et al., 2012).

Applications of NGS in human health

NGS has changed the way of observing human disease mechanisms and impacted both basic and clinical research. At one end the basic research sector is fairly driven by direct use of NGS to sequence novel variations or by the information generated through NGS, which is to be used with traditional experiments. Clinical research involves high-throughput genetic testing with higher resolution and clinically relevant genetic follow-ups. Few key areas where NGS has made a great impact can be summarized below.

Cancer research

The area of cancer research has been specially revolutionized by NGS. As cancer research has traditionally been complicated by the fact that there is no clear-cut mechanism for all types of cancers and we need to analyze a large number of genetic variations in human genome that can be associated with cancer phenotypes. A large number of cancer as well as healthy individuals need to be studied for a comparison of their genetic make-ups with several genetic targets. With the introduction of NGS, this area has primarily been benefited, as several genomes can be sequenced simultaneously within few weeks' time. Targeted DNA sequencing, especially exome sequencing, allows even higher throughput at a reduced cost per sample. This has facilitated analyzing genomic variations such as millions of SNPs to be associated with a particular phenotype in genome wide association studies (GWAS). World-wide collaborations to catalogue mutations in multiple cancer types are underway. In April 2008, leading teams of international scientists constituted the International Cancer Genome Consortium with the aim of generating high-quality genomic data from 53 tumor types over the next decade, which will help in new discoveries related to diagnostics, prognostics, and therapeutics.

Genetic disorders

Recent efforts have demonstrated a significant opportunity for the use of NGS in the diagnosis of genetic disorders making NGS a practical, attractive, and economically feasible technology for clinical applications. NGS has significantly improved the identification of disease-associated mutations and genetic alterations. NGS facilitates researchers with required capacity to analyze large panels of genes for each individual. In the last few years, technological advancements in NGS, specially, target enrichment methods, have resulted in the identification of variations responsible for more than 40 rare disorders. These include numerous diseases such as Schinzel–Giedion syndrome, Sensenbrenner Syndrome, Neonatal Diabetes Mellitus, Miller syndrome, Kabuki syndrome, Fowler Syndrome, Hereditary Deafness, Parkinson's disease, and others. Identification of genetic causative agents in mental disorders has also been improved by the use of NGS such as identification of mutations responsible for hyperphosphatasia mental retardation syndrome.

Human microbiome

Microbial organisms have a close association with human body, both beneficial and harmful. These organisms may primarily be bacteria, but also involve Archaea, yeasts, single-celled eukaryotes, helminths and viruses. Many of these organisms have not been successfully cultured, identified, or otherwise characterized. Few important site of micobial colonization in human body include colon, stomach, vagina, skin, esophagus, hair, nose, and mouth (oral cavity). In 2008 the Human Microbiome Project (HMP) was initiated by United States National Institutes of Health to identify and characterize the microorganisms that are found in association with healthy and diseased humans.

NGS technologies have become a valuable tool in the study and analysis of microbial communities in diverse environments, including the human body. It is useful in identifying microbial colonization, polymicrobial infections, and microbial communities with a better resolution. With the help of NGS, one can identify new species of colonized microbes through metagenome sequencing approach, by employing 16S rRNA gene amplicon sequencing from a mixed population or by whole genome sequencing approach. In the latter case NGS technologies producing long read have an advantage as it is better assemble longer reads to form a suitable genome from reads of diverse organisms.

Pre and postnatal diagnoses

One of the very valuable applications of NGS technology is molecular genetic testing in pre and postnatal diagnostics. Traditionally invasive methods have been used to draw sample and detect chromosomal abnormalities with high risk for mother and fetus, which can provide definite genetic information about the fetus. The NGS-based detection of fetal aneuploidy in high-risk-pregnancies is very promising and has successfully been used for the detection of chromosomal aneuploidy in fetal DNA from cell free DNA fragments in maternal plasma (Fan et al., 2008, Ashoor et al., 2012). The advantage of NGS over traditional prenatal diagnosis is that it allows noninvasive testing of fetal abnormalities with high sensitivity and specificity.

Infectious diseases

Because of the constantly decreasing costs of DNA sequencing, next-generation technologies are continuously becoming an integral part of the genetic and infectious diseases research and diagnostics. It has primarily been used in viral research for ultra-deep whole viral genome sequencing for influenza viruses, for detection of human immunodeficiency virus (HIV) genome variability and evolution within the host, human hepatitis C virus quasi-species. It has also been used in monitoring of antiviral drug-resistance mutations.

NGS has emerged as an extremely powerful tool to study bacterial genomics, viral dynamics, and host-response and other aspects in infectious disease biology, which were previously inaccessible. Due to the capability of NGS to sequence individual molecules in massively parallel manner, it is possible to segregate and sequence individual genomes, which is useful in studying the evolution of infectious agents though the history, monitoring emergence of drug resistance, discovering novel viruses in population.

Personalized medicine

Every individual is different in its genetic make-up. Therefore its susceptibility to different diseases, infections, and disorders are also different. Moreover every individual needs different treatment and management of illness. One should know what is his/her genetic make-up to decide accurate and proper care for what is going to happen with him and need to know more about his genes and their impact on his lives. Our personal genome can provide information whether there is increased risk for a selection of hereditary diseases. NGS technologies can provide information on all of the different types of disease-causing alterations in individuals in the short turn-around time required to screen patients for either clinical trials or for diagnostics in clinical settings. It is beneficial in identifying and developing panels for biomarkers, individual genes associated with a particular type of health condition.

Clinical correlation

NGS technology has expanded to clinical research and healthcare. The ultimate use of genomics being into patient benefit has been largely smoothed with the use of fast-growing high-end technologies. NGS has now become an established testing method of many genetic conditions. One of the advantages of NGS is to interrogate several genomic targets at the same time on the scale of thousands or even millions of genomic targets. Such capacity gives NGS huge potential application in clinical settings. Different workflows are used according to the requirements of genetic tests such as exomes, custom targeted panels and sometime whole genome, if required. Laboratories develop custom panels as per the need of the test with genes involved, or supposed to be involved, in clinical disorders. While cancer sample analysis requires a

much broader gene target lists for germline and somatic mutations, other inherited disorders such as various immunodeficiencies, genetic deafness, congenital blindness, multiple neurological disorders, cardiomyopathies, and others require various targeted panels with fewer number of genes. For more complex disorders, or conditions where no definitive detection could be made, exome sequencing offers as a better option. Clinical applications of NGS involve several aspects, such as identifying and choosing what genetic mutations need to be investigated. This is usually based on what disease or diseases the particular NGS testing is designed for, various guidelines involved in such type of testing and to identify the targeted gene mutations that are included in the guideline. For example in non-small cell lung cancer, the guidelines indicate that EGFR, KRAS, BRAF, MET, RET, and Her2 mutations, and ALK and ROS1 translocations are clinically significant and need to be tested.

The technology is growing and being used in applications other than cancers, which also involves reproductive genetic testing. Prenatal testing for various disorders by NGS facilitates detection of abnormalities in the fetus, which can be managed if detected at an earlier stage. In in vitro fertilization process, NGS-based genetic testing is growing its usage as a preimplantation genetic testing and diagnosis, where different embryos are tested for presence and absence of genetic defects before implantation into the uterus. This leads to more successful pregnancy and less chances of genetic defects in the baby. In postnatal analysis, NGS tests are being performed to detect genetic defects in the patient, where whole exome sequencing presents a better option, involving child, mother, father, and sometime grandparents.

Newer applications of NGS into clinical settings involve circulating tumor DNA in cancer. In ctDNA applications genetic defects are detected in cell-free DNA circulating in the blood stream omitting the need to take biopsy from solid tumors. Very sensitive library preparation and detection system in NGS is required to perform such analysis, which has been made possible with new methodologies and use of unique molecular barcodes to remove false positives and detection of variants present in lower allele frequencies. ctDNA, which is composed of small fragments of cell-free DNA, supposed to come from tumor in the body and carries tumor-specific genomic mutations. It has been shown that ctDNA is a sensitive and specific biomarker for the detection of early and late-stage human malignancies including lung cancer. It is being used in the form of liquid biopsy to monitor treatment response and detect resistance to chemotherapy. NGS analysis of ctDNA has been accepted as a good noninvasive method for detection of early cancer mutation.

Similar tests are also performed for prenatal testing to detect genetic defects in fetus without the need of taking sample from invasive methods such as amniocentesis and chorionic villus sampling. Here the circulating fetal DNA is targeted from the mother's blood and used for NGS. Such test is called noninvasive prenatal testing (NIPT), which offers early genetic screening for chromosomal conditions as early as 10 weeks of pregnancy. Noninvasive testing provides high detection rates, low false-positive results, and has advantage as there is no risk to mother and baby. However invasive tests are sometimes required as a definitive diagnosis where defects are found in NIPT. It is used to screen common chromosomal variations such as trisomy 21 (Down syndrome), trisomy 18 (Edwards syndrome), and trisomy 13 (Patau syndrome). The American Congress of Obstetricians and Gynecologists (ACOG) and International Society of Prenatal Diagnosis (ISPD) along with other professional societies have also stated that NIPT is an available screening option for all pregnant women.

NGS can also be used for transcriptome analysis to investigate various changes at expression level. This includes whole RNA in a given cell type in a given condition including mRNA, tRNA, rRNA, micro RNA, and non-coding RNA. Unlike DNA sequencing, this is called RNA sequencing or RNA-seq. RNA-seq is used to detect expression changes as well as to detect fusion genes important in certain cancers and disorders. It is also used in association with detection of structural changes by DNA seq.

Variant interpretation from such sensitive applications is a complex process, which requires specific alignment, variant detection, and filtering tools. Tests that involve larger genomic regions to cover and those with sequences at a higher depth provides greater probabilities of detecting rare or novel variants. There are guidelines from different scientific and medical agencies such as American College of Medical Genetics (ACMG), College of American Pathology (CAP), Association of Molecular Pathology (AMP), and so on, which assign evidence for various criteria regarding a particular variant to classify them as pathogenic, likely pathogenic, uncertain significance, likely benign, or benign. It is important to filter the variants according to quality, frequency, structural significance, and patient's phenotype to make sense of NGS data.

In summary, NGS provides a powerful tool in the field of medical and clinical genetics, allowing us to perform multigene analysis and to sequence several genes at a time, entire exomes, transcriptomes, or genomes. The generated high-throughput data are particularly suitable for understanding the genetic bases of complex, multigene diseases, such as cancer and other genetic disorders. In this scenario, NGS technologies

offer a powerful tool for the discovery of novel factors involved in various clinical conditions.

Translational significance

Translational research pertains to the process of translating scientific discoveries into practical (clinical) applications. NGS is playing a transformational role in cancer discovery and genetic disorder research. Providing new insights into disease mechanisms and metabolic and signaling pathways are the examples of progressions made through NGS that were previously unfeasible. The information is being used in improving diagnostics, more effective and more personalized treatments for disease and patient care.

Further with targeted NGS, it holds great potential for speedy, wide-range mutational analysis to unravel complex tumor signatures variations, improving the cost-effectiveness of sequencing by focusing on the portions of the genome that are relevant for the question of study. With the down going cost and improving technology, NGS has a potential to translate the enormous information in almost all aspects of health, development, and disease.

Ethical issues

The NGS allows simultaneous sequencing of enormous amounts of DNA. Taking advantage of tremendous power of the technology, NGS has been started to be used in understanding of human genetic diseases. However important ethical and social issues need to be addressed before its routine clinical diagnostic applications. The transition of NGS technology into the clinic is one of the most important aspects but will also have significant ethical issues regarding the ownership and privacy of patient genetic information. Clinical genetic investigator may also face issues such as return of genetic information of a patient after being analyzed by NGS. For example, in 2010, members of NHGRI's ClinSeq study found a patient had passed away, whose exome had already been sequenced and analyzed. In this case, the question arises that should the results of genetic studies be returned to the patient, if yes, what information should be returned and to whom, especially if the patient dies. The NHGRI team then concluded that certain results such as variants with well-established clinical significance should be returned to the family, which is relevant to the family's health with a face-to-face genetic counseling.

Ethical issues also cover to conceal the identity of an individual from publicly accessible deidentified data. It would also be essential to confirm that would-be participants are aware of the risks before they decide whether to participate and whether participants or their family members should be informed of incidental genotype findings specially if such findings have some adverse effect on family health (Devarakonda et al., 2012).

Future perspectives

NGS being relatively in-expensing technology in view of generating sequence information at genome-wide level has revolutionized nearly each and every area of biotechnology. Traditional methodologies in medical biotechnology such as novel pathogen identification, biomarker discovery, SNP association with diseases, stem cell biology, and so on; and of animal biotechnology such as transgenics, production of probiotics and prebiotics, enzyme technology, animal cloning, gametes and embryo production, artificial insemination, gene−gene and gene−environment interactions related to environmental conditions could be studied quantitatively using modern bioinformatics tools.

In future sequencing of individual genomes of interest under different living, nutrition, or treatment conditions will benefit the medical and agricultural community by providing guidance for disease control and prevention. Sequencing the genome of animals is supposed to enable scientists to more accurately identify the genetic markers useful in economically important traits. While medical community is supposed to be benefited by the information generated by NGS in terms of better diagnostics and therapeutics, the information is also supposed to help agriculture community to breed healthier dairy cattle producing more and higher quality of milk as well as beef cattle that produce better quality beef. The purpose of sequencing agricultural animals has moved far beyond the original goals of serving as a model for studying human health issues. At present sequencing of animals have goals of, but not limited to, studying traits of economic interest to raising livestock production, study of effect of domestication, selection of high fertility breeds and understanding of genotypic and phenotypic changes due to environmental factors like nutrition.

The biotechnology is entering the postgenome era. NGS technologies are well able to help scientists and clinicians study genomes of individuals much faster than earlier. Sequencing microorganisms and parasites in agricultural animals' organs can also help veterinary scientists to develop new vaccines and therapeutics (Bai et al., 2012).

Challenges

Sequencing whole genomes by these massively parallel sequencing technologies generates huge amounts of data that must be properly managed, stored, and analyzed. As the reagent cost of sequencing decreases with the development of better reagents and protocols, a number of sequencing projects are running simultaneously and generating enormous data of millions and billions of DNA base pairs. While sequencing projects of many animals and plants are still running and many scientists have historically conducted high-end sequencing of various animal and plant samples, the lack of computing skills, required hardware, storage and network necessary to manage the massive data sets generated by larger scale whole genome sequencing studies is often being felt. As sequencing studies are continuously increasing in numbers, the cost and complexity of data analysis and management is emerging as the primary limiting factor among researchers.

Web resources for NGS

The websites relevant for information about different types of NGS platform/technologies are given at the end of reference section. These websites have enormous information about technologies, chemistries and applications of various NGS platforms as most of these are from sources of technology developers and manufacturers. However comparison of significant research outputs, applicability and relevance to clinical applications should be made through various publications from independent research groups, which are supposed to be unbiased and impartial in application and usability of individual technology. Few websites, which are focused on NGS applications and are useful for NGS users are given here:

1. www.seqanswers.com: This website addresses several issues related to workflow or protocol, which are not necessarily given in the manuals or other webpages. The NGS users make their account and share the issues they face while performing the actual experiments as a thread. The issue is then discussed by a number of NGS users who share their unbiased experiences and try to provide the resolution of the issue.
2. www.genomeweb.com: This website is useful to get recent updates about different technologies/platforms available. This website also addresses other genomics techniques such as array, clinical genomics, PCR, bioinformatics, and so on. Under the tab 'sequencing', the visitor gets most recent information about new products for novel applications in NGS launched/introduced by different NGS firms. Although it also has news related to marketing updates, it is useful to understand what new technology is being introduced and available for the user.
3. http://en.wikipedia.org: Wikipedia is a good encyclopedia for everything. It also contains a lot of information about nearly each type and application of NGS, especially for those, who are very new to the technology and want to get through the basic idea of a particular technique, its principle, history, workflow, etc.

World wide web resources

1. www.454.com
2. http://my454.com/products/technology.asp
3. http://www.illumina.com
4. www.appliedbiosystems.com
5. www.pacificbiosciences.com
6. www.helicosbio.com
7. www.en.wikipedia.org
8. www.iontorrent.com
9. http://www.nature.com/news/nanopore-genome-sequencer-makes-its-debut-1.10051.
10. http://www.polonator.org
11. www.completegenomics.com
12. www.raindancetech.com
13. http://www.nimblegen.com
14. http://www.agilent.com
15. CSIRO. http://www.csiropedia.csiro.au/display/CSIROpedia/Cattle + genome + project
16. http://www.dnasequencing.org/history-of-dna
17. http://massgenomics.org
18. www.sanger.ac.uk
19. www.icgc.org
20. www.seqanswers.com
21. www.genomeweb.com/sequencing
22. https://www.genome.gov/sequencingcostsdata/
23. https://en.mgitech.cn/
24. https://nanoporetech.com/
25. https://www.agilent.com/en/products/genomics-agilent

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Glossary

Adaptors Adaptors are short DNA sequences, which are attached to the unknown DNA fragment so that DNA with known sequences flanks the unknown DNA for priming. For example, sequencing adaptors.

Assembly Assembly refers to merging small fragments of a DNA sequence to reconstruct the original much longer sequence. This is needed as DNA sequencing technology/instrument cannot read whole DNA in one attempt, but reads the DNA in small pieces of between 20 and 1000 bases, depending on the technology used. For example, human genome assembly from shotgun reads.

De novo assembly De novo assembly refers to assembling short reads to create novel full-length DNA sequence with no prior reference sequence available. For example, de novo assembly of plant genomes.

Di-deoxy nucleotides Di-deoxy nucleotides are chain-terminating nucleotides, used in the Sanger method for DNA sequencing. The absence of the 3′-hydroxyl group means that, after being added to a growing nucleotide chain, no further nucleotides can be added to them as no phosphodiester bond can be created.

DNA Sequencing DNA sequencing is a process of determining the order of nucleotides within a DNA molecule. It includes a method or technology that is used to determine the order of the

four bases: adenine (A), guanine (G), cytosine (C), and thymine (T) in a strand of DNA.

Emulsion PCR An emulsion is a mixture of two or more liquids that are normally immiscible. Emulsion PCR isolates individual DNA molecules in aqueous droplets within an oil phase along with other PCR reagents for amplification. A PCR reaction within each droplet then makes clonal copies of the DNA molecule followed by immobilization on beads for later sequencing. For example, emulsion PCR used in 454 sequencing.

Fragmentation In terms of sequencing processes fragmentation of DNA means physical shearing of DNA to produce smaller fragments of desired size range with the help of pressurized gas. For example, nitrogen (nebulization), sonication or enzymatic fragmentation.

Genome The genome is the total nucleotide content of an organism, generally DNA, but in some viruses, RNA. The genome includes both the genes and the non-coding sequences of the DNA/RNA.

Genomic variation Genomic variations pertain to changes in the sequence of genome as compared to the reference genome of the same species. It can be single nucleotide changes, insertion, deletion of a segment of DNA or large chromosomal changes.

GWAS Genome-wide association study (GWAS), is an examination of common genetic variants in different individuals of a population to analyze which variant especially SNP is associated with a trait or disease.

High-throughput A high-throughput process is a scaled-up, parallelized, and automated process with rapid and greater output as compared with conventional methods.

Library preparation In terms of NGS library preparation pertains to preparing the input nucleic acid (DNA or RNA) for downstream sequencing. It is done by fragmentation. For example, adaptor ligation, index ligation, purification, quantification, and so on.

Mapping Mapping is aligning reads against an existing genome sequence to find out genomic variations. Mapping assembly is building a sequence from reads with the help of an existing genome sequence.

NGS platform An NGS platform is a particular NGS technology with the instrument and reagent, which does the next generation sequencing. For example, Roche 454, Illumina-HiSeq, ABI-SOLiD, and so on.

Polony Polony is a contraction of "polymerase colony," a small colony of DNA. Polonies are discrete clonal amplifications of a single DNA molecule.

Read length The length of a DNA fragment or piece, which a particular sequencing technology/instrument can sequence (read). Later these reads are aligned together to form a larger DNA construct.

Abbreviations

BAC	Bacterial Artificial Chromosome
cDNA	Complementary DNA
ChIP	Chromatin ImmunoPrecipitation
CNV	Copy Number Variation
cPAL	Combinatorial Probe Anchor Ligation
DNB	DNA Nano Ball
EBV	Estimated Breeding Value
EST	Expressed Sequence Tags
GB	Giga Bases
GWAS	Genome Wide Association Studies
Indels	Insertions and Deletions
MB	Mega Bases
NGS	Next Generation Sequencing
rDNA	Ribosomal DNA
SMRT	Single Molecule Real Time (Sequencing)
SNP	Single Nucleotide Polymorphism
TB	Tera Bases
TE	Target Enrichment
ZMW	Zero Mode Waveguide

Long answer questions

1. Define next generation sequencing and its applications in Animal Biotechnology.
2. What are different generations of DNA sequencing? Describe their principle and applications.
3. Describe workflow of next generation sequencing in different applications.
4. Describe use of next generation sequencing in human health and its translational significance.
5. Describe various next generation sequencing technologies, their advantages, limitations and specific uses.

Short answer questions

1. What is next generation sequencing and how it is different from Sanger sequencing.
2. Give a brief description about different generations of next generation sequencing.
3. Give a general workflow of next generation sequencing indicating differences for various applications.
4. What do you understand with sequence assembly? Define de novo and mapping assemblies.
5. What is targeted sequencing. Describe in brief its use in medical research.

Answers to short answer questions

1. Next generation sequencing (NGS) is a term given to sequencing technologies post-Sanger sequencing methods. NGS can produce huge amount of sequencing data at incredibly low cost.

   While sanger sequencing works on the principle of chain termination, whereby the growing DNA strand is terminated by the incorporation of dideoxy nucleotides (ddNTPs), A, T, G, and C in different reactions, Next generation sequencing works on the principle of sequencing millions of DNA fragments simultaneously in a massively parallel mode. Sequencers based on Sanger

sequencing produce total sequencing data output of few hundred base pair at a time, NGS can produce sequence data in megabases or gigabases.

2. The "Generation" refers to the chemistry and technology used by the sequencing process. The first generation sequencing was the Sanger and Maxam–Gilbert sequencing, which were able to sequence few hundred base pairs at a time, used for individual gene sequencing. Sequencing 3 billion base pairs of the human genome would take a very long time with Sanger sequencing, as it needs to sequence about 6 million fragments of DNA with 500 bp length.

   Next generation sequencing technologies, also termed as second-generation sequencing, have been able to parallelize the sequencing reaction in a massive manner, thus generating huge amount of data very rapidly at a modest cost, thus revolutionized the field of genomics. It includes Roche 454, Illumina Solexa, and ABI-SOLiD technologies.

   The term third-generation sequencing or next-next generation sequencing is given to technologies which interrogate single molecules of DNA, without amplifying them through PCR, thereby overcoming issues related to the biases introduced by PCR amplification and dephasing. However the third-generation sequencers were developed with a vision of making sequencing cheaper than second-generation sequencing. Third-generation sequencers includes those of PacBio and Helicos.
3. Next generation sequencing can be used in a number of ways depending on the application. In every type of sequencing, the major changes occur in the sample processing and library preparation steps. Once the library is prepared, a particular sequencing platform uses the same chemistry to sequence the fragments. Major types of sequencing used for different types of applications are shown in Fig. 20.3. An outline of the protocols used in various applications is summarized in Fig. 20.4.
4. Assembly is the process of using various computer programmes to align multiple sequencing reads that are overlapping with one another to reconstruct the long DNA fragment, may be the whole genome, as DNA sequencing cannot read whole genomes in one attempt, but reads small pieces of different length, depending on the technology used. De novo assembly refers to assembling reads to build a new sequence, where no existing sequence of same or related organism/ species is available. Mapping pertains to assembling reads against an already existing backbone sequence of same or related organism/species to build a sequence that is similar but not necessarily identical to the backbone sequence. Mapping is generally used to figure out genomic variation in the sequenced DNA compared with the reference sequence such as SNPs, insertions, deletions, and structural variations.
5. Targeted sequencing refers to sequencing regions of interest in the genome rather than sequencing whole genome. The most popular one is sequencing the exon part of the genome to get rid of unwanted introns. Targeted resequencing can be used to examine all the exons in the genome, a part of the genome, specific gene families that are supposed to constitute known drug targets or regions and are thought to be involved in disease or pharmacogenetics effects through genome-wide association studies.

Yes/No type questions

1. Is Sanger sequencing is also called “Next Generation Sequencing”?
2. Is Illumina’s reversible terminator sequencing technology a “short read” next generation technology?
3. Is Pac-Bio also a short read technology?
4. Is data interpretation for biological significance called Tertiary analysis?
5. Is targeted sequencing done without a reference genome?
6. Is the first-time sequencing of an organism without a reference genome available called “de novo” sequencing?
7. Is RNA-seq also used to detect novel fusion transcripts?
8. Is Exome sequencing also called RNA-seq?
9. Can metagenomics be done with WGS and 16-S amplicon sequencing?
10. Can NGS also be used for animal breeding and livestock productivity?

Answers to Yes/No type questions

1. No—Sanger sequencing is conventional sequencing, which is not massively parallel.
2. Yes—Illumina produces short reads in the range of 250–350 bp.
3. No—Pac-Bio produces reads in the range of several KB and known as long-read technology.
4. Yes—Primary analysis is base calling, secondary is alignment and variant calling, and tertiary is data interpretation.
5. No—For targeted sequencing a reference genome is needed for alignment.

6. Yes—As newly sequenced organisms don't have reference genome, alignment of their sequencing data is done first-time, that is de novo.
7. Yes—RNA-seq is used to detect mutations, gene expression, and fusion transcripts.
8. No—Exome sequencing is done with DNA by targeting exons- regions which encodes for proteins.
9. Yes—Metagenomics is a mixed population study which can be done by either of the above methods.
10. Yes—NGS is transforming animal breeding, due to low cost involved in WGS studies facilitating identification of genetic markers at a genome wide level.

CHAPTER

21

Biomolecular display technology: a new tool for drug discovery

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Summary

The identification of molecules with desired function is of a great significance in biology and medicine. Display technologies represent a new tool for the drug discovery by facilitating the screening of novel biomolecules against any target of choice. This chapter reviews the development of in vitro display technologies and their application into drug discovery, focusing on challenges and perspective for the rapid and efficient modern drug discovery process.

What you can expect to know

Discovering and developing safe and effective drugs is one of the most challenging scientific endeavors. However, the current decreasing number of approved drugs produced by the pharmaceutical industry and the increasing number of new, emerging and re-emerging diseases, demands a technology-based paradigm shift toward developing a new generation of therapeutics drugs based on the understanding the mechanisms at molecular level. Most drug development fails because of unacceptable toxic side effects of a drug and a barely of our knowledge to understand the binding of an arbitrary drug to an arbitrary target (diseased protein). A new generation so-called biomolecular drugs, especially peptide aptamers, was born to meet the demands. The peptides aptamers are combinatorial protein reagents characterized by high specificity and strong affinity to their targets and some other advantageous properties such as a versatile selection process, ease of chemical synthesis and small physical size, which collectively make them a potential therapeutic agent. Recently, peptides have shown a significant impact on the success rate of drug discovery. This chapter is focus on the technological advancement and is particularly emphasized on the significance of "display technologies" toward developing peptides aptamer as a novel tool to discover the target, biologically relevant druggable sites and hit drug compounds.

Box

Test-tube evolution that used display technology awarded The 2018 Chemistry Nobel Prize.

Introduction

The global war against diseases appeared to be on the road to victory when Dr Edward Jenner discovered vaccination in 1796, one of the greatest discoveries in medicine. The next revolution was a period of accidental discoveries in the middle of the 20th century where the discovery of a drug was happened primarily by observing the therapeutic effects followed by chemical isolation. Aspirin and penicillin are the well-known examples of these fortuitous discoveries. The further revolution occurred in the latter half of the 20th century where the discovery of a drug was driven by introducing force screening of large libraries of chemical compounds with some preliminary knowledge. Statins are one of the examples that lower cholesterol levels in patients with heart disease. However, it is

Animal Biotechnology.
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recent that we identified two most challenging yet indispensable issues in the healthcare field: first, the advent of new emerging infectious diseases and the re-emergence of old diseases, combined with the rapid spread of pathogens resistant to drugs and of disease-carrying insects resistant to insecticides. Swine flu is the most recent example. It is thought to be a mutation, more specifically, a reassortment of four known strains of influenza A virus subtype H1N1. Second issue is "tailoring care" to the individual patient by treating the disease specifically and not by following "one-treatment-fits-all" formula. Sickle-cell anemia is the best example where patients have a similar genetic lesion in the beta-globin gene, but their phenotypic diversity ranges from life-threatening to symptom-free states. Now, we are entering an era of personalized medicine that has already became clearer in 2003 when the international Human Genome Project completed a blueprint of the human genome and conveyed the fact that no two individuals are exactly alike, especially in terms of medicine. Therefore a paradigm shift in medicine is essentially required with an increased interest in the discovery of new drug candidates. However, the productivity crisis of the pharmaceutical industry currently introduces fewer drugs onto the market than was the case in late 20th century (Kola and Landis, 2004; Pammolli et al., 2011). Drug candidates with the lack of efficacy and too high toxicity mainly caused by insufficient validation of therapeutic targets and/or insufficient specificity of drug candidates were identified as the major causes of this attrition. Therefore a clear technology-based shift is essentially required to address major causes of failure and to offer a powerful drug discovery approach, enabling a seamless process from target identification and/or validation to the identification of hit drug candidates.

The completion of Human Genome Project in 2003 provides an unprecedented opportunity to elucidate the genetic basis of human disease at the molecular level. This information on gene and their link to disease(s) is comprehensively stored in several public databases (George et al., 2008). Among these, GeneCards database has identified 3204 genes that are associated with human diseases (total number of human genes are estimated near 25,000). Out of these, 523 genes that are reported to encode disease-related proteins are druggable (druggablity is the likelihood of being able to modulate a target with a drug, of Human Genome) (Russ and Lampel, 2005). Near 215 of these disease genes are included in DrugBank database with drugs availability and only 89 of the disease genes from this dataset have Food and Drug Administration (FDA)-approved drugs available (see Fig. 21.1) (Sakharkar et al., 2007). Therefore with near 85% of the human disease genes representing druggable targets with no drugs available, it is important to develop a powerful approach with a significant impact on the success rate of drug discovery.

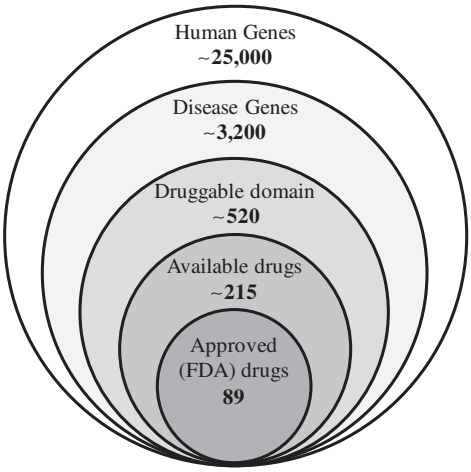


FIGURE 21.1 Cartoon representation of the current status and need of a paradigm shift in modern drug research and development.

Principle

The discovery and development of a new drug has become one of the most challenging scientific endeavors. How an individual new drug discovered and brought to the public is a very long, complicated process that typically cost close to $1 billion and takes an average of 10–15 years (see Fig. 21.2).

The drug discovery process starts by identifying cellular and genetic chemicals in the body that play a role in specific diseases, called "Target identification." After choosing a potential target, "Target validation" step is followed to insure that it actually is involved in the disease and can be acted upon by a compound (drug). In next, compounds are then identified that have various interactions with these targets and screened based on their comparative association with a desired change in the behavior of diseased cells. This is followed by the next step of "Lead identification." A lead compound is one that is believed to have potential to treat disease. Testing and optimization are then done on each of the lead compound by performing in vitro (testing on cells in the test tube) and in vivo (testing on living organisms) confirmation followed by clinical evaluation. In the preclinical step, the lead compound is tested extensively to ensure it will be safe to administer to humans. Testing at this stage can take from 1 to 5 years and must provide information about the pharmaceutical composition of the lead compound (drug), its safety, how the drug will be formulated and manufactured, and how it will be administered to the first human subjects. In the final step, the clinical testing of drug is

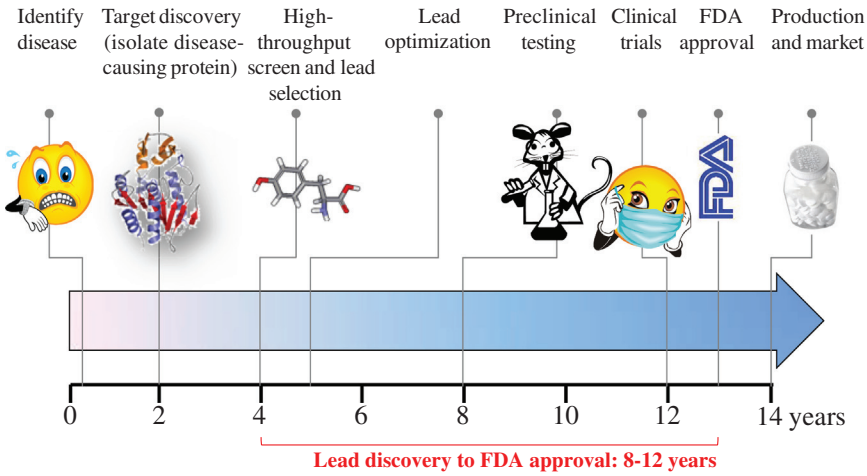


FIGURE 21.2 Stages and timeline in the drug discovery process. The initial phase, where promising targets are first discovered and linked to a disease, is shown here as taking 1–2 years, but it can require several years.

done by starting administration to healthy volunteers or patients. This is usually consisting of Phase I, Phase II, and Phase III where in each successive phase, increasing numbers of patients are tested. After the conclusion of successful preclinical and clinical testing, the drug is processed to FDA for marketing authorization. In general, out of several thousand new compounds identified during the discovery process, approximately few are considered safe for clinical testing in patients, and only one of these compounds on average is ultimately approved as a marketed drug for treatment.

Therefore drug discovery is plagued by time-consuming and costly processes, with extremely high failure rates. The FDA also estimated that just a 10% improvement in the ability to predict drug failures before clinical trials could save US$100 million in development costs per drug. The high failure rate is in principle originated during the lead generation and optimization steps. It may not be possible to speed up clinical trials, but bottlenecks in lead discovery can be tackled. What is important in lead discovery process is that understanding principles is much more critical than calculating accuracy. For example, instead of measuring binding affinity to few decimal places, calculating relative trends in binding affinity could be more meaningful. Therefore explaining "why" (e.g., why a small change in a drug causes a large change in its activity or why one enantiomer causes side effects while another does not) in turn can reduce the failure rate and drive better drug development. Needless to say that selecting the most efficient compounds and introducing high-throughput screening system will dramatically change the picture.

Necessity: small molecule versus biomolecular (biologics) drugs

A systematic search of a drug was first advocated by Paul Ehrlich, the founder of chemotherapy, who received the Nobel Prize for Medicine in 1908 for his work on magic bullet concept (where a compound is used to target a particular biomolecule of interest). Essentially, this biomolecule is an intracellular or membrane-bound protein identified as contributing to a disease state. Targeting these proteins by chemical agents led the discovery of organic small molecules for the treatment of various types of diseases including infectious diseases and cancer. However, the entries of newly approved chemical drugs are decreasing recently due to the lack of efficacy and very high toxicity of drugs. Since most of all biological processes are mediated by proteins owing to their remarkable capability of molecular recognition and specific interactions with other molecules, it is natural that the protein-based biopharmaceuticals have emerged as a new generation of therapeutics drugs. Among them, monoclonal antibodies such as "Trastuzumab" or "Centuximab" have gained the remarkable success in biomedical area. However, several disadvantages have become apparent with the increasing application of antibodies including low bioavailability due to large size, limited shelf life, difficult to produce biologically, viral or bacterial contamination during manufacturing process, and immunogenic effects. To reduce the immune response they inherently trigger, efforts were devoted to design new generation of antibodies switching from murine antibodies to chimeric (human 60%, murine 40%, e.g., *Centuximab*; human 90%, murine 10%, e.g., *Trastuzumab*; human 100%, e.g., *Panitumumab*). Meanwhile, insight into molecular biology was led to the invention of systematic evolution of ligands by exponential enrichment (SELEX) process for in vitro selection of high-affinity oligonucleotides (Gold and Tuerk, 1990; Ellington and Szostak, 1990). A number of DNA and RNA aptamers (aptamers are short biomolecules such as DNA/RNA/peptides chain, capable of identifying a target molecule with high affinity and

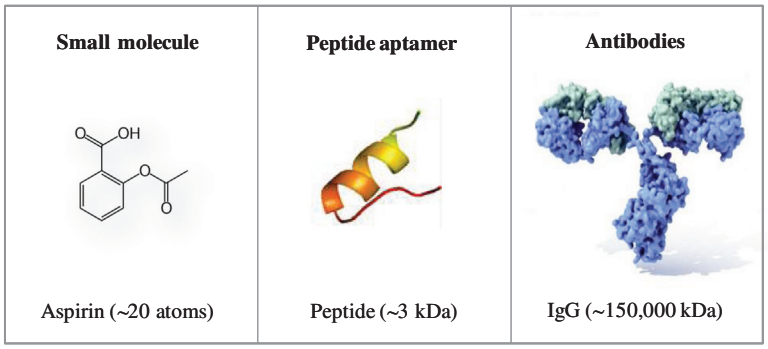


FIGURE 21.3 Comparison between major types of therapeutics.

	Antibody	Peptide aptamer
Size	30 ~180 Kda	1~5 Kda
Dissociation constant (K_d)	10 nM	10 nM or can be improved
Library size	10^9	$10^{12–15}$
Cost	Expensive	Inexpensive
Quality control	Unstable	Stable
Immunogenicity	High[11]	Less
Chemical modification	Difficult	Possible (unnatural amino acid)
Optimization	Difficult	Easy
IP Problems	Unsolved	Solved
Shelf life	Limited and sensitive to temperature	Long and stable
Production	Difficult to synthesize, requires animals, suffer from batch to batch variation	Produced by chemical synthesis (no animal) resulting in little or no batch to batch variation and extremely simple and robust

specificity) have been generated that can bind to multiple targets in a similar mode to that of antibodies. The first RNA aptamer-based drug *Pegaptanib* was approved for therapeutic use by FDA (Gragoudas et al., 2004). Very recently, the peptide aptamers have also been emerged as an attractive alternative of antibody therapy. Unlike antibodies, the peptide aptamer sequences are short, easy to synthesize, chemically stable and less immunogenic. However, the most clinically used drugs are small molecules or proteins, but each of these differ much in their properties (Fig. 21.3). Small molecules, because of their size, are advantageous for their accessibility (cell permeability and tissue penetrations), whereas proteins provide high binding affinity and target specificity. On the other hand, peptide, as shown in the middle of Fig. 21.3, can potentially combine favorable properties of both small molecules and proteins. Such as, peptide can bind as tightly and specifically as antibodies while being small enough to enter into the cells and tissues.

Recent technological advances allowed the development of peptide aptamers that can bind to protein targets with high affinity and inhibit the function with high specificity (Crawford et al., 2003). In contrast to the inhibitory abilities of peptide aptamers, some peptide aptamers could be screened to activate the function of their cognate target proteins (Nouvion et al., 2007). Therefore peptide aptamers have big advantages over small molecules in terms of specificity and affinity for targets, and over antibodies in terms of size and thus great potential to further explored as future therapeutic drugs. The recent advancement of high-throughput screening systems and in vitro molecular selection technologies can allow the identification of novel and potentially commercially relevant peptides. These will be discussed in following sections.

Methodology: biomolecular display technologies

The drug discovery process to develop new pharmaceutical compounds has depended traditionally on empirical approach of screening. However, rising the R&D costs, short product life cycle and the public's ever increasing demand for new, high-quality treatments, the pharmaceutical companies are encountering pressure to develop new drug faster, more cost-effectively and in

greater numbers than ever before. These pressures have dramatically transforming the process by which new drug are being discovered and developed and motivated the pharmaceutical companies to collaborate biotechnological companies for technological solutions to the need to enhance and accelerate the drug discovery process. Therefore the discovery of highly functional and biologically active molecules is necessarily required for future therapeutics.

Biomolecular display technologies, which allow the construction of a large and diverse pool (millions to trillions) of biomolecules, their display for property selection, and rapid characterization (decoding) of their structures, are particularly useful for accessing and identifying a novel and potential candidate for drug discovery. These display technologies, basically, mimic the process of natural evolution and, in general, rely on a common principle of coupling (linkage) of individual biomolecules (DNA/RNA) with its encoded product (peptide or protein). A typical display module in biomolecular display technologies is consist of three major components: displayed entity, a linker, and the corresponding genetic code (Fig. 21.4A).

A number of display technologies have been developed and provided essential tools to enrich biomolecular drugs including nucleic acids and proteins (peptides) with desired functions based on evolutionary molecular engineering and combinatorial chemistry (Li, 2000). Over the past two decades, many display formats have been developed, which use different types of displayed entities, linkage formats, and coding strategies. These all can be systematically classified into major two categories based on their expression system, cell-based type or cell-free type (Fig. 21.4B).

Phage display is one of the first methods described by George P. Smith in 1985 by displaying a foreign peptide on the coat of filamentous phage (Smith, 1985). Since then, phage display and two other introduced bacterial and yeast surface display technologies have been used as a widely adopted technology (Francisco et al., 1992; Boder and Wittrup, 1997). However, cell-based type display technology has its limitations and includes slow speed (few days to weeks), limitation of library size due to the transformation efficiency of host (approximately 10^8), and the toxicity to the host cells caused by the expression of exogenous proteins. This

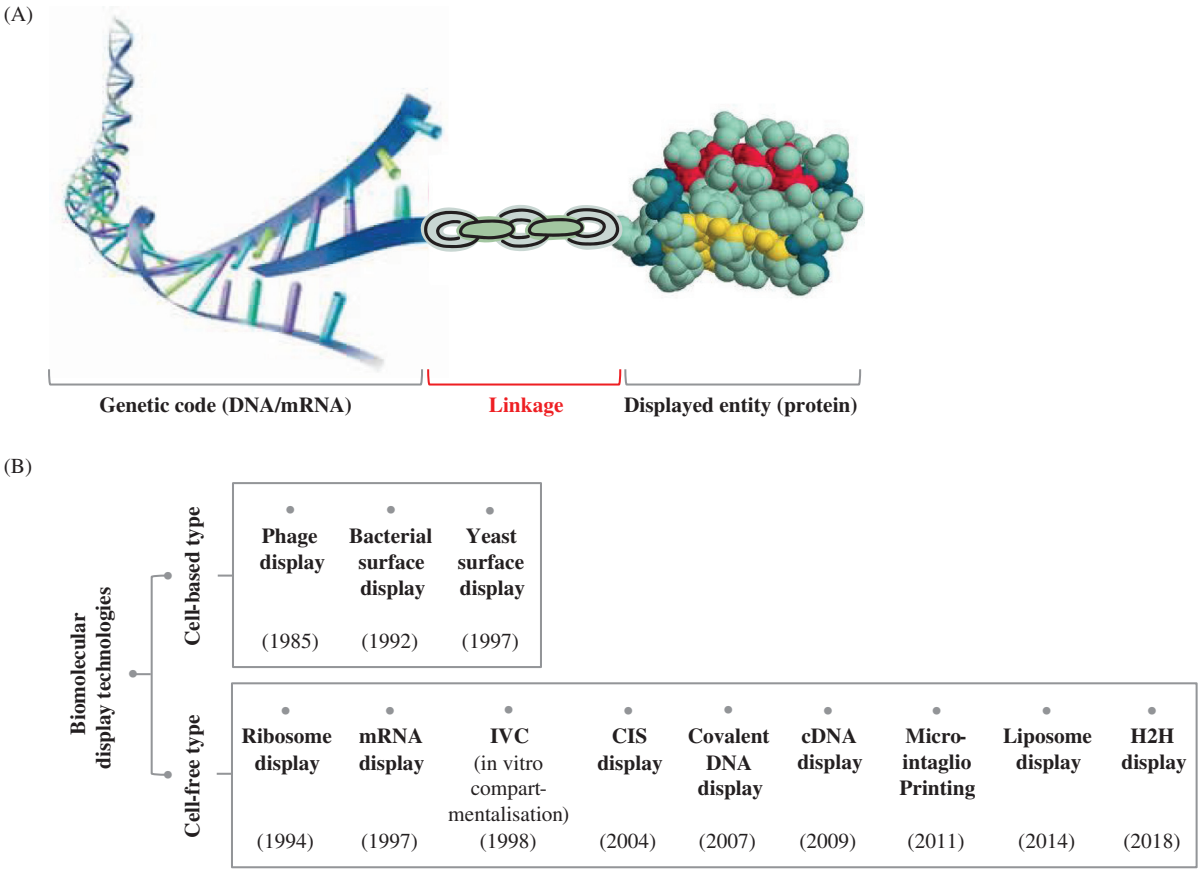


FIGURE 21.4 An illustration of a typical display module used in biomolecular display technologies (A) and a timeline of the different biomolecular display technologies developed till date (B).

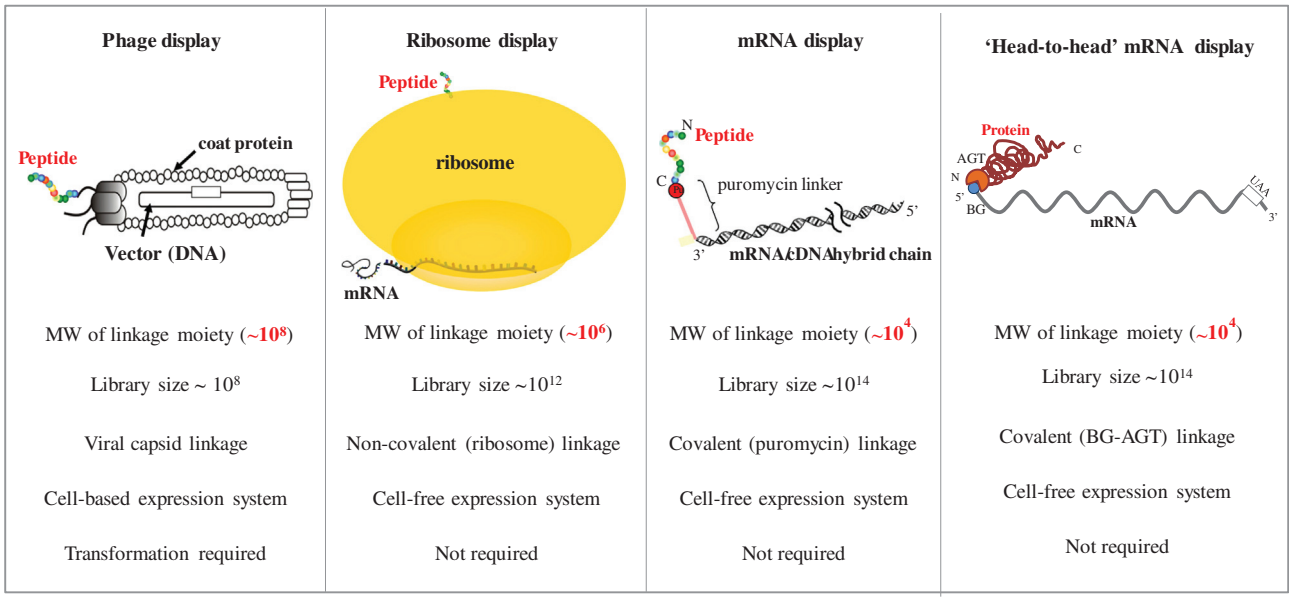


FIGURE 21.5 Illustration and comparison of the most common biomolecular display technologies. In phage display, an indirect linkage (physical) between the gene and gene product is provided by the viral capsid. In ribosome display, a noncovalent linkage is achieved by producing ternary complexes of RNA, ribosomes, and associated nascent peptide. In the mRNA display system, a covalent linkage between C-terminus of protein and 3′-terminus of mRNA is generated through a puromycin molecule attached to the encoding mRNA via a short DNA linker molecule and in the "head-to-head" mRNA display, a covalent linkage between N-terminus of protein and 5′-terminus of mRNA is generated through a BG-AGT chemistry.

led to the generation of cell-free type display technologies, which avoid the need for transformation and enable more of the sequence landscape to be displayed and an increased probability of higher-affinity hits. The very first of cell-free type display technology, ribosome display, was originally developed in 1994 using *Escherichia coli* lysate for the display of peptides onto the ribosomes (Mattheakis et al., 1994). During the cell-free expression, the ribosomes were stalled on the mRNA template and the nascent peptide remained in a complex, which could then be recovered by EDTA. In next, a related technology, termed mRNA display (or in vitro virus), was reported by the formation of a covalent linkage between the mRNA template and the expressed protein via puromycin and that gives advantages of speed and stability over ribosome display (Nemoto et al., 1997; Roberts and Szostak, 1997). In 1998, Tawfik and Griffiths introduced man-made cell-like compartments using water-in-oil droplets, termed in vitro compartmentalization, which provides an alternative way of linking phenotype and genotype to mimics the natural compartments of living organisms (Tawfik and Griffiths, 1998). Later on, some other display technologies were introduced to demonstrate their potentiality for unprecedented library size and stability issues: CIS display (Odegrip et al., 2004), covalent DNA display (Bertschinger et al., 2007), cDNA display (Yamaguchi et al., 2009), micro-intaglio printing (Biyani et al., 2011), Liposome display (Fuji et al., 2014), "head-to-head" display (Sharma et al., 2018).

The aim of this chapter is to focus on the recent advancements in those display technologies that are particularly emphasized on the development of peptide aptamers and their prospective advantages over the antibodies-based target disease diagnosis and therapy. The three major display technologies including phage display, ribosome display, mRNA display and its extended form "head-to-head" mRNA display are schematically shown in Fig. 21.5 and are discussed in following sections.

Phage display

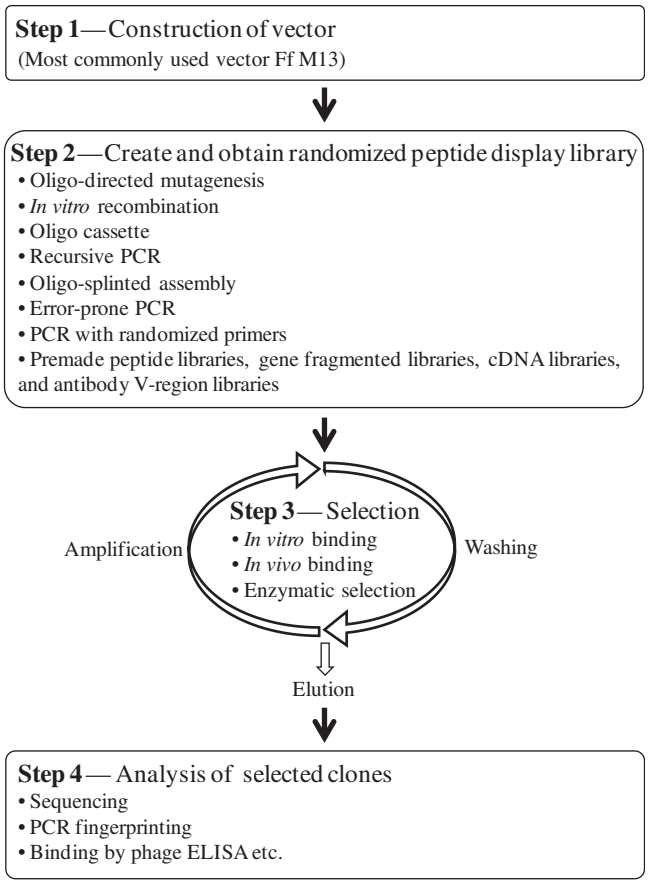
Phage display is one of the first methods that introduces an indirect linkage (physical) of a protein with its DNA sequence and the widely adopted technology so far for the discovery of novel drugs (Smith and Petrenko, 1997). To provide the linkage of protein and the encoded nucleic acid, it uses a bacterial virus. First, a gene library is genetically fused to a filamentous bacteriophage so that on co-infection by the phage, newly emerging phage particles encase individual gene sequences while displaying the corresponding gene-encoded polypeptides on their outer surface (see Fig. 21.5). In next, the recombinant phage DNA is transformed into *E. coli* cells resulting in large numbers of bacterial clones, each containing the coding

sequence for unique library member. The expressed protein is incorporated into the viral coat, and cloned library DNA is packed within the virion. For the selection of potential candidates, the diverse pools of recombinant phage particles are incubated with an immobilized target molecule. Nonbinding particles are washed away, and those library candidates that bind specifically to the target are eluted and amplified by infection into fresh *E. coli* cultures. In this way, potential library members are enriched from very large libraries based on their binding affinity to target molecule (antibodies, enzymes, cell-surface receptors). The phage display technology provides the flexibility that selection may be performed in vivo and in vitro conditions.

Flow Chart 21.1

A methodology of phage display technology is involved in the following steps:

1. In step 1, the filamentous phage M13 is used commonly for the vector construction.
2. In step 2, the library is generally created or obtained through various methods as described in the flow chart. The diversity of library size from 10^{10} to 10^{11} can be generated within easy reach. The DNA library is inserted into N-terminus of either the major coat protein, Gp8, or a minor tip protein, Gp3, of filamentous phage. Then, the target protein or the peptide is displayed as a fusion protein with Gp8 or Gp3 on the surface of filamentous phage.
3. In step 3, phage displayed library is selected through in vitro binding (the most common selection method) to a target, washed, and then the retained phages are eluted. This process is also referred to as "sorting or biopanning." The eluted phages are used to re-infect *E. coli* for the preparation of new phage (amplified to allow further round of selection).
4. In step 4, the selected clones are identified and determined the properties of displayed proteins. The most common technique for identifying selected clones is simply to sequence them and alternative approach is PCR fingerprinting (insert is amplified and digested with a frequent-cutting restriction enzyme. Pattern of bands is specific for each clone). The phage ELISA is the most popular and common technique for the selection of clones.



FLOW CHART 21.1

Selection of peptides

The phage display has been successfully applied for the isolation of peptides that bind protein targets with high affinity and specificity. Few examples include: peptides that bind angiotensin-converting enzyme 2 with a Kd ranged from 140 to 3 nM (Huang et al., 2003); peptides that bind each of three members of the inhibitor of apoptosis family with the Kd ranged from 160 to 440 nM (Franklin et al., 2003); peptides that bind to protein kinase Cα, an intracellular target, only under activation conditions (Ashraf et al., 2003); peptide (HTMYYHHYQHHL) that binds to the VEGF receptor kinase domain-containing receptor and slows the growth of breast carcinoma BICR-H1 tumors in mice (Hetian et al., 2002); and peptide that binds to recombinant human ErbB-2 tyrosine kinase receptor, which is implicated in many human malignancies, with Kd ranged from 3 nM to 5 μM (Huang et al., 2003). Most often, one or more families of related peptides are found. The common motif of one family can then be built into a secondary library and higher-affinity peptides selected. In most cases, these peptides have stronger affinities in the range of 1–10 nM. Usually, several families of binders are found to not compete for binding to the target, presumably because they bind different sites. However, when two of these molecules that bind at nonoverlapping sites are joined with a suitable linker, such as several units of PEG, the affinity of the heterodimer is usually much greater than the affinity of either component. An added advantage of such a heterodimeric molecule is that it covers a larger area of the active site on the target and is more

likely to interfere with protein–protein interactions. Phage display method has been widely used display method so far for the discovery of diagnostic and therapeutic molecules; however, the technology has the limitations. The major drawbacks of this technology are the limitation of library size by the transformation efficiency of bacteria (generally 10^{7-10} members) and the toxicity to the bacterial cells caused by exogenous protein expressions.

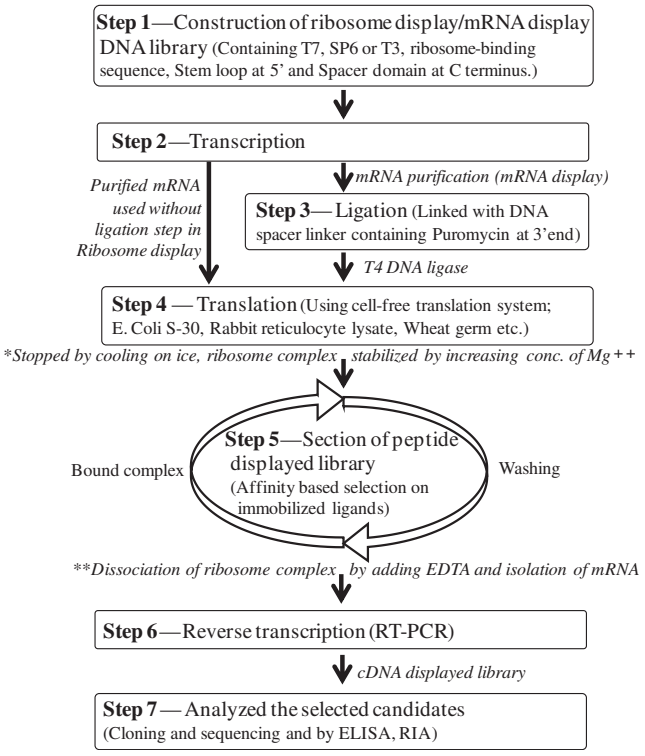
Ribosome display

Ribosome display is the first described entirely in vitro display technology (Mattheakis et al., 1994). It has been developed by generating protein–ribosome–mRNA ternary complex for selection in cell-free translation system (see Fig. 21.5). The key idea is to translate a library of mRNA molecules that has no stop codon using cell-free system. As the translation processes, the ribosome will run and stall to the very end of the mRNA molecule and does not release the encoded protein since the last amino acid of protein is still connected to the petidyl-tRNA. Thus the ribosome that translates mRNA without stop codon will be trapped in a form where the protein has emerged from the ribosome and the mRNA is also still connected to the ribosome. The resultant mRNA–ribosome–protein ternary complexes are then used for affinity selection on an immobilized target. mRNA of bound complexes is recovered after washing steps, reverse transcribed, and amplified and identified.

As this system is performed entirely in vitro, there are two main advantages over cell-based methods. First, the diversity of the library is not limited by the transformation efficiency of bacterial cells, but only by the number of ribosome (can be up to 10^{14}/mL) and the number of mRNA molecules (a huge potential diversity of above 10^{12} members can be generated easily by PCR) available in the reaction test tube. Thus the larger accessible library renders ribosome display a superior opportunity to select rare sequences with high-affinity properties. Second, random mutations can be introduced easily during PCR steps since no library need to be transformed after any diversification step. Thus further diversity can be continuously introduced into the DNA pools after selection, making this technology as an efficient route for facile-directed evolution of biomolecules.

Flow Chart 21.2

A methodology of ribosome display/mRNA display technology is involved in the following steps:



FLOW CHART 21.2

1. In step 1, a random DNA library containing T7 promoter, ribosome binding site, and stem-loop and encoded polypeptide of interest is constructed and amplified by PCR.
2. In step 2, the constructed DNA library that does not carry the stop codon is transcribed into mRNA and purified mRNA (without ligation in ribosome display and with ligation in mRNA display as shown in the flow chart) is used for cell-free translation reaction.
3. In step 3, which is specifically for mRNA display method only, the resultant mRNA is covalently linked to a puromycin-attached linker DNA by hybridization and T4 RNA ligation reaction.
4. In step 4, mRNA is used as a template for in vitro translation to generate the ribosome display or the mRNA display protein fusion (*for enhancing the stability of ribosome complexes, the concentration of Mg^{++} is increased and the purified mRNA–protein fusion is stabilized by converting mRNA into cDNA using reverse transcription reaction).
5. In step 5, the desired ribosome complexes or the cDNA/mRNA–protein library are followed by affinity-based selection reaction. The library is allowed to bind with the immobilized target protein and those library components that bind the target weekly or non specifically are removed by washing steps (**the bound ribosome complexes can be dissociated by EDTA or whole complexes can be

specifically eluted with target and then mRNA is isolated). The recovered library is then enriched for target binding sequences.

6. In step 6, isolated mRNA is reverse transcribed to cDNA (in ribosome display). The cDNA (in both ribosome display and mRNA display) is then amplified by PCR and used for next round of selection. In general, 3–6 rounds of selection are required to select the peptide with low nanomolar or sub-nanomolar affinity to target, depending on library complexity and target protein. To select even higher affinities (pM), special strategies (off-rate) are required.
7. In step 7, the selected candidates are identified by cloning and sequencing and the binding affinity to target protein is determined by the most common methods such as ELISA or SPR.

Selection of peptides

Ribosome display has successfully applied for in vitro antibodies selection, evolution, and humanization. Few examples include: a 10-mer peptide that bind with dynophin B with 0.29 nM affinity (Mattheakis et al., 1994); a 15-mer streptavidin-binding peptides with an affinity of about 4 nM (Lamla and Erdmann, 2003); and a 20-mer peptide that bind with prostate-specific antigen, a tumor marker (Gersuk et al., 1997). Despite encouraging results from ribosome display selection, it is suffered with mechanistic challenges such as a larger size of ribosome moieties are to hinder the specific binding of displayed peptides to the target protein. Another issue is the inherent instability of the resulting protein–ribosome–mRNA ternary complexes that makes it difficult to keep this ternary complex intact during the selection step and thus restricts the choice of experimental conditions.

mRNA display and "head-to-head" mRNA display

A more robust cell-free method was devised for the display system and termed as mRNA display or in vitro virus (Nemoto et al., 1997; Roberts and Szostak, 1997). Like ribosome display, mRNA display uses a complex between mRNA and the polypeptide encoded by that mRNA as the basic unit of selection. What makes mRNA display more robust from ribosome display is the covalent nature of the linkage between the mRNA and the protein in the mRNA–protein complex. This is achieved by bonding the two macromolecules through a small adaptor molecule, typically puromycin, which are ligated in advance at the end of the mRNA molecule through a short linker DNA molecule (see Fig. 21.5). Puromycin serves as a chemically stable small molecule mimic of aminoacyl tRNA. So, in the case of no stop codon and when the ribosome reaches at the end of the mRNA template, puromycin enters the peptidyltransferase site to form a covalent bond with the nascent peptide chain and makes mRNA–protein fusion.

All aforesaid display methods have been useful for use in affinity-based selection. However, to identify the functional molecules that are, for example, inhibiting/activating, agonistic/antagonistic, catalytic, or of all these types, in principle, there is a need to employ the cloning process of the affinity-selected products and to examine the cloned products one by one; this requires multiple processes and is very time consuming in general. To circumvent the necessary cloning processes, various inventions have already been made, such as compartmentalization methods [water-in-oil method (Tawfik and Griffiths, 1998; Lu and Ellington, 2013), Micro-intaglio printing method (Biyani and Ichiki, 2015), liposome method (Fuji et al., 2014), and novel concept microarray with manageable volumes (MMV), which enable us to introduce function-based selection (Sharma et al., 2014)], "selection-by-function," which involves the breakage of a link caused by the selection product (nuclease or protease), and genetic selection. In this stream, a novel approach has recently been devised for function-based selection beginning with a large library size (10^{13}): mRNA display-cooperated compartmentalization method (termed MMV Clop; to appear elsewhere), which demands a "head-to-head" linking in the mRNA display construct (i.e., heads mean 5′-terminus of mRNA and N-terminus of protein), so as to realize the iterative translation of proteins (Fig. 21.5) (Sharma et al., 2018). In other words, this linking method, as opposed to the conventional mRNA display (i.e., "tail-to-tail"), enables two roles for this construct: the linking of genotype (mRNA) and phenotype (protein) and generation of multi-copied proteins. The latter is essential for the detection of protein functions and is unavailable for the conventional tail-to-tail method due to the removal of the stop codon. Therefore the most recently reported "head-to-head" mRNA display enables the direct amplification of each protein of a library captured in a compartmentalized space and thus allows a functional assay to be performed in situ, owing to a detectable amount of protein. It also makes a diversity of post-translational modifications of the C-terminus such as C-terminal methylation. Thus the H2H mRNA display deserves to be pursued for the progress of in vitro evolution of functional proteins.

This flow chart is collectively described in the flow chart given in the "Ribosome display" section.

Selection of peptides

Streptavidin-binding peptides that contain at least one copy of histidine–proline–glutamine was successfully selected by mRNA display with a Kd of 2.5 nM, which is in contrast much stronger than the peptide selected by phase display with a Kd of 13–72 μM (Wilson et al., 2001). A disulfide-constrained library based on EETI-II, a knottin trypsin inhibitor, was constructed by randomizing the six residues of the trypsin-binding site, and mRNA display was used to select new trypsin-binding peptides. The selected peptides were highly homologous or identical to wild-type EETI-II and their dissociation constants from trypsin ranged from 16 (for the wild type) to 82 μM (Baggio et al., 2002). Recently, peptide aptamers have been selected that bind with high affinity and specificity to the internal ribosomal entry site of hepatitis C virus mRNA (Litovchick and Szostak, 2008).

The mRNA display system is apparently the most widely used in vitro selection method for not a few reasons such as being in vitro translation, which enables both an expanded range of experimental conditions and unnatural amino acids to be incorporated by the exploitation of suppressor codons and being of a greater diversity in the molecular library. However, still there are few other challenges remain to be solved. In mRNA display, the purification of protein–puromycin–mRNA adducts from the ribosome presents a topological puzzle. After translation, the protein folds outside of the ribosomal tunnel to a globular domain. At the other end of the tunnel, the puromycin–mRNA reagent reacts with the polypeptide. Thus a folded domain sits at one end of the tunnel, while the long mRNA is connected to the peptide at the other end. Whereas the purification is performed under conditions expected to dissociate the ribosome, no direct evidence is yet available that an "opening" of the tunnel takes place. Alternative explanations are that (1) the mRNA passes through the protein exit tunnel or (2) the protein denatures and goes "backward" through the tunnel. If such denaturation of the displayed protein is required, that might limit the application of mRNA display to proteins with robust refolding properties.

Other display systems

Several other strategies linking the displayed protein directly to the encoding DNA including CIS display, covalent DNA display, cDNA display, have been described. These technologies are in principle analogous to the mRNA display but using the DNA molecule instead of mRNA molecule could be advantageous due to the higher stability of the DNA molecules in comparison to labile mRNA molecules. It is not evident that one selection system is clearly advantageous over the others, since they all have their advantages and limitations (Fig. 21.5). Thus there is a high demand for an efficient, sophisticated and combined in vitro evolution method that can address following shortcomings to generate novel functional peptide aptamers for drug discovery:

1. The high diversity and the quality of library are the vital strengths for the success of an in vitro selection method. Various methods have been reported, including the most popular error-prone PCR and DNA shuffling methods for the construction of primary library and subsequent libraries. It is yet to be a puzzle how to choose a mutation method for making suitable and efficient primary and subsequent libraries. Therefore a method is required that has ability to generate and combine a wide range of sequence diversity.
2. All in vitro selection methods discussed above are advantageous especially in finding the peptide aptamers of high affinity from huge molecular diversity. Ideally, for the discovery of therapeutic agents, a method can be more advantageous if it can select the molecules based not only on their binding but also on their functional abilities.
3. The various methods have been developed so far to identify mainly enzyme inhibitors. However, there is no such general method developed to identify activators despite their emerging opportunities in drug discovery.
4. A general platform that can be widely applied for any types of protein targets is desirable, especially in the age of personalized medicines when diversities of drugs are required.

A general method for discovery of functional peptide aptamers

To tackle all challenges discussed in above sections, Nishigaki group has recently developed a method called evolutionary rapid panning analysis system (eRAPANSY) (Kitamura et al., 2009) for acquiring protease-inhibiting peptide aptamers. This method consists of primary library construction and selection and then construction and enrichment of the secondary library using the primary library selection products. It enabled us to obtain cathepsin E-inhibiting peptides with higher affinity and activity. What is highlighted here that, in the course of evolution, the "information" acquired in the preceding rounds of selection should be preserved as a kind of module to be used as the building blocks for successive rounds of selection. It is just as similar as circuit modules in electronics that can be used as a unit preserving its specific function or words composed of letters in linguistics that can constitute different sentences of different meanings (function). Each elementary unit like resistors and condensers in electronics or letters and

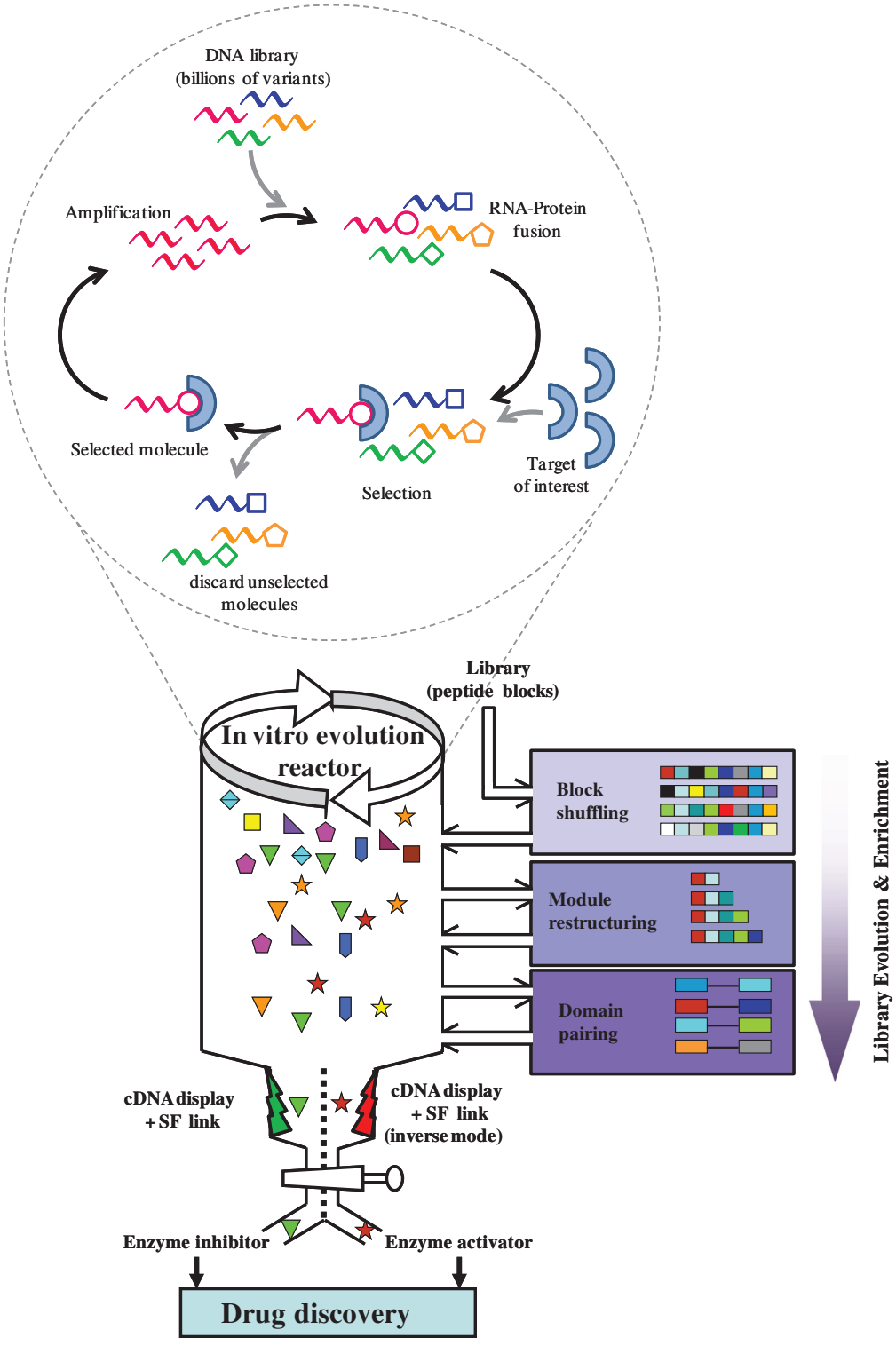
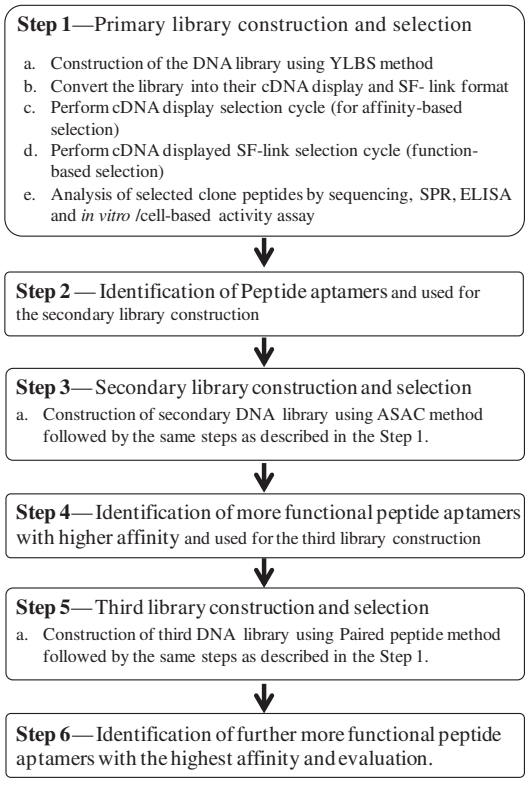


FIGURE 21.6 A schematic drawing of a general method for in vitro evolution of functional peptide aptamers using PLM method.

symbols in linguistics does not convey any meaning but the circuit modules and the words do and they can construct higher ordered structure of novel functions. Therefore such an attempt to construct artificial modules in protein engineering is conducted in eRAPANSY. In the further work, eRAPANSY was advanced as a general approach by devising a paired peptide method, termed as "progressive library method (PLM)" for acquiring highly functional peptide aptamers (Fig. 21.6) (Kitamura et al., 2012).

The whole process of PLM consists of three successive library constructions {using basic techniques such as the block shuffling for the primary library construction [Y-ligation block shuffling (YLBS)], the module-restructuring for the secondary library construction [all-steps-all-combinations (ASAC)], the module (domain) pairing for the third library construction (p&p)} and selections (cDNA display for affinity-based selection and SF link for function-based selection). Thus the whole process in PLM is highly integrative, sophisticated, and closely resembled to the process of natural evolution of proteins. Proteases involve in various human diseases and an attractive target for discovery of therapeutic candidates. Intriguingly, some proteases, for example, cathepsin E, are found to have antitumorigenic roles (Kawakubo et al., 2007). The PLM method was successfully applied to screen protease-regulating peptide aptamers (Biyani et al., 2011). In addition, PLM was used to develop a novel peptide aptamer-based technology, termed as "pep-ELISA" (as a promising substitution for antibodies-based method) and successfully demonstrated with sufficient sensitivity (10 μg/mL) for the detection of cathepsin E in cancer tissues and plasma (Kitamura et al., 2011). Therefore we expect that PLM holds a great promise on the drug discovery field including diagnostics and therapeutics as well as protein science due to its powerful property to identify de novo functional structures of proteins and peptide-to-peptide interactions.



FLOW CHART 21.3

Flow Chart 21.3

The methodology of PLM is mainly developed for the in vitro evolution of peptide aptamers using cDNA display (Yamaguchi et al., 2009) and SF-link method (Naimuddin et al., 2007). The overall strategy of PLM is comprised of three successive library constructions and selections as described below:

1. Primary library construction (YLBS) and selection: the primary library is essential in the identification of bioactive molecules with novel functions. The primary library selection is equal to module finding. The primary library is constructed using YLBS. Briefly, the whole cycle of YLBS is composed of hybridization of two sequences (5′-half and 3′-half), T4 RNA ligase ligation of two variable sequences (equal to blocks), PCR amplification of the ligated DNA, regeneration of 5′- and 3′-half precursor DNAs by restriction cleavage, and recovery of single-stranded DNAs. By repeating these steps, the number of ligated blocks and the diversity of resultant products increase exponentially. Then, the cDNA display and SF-link method are applied to the library to generate a cDNA-encoded peptide library (used for the affinity-based selection) and a cDNA-displayed peptide library tagged with enzyme−substrate sequence (used for the function-based selection). In affinity-based selection, the selected library products contain all of the inhibitory, activating and function-free neutral binding peptides while in the function-based selection, peptides can be differentiated in an inhibitory and an activating peptide by employing the inverse mode of the operation using the SF-link method.
2. Secondary library construction (ASAC) and selection: the secondary library construction and selection allows further refinement of the molecules selected from the primary library. The secondary library selection is equal to module shuffling. The secondary library is constructed by employing the YLBS method with slight modifications. The peptide sequences obtained by the primary library selection were cluster-analyzed and used to design the blocks to be constructed (tetramer). Using these blocks, YLBS shuffling is performed. The resulting library contained all of the blocks arbitrarily shuffled with a different number of blocks (2−8 blocks) and therefore it was termed as ASAC library. Then, cDNA display and SF-link methods are applied to the library as described above for the primary library and used for selections.

3. Third library construction (pair peptide library/p&p) and selection: the third library plays a very important role to enhance the affinity and function at the highest level. The third library selection is equal to module pairing. The third library was constructed by combining two peptides selected from the secondary library. The YLBS method was employed to obtain paired peptides with a linker sequence separating them. In brief, a set of peptide blocks (5′-halves) were combined arbitrarily with a set of linker blocks and then elongated by combing with another set of peptides (3′-halves), which consisted of the same elements as those of the 5′-halves. The diversity of library constructed was nominally $\sim 10^4$ and then sequenced to check the conformation of library. The same protocol is adopted for the affinity- and function-based selections of the paired peptide library as described for the primary and the secondary library selections.

Translational significance

Drug discovery is a wide translational area involving contribution from academic institutions to biotech companies and large pharmaceutical corporations. With the increasing adoption of technological advancements, novel integrated approaches are emerging to generate new therapies. Biomolecular display technologies have the potential to provide the must-needed interface between modern genomics/proteomics-based therapeutic challenges and the screening of potential lead candidates for the development of next-generation novel drug compounds. During the past two decades, several display technologies that mimic the process of natural evolution have been developed and applied to tackle several key issues in the process of drug discovery including diversity and abundance of drug candidate libraries, speed and cost-effectiveness, automation and rapid optimization of lead candidates. Furthermore, a greater collaboration between academia, biotech companies and pharmaceutical corporations can bring the speedy progress and growth of new drugs to market.

World Wide Web resources

The two important sites that can be consulted for the topics covered under this chapter are https://www.chemistryworld.com/news/what-is-directed-evolution-and-why-did-it-win-the-chemistry-nobel-prize/3009584.

TABLE 21.1 Some of therapeutic peptides that have reached in pharmaceutical market.

Name	Length	Target	Indication	Company
Hematide	Dimeric	Erythropoietin	In the treatment of chronic kidney-related anemia	Affymax
POT-4	cyclic	C3 convertase	In the treatment of age-related macular degeneration	Potentia Pharmaceuticals Inc
Acthrel	41 aa	ACTH	Diagnosis of ACTH-dependent Cushing's syndrome	Ferring Pharms
Sarenin	8 aa	Angiotensin II receptor antagonist	In the treatment of Hypertension	Norwich-Eaton Pharms; Procter & Gamble
Byetta	39 aa	Antidiabetic agents	Glycemic control in patients with type 2 diabetes mellitus	Amylin Pharms
Fuzeon	36 aa	Anti-HIV	In the treatment of AIDS/HIV-1 infection	Roche
Acticalcin	32 aa	Calcitonins	In the treatment of postmenopausal osteoporosis	AstraZeneca
Angiomax	20 aa	Cardiovascular	Anticoagulant in patients with unstable angina undergoing PTCA	Nycomed Pharma
Takus	10 aa	Cholecystokinin diethylamine	Diagnosis of the functional state of the gallbladder and pancreas	Parmacia and Upjohn
Geref	29 aa	GHRH and analog	Growth hormone deficiency, diagnosis evaluation of pituitary function	Serono Labs
Bigonist	9 aa	GnRH and analogs	In the treatment of advance prostate cancer	Sanofi-Aventis
Antocin	9 aa	Oxytocin, antagonist	Delaying the birth in cae of threat of premature birth	Ferring Pharms
NeoTect	10 aa	Somatostatin	Diagnosis of lung tumors	Amersham H.
Pitressin	9 aa	Vasopressin	Central diabetes insipidus	Monarch
Naaxia	2 aa	Spaglumat	Allergic rhinitis and conjunctivitis	Laboratoire Thea

Adapted and updated from Vlieghe P, Lisowski V, Martinez J and Khrestchatisky M, 2010 Synthetic therapeutic peptides: science and market. Drug Discov. Today 15, 40–56.

article. This sight gives a detailed description of what is directed evolution and why it won the Nobel prize. The other site is https://bioseeds.jp/ which provides novel tools invented through micro-nanotechnologies and can be checked for developing peptide-based diagnostics and therapeutic reagents.

Conclusion and future perspective

Biomolecular display technologies have great potential in providing the biological active molecules for the various applications such as in discovery of novel therapeutics, diagnostics, medical imaging molecules and research reagents. These technologies also have the potential to provide the crucial interface between modern therapeutic target discovery using genomics, proteomics and bioinformatics methods and development of new and potent drugs. For example, molecular probes generated from molecular display technologies for the proteins that having the relation with a disease can be useful for rapid validation of those proteins as viable therapeutic targets. Therefore these technologies have been receiving more interest by the pharmaceutical companies for rapid generation of such molecules.

Cell-free type display technologies, namely ribosome and mRNA/cDNA display have several features that should make them more amenable to standardization and automation than in the case with phage display. They comprise fast selection cycle, allow processing of huge libraries and are not limited by cellular transformation. Despite the several advantages of cell-free type display technologies, still there is demand for sophisticated, integrated and a general method that can become the in vitro display technology of choice for many applications. Antibodies and DNA/RNA aptamers obtained from molecular display technologies have shown a great progress for the use in the therapeutic field and analytical methodologies such as affinity chromatography, capillary electrophoresis, mass spectrometry or biosensors for diagnostic assays. However, peptide aptamer tool is still in an infantile stage of evolution. As reported above, the good success (reaching to the clinical stages of development and to the pharmaceutical market, see Table 21.1) and high number of recently published papers on peptide aptamers selection using molecular display technologies represents that peptide aptamers can be promising molecules for therapeutic and diagnostic applications in near future.

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Glossary

Small molecule drug A drug made of organic molecules of small molecular weight (less than 1000 Da), which possess a biological activity against a protein or molecular target responsible for causing the disease. Such as, acetylsalicylic acid (aspirin).

Biomolecular drug (biologics) A drug made from living organisms and their products, such as a serum, toxin, antitoxin, or analogous product applicable to the prevention, treatment, or cure of a diseases.

Peptide aptamer A new class of biomolecules, with a peptide moiety of randomized sequence, which are selected for their ability to bind to a given target protein under intracellular conditions.

Affinity Descriptive, qualitative term that indicates the relative tendency of one molecular entity to associate or interact with another.

Directed evolution A method used in protein engineering to harness the power of natural selection to evolve proteins or nucleic acids with desirable properties not found in nature.

In vitro selection Selection for phenotypes (traits) in test tubes expressed at the cellular or callus level, which usually possesses genetic changes that control the trait.

Combinatorial library Set of compounds prepared by combinatorial chemistry. It may consist of a collection of pools, or sub libraries.

ELISA Heterogeneous assay in which an antibody linked to an enzyme is used to detect the quantity of antigen present in a sample.

Epitope Any part of a molecule that acts as a an antigenic determinant. A macromolecule can contain many differerent eptiopes each capable of stimulating production of a different specific antibody.

Functional assay Assay in which the biological or physiological activity of the target is measured.
In vitro In the test tube, referring to a study in the laboratory using involving isolated organ, tissue, cell, or biochemical systems.
In vivo In the living body, referring to a study performed on a living organism.
Lead identification A process that is targeted toward the generation of at least one compound series that meets the requirements for progression to lead optimization.
Lead optimization A process in which the drug-like properties of an initial lead or lead series are improved.
Library A set of compounds (samples) produced through combinatorial chemistry or other means that expands around a single core structure.
Druggable site A portion of genome that can be targeted by a drug.

Abbreviations

ACE2	Angiotensin-converting enzyme 2
ASAC	All-steps-all-combinations
DNA	Deoxyribo nucleic acid
E. coli	*Escherichia coli*
EDTA	Ethylene diamine tetraacetic acid
EETI-ii	Ecballium elaterium trypsin inhibitor
ELISA	Enzyme-linked immuno sorbent assay
eRAPANSY	Evolutionary rapid panning analysis system
ErbB-2	Erythroblastic leukemia viral oncogene homolog
FDA	Food and Drug Administration
H1N1	Hemagglutinin type-1 and neuraminidase type-1
IAP	Inhibitor of apoptosis
IRES	Internal ribosomal entry site
IVC	In vitro compartmentalization
Kd	Kilo Dalton
KDR	Kinase domain-containing receptor
mRNA	Messenger ribo nucleic acid
nM	Nanomolar
P&P	Pair and peptide
PCR	Polymerase chain reaction
PEG	Poly ethylene glycol
PKC	Protein kinase Cα
PLM	Progressive library method
PSA	Prostate-specific antigen
RNA	Ribo nucleic acid
SELEX	Systematic evolution of ligands by exponential enrichment
tRNA	Transfer ribo nucleic acid
VEGF	Vascular endothelial growth factor
YLBS	Y-ligation block shuffling

Long answer questions

1. What are the various stages of drug discovery process? Give a brief description of each stage.
2. What is the most expensive part of drug discovery process and why? What is the solution/approach to deal with this issue based on recently developed biotechnologies?
3. What is the directed molecular evolution and what is the significance of library enrichment in the process of directed molecular evolution?
4. What is biomolecular display technology? Describe the various types of available display technologies and compare their significance with each other.
5. What is the significant of structural biology in drug discovery process? How do biomolecular structures interact with each other and drug molecules in their environment? Explain with examples.

Short answer questions

1. Differentiate small molecular drug with biomolecular drugs.
2. What is the mRNA display technology and how it is different from the ribosome display technology?
3. What is eRAPANSY and PLM?
4. What are aptamers? Describe their types with examples.
5. What are the difference between antibodies and peptide aptamer-based therapeutics?

Answers to short answer questions

1. The small molecular drug and biomolecular drugs can be differentiated as follows:

Small molecular drug	Biomolecular drug
Organic compound in nature (e.g., aspirin)	Biological product in nature (e.g., nucleic acids, antibiotics, peptides)
Low molecular weight size (<800 Da)	Smaller (e.g., 3 kDa peptide) to larger (150,000 kDa antibiotic) size
Chemically synthesized and produced	Both, chemically and biologically synthesized and produced
Traditional drugs	New generation drugs
Toxicity is high	Low in toxicity

2. The mRNA display is a cell-free system for the in vitro selection of the proteins and peptides. It uses the principle linkage between nascent proteins (phenotypes) and their corresponding mRNA (genotype), through the formation of mRNA–polypeptide complexes.
   A. The mRNA display technology has some following advantages that differentiate it from the ribosome display technology.
   B. Linkage: the mRNA display utilizes covalent mRNA–polypeptide complexes linked through puromycin, whereas ribosome display utilizes stalled, noncovalent ribosome–mRNA–polypeptide complexes.
   C. Library size: mRNA display can screen a library of size near 10^{14} or more while it is 10^{12} or less in ribosome display technology.

D. Selection pressure: in the ribosome display, selection stringency is limited to keep ribosome−mRNA−polypeptide in a complex because of the noncovalent ribosome−mRNA−polypeptide complexes, but this is not restricted in mRNA display method.

A. Molecular weight of linkage moiety: the polypeptides in a ribosome display system are attached to a ribosome, which has a molecular weight of $\sim 10^6$ Da. There might be some unpredictable interaction between the selection target and the ribosome, and this may lead to a loss of potential binders during the selection cycle. In contrast, the puromycin-modified DNA spacer linker is used in mRNA display technology, which is much smaller comparing to a ribosome. This linker may have less chance to interact with an immobilized selection target. Thus mRNA display technology is more likely to give less biased results.

3. eRAPANSY is a systemic in vitro evolution method to generate the protease-inhibiting peptide aptamers. It is introduced by Nishigaki lab in 2009. This method consists of the primary library construction and selection and then construction and enrichment of the secondary library using the primary library selection products. This method is successfully reported to select out the high affinity and inhibit peptide aptamers for the protease cathepsin E and thus their significant role in cancer therapeutics. PLM is an advanced version of eRAPANSY by devising a paired peptide method for the third library construction to obtain highly functional peptide aptamers.

4. Aptamers are short oligonucleic acid or peptide molecules that bind to a specific target molecule with high affinity. Aptamers are usually generated by selecting them from a large random sequence pool in in vitro selection methods such as SELEX and display technologies. Aptamers can be used for both basic research and clinical purposes as macromolecular drugs. These compound molecules have additional research, industrial and clinical applications.

Aptamers can be classified as:

E. Nucleic acid aptamers (DNA or RNA): nucleic acid aptamers are engineered through repeated rounds of in vitro selection or equivalently, SELEX to bind to various molecular targets. Example: *Pegaptanib*, an anti-VEGF aptamer for the treatment of human ocular vascular disease.

F. Peptide aptamers: the peptide aptamers are short amino acids sequences (10−30 amino acids). They can be selected from combinatorial peptide libraries constructed by phage display and other display system such as ribosome display and mRNA display. Example: *Fuzeon*, an anti-HIV aptamer for treatment of AIDS infection.

5. Please follow Fig. 21.3.

Yes/no type questions

1. Can H2H mRNA display method play a critical role in drug seeds discovery? Please justify in one-line answer.
2. Do you think that peptide aptamer technology is more potent than small molecules and antibodies? Please explain the one most important property of peptide aptamer that separates it from others.
3. Can diversity and quality of a library strength the success of an in vitro selection method? Explain in one-line answer.
4. Is the lack of technological advancement is the major reason for the fewer drugs introducing in the market? What is your explanation?
5. Can display methods more advantageous if it can select the molecules based on their functions? Please justify.
6. PLM method is based on progressive library construction method. Is this concept inspired from natural evolution of protein?
7. Do you think H2H mRNA display technology is more powerful than conventional (tail-to-tail) mRNA display technology? Explain one-line reason for justification.
8. Can H2H mRNA and MMV technologies make the drug discovery fast or still advancement is needed?
9. Why did the pair peptide library show its potent effect in enhancing the function and affinity than previous library?
10. What is the role of SF-link method in cDNA display method?

Answers to yes/no type questions

1. Yes—H2H mRNA display method can select the molecules based on their function and thus H2H mRNA display is advantageous over other nonfunctional screening methods for discovery of therapeutic molecules.
2. Peptide aptamer combines the favorable properties of both antibodies and small molecules such as affinity and specificity like antibodies and shorter of size like small molecules.
3. Yes—Because the method for construction of library is very important to generate and combine

the wide range of sequence diversity and quality of molecules in library.

4. Yes—Lack of technological advancement is one major cause of drug attrition, which can cause insufficient validation of therapeutic targets and inefficient specificity of drug candidates.
5. Yes—Because functional based selection is more advantageous for the discovery of therapeutic molecules.
6. PLM method follows the method of evolution that the nature used for protein evolution such as recombination and point mutation methods.
7. Yes—H2H mRNA display enables the use of stop codon and so thus rapid synthesis and accurate linkage (covalent) of mRNA/cDNA and peptide conjugate can be achieved.
8. Yes—Both technologically sound and efficient to find the therapeutic molecules, however, further validation for other targets is warranted.
9. As we can assume the coordination of two binding sites with two peptides has greater effects for increasing the affinity with target protein in compared to individual effect.
10. SF link is the attached enzyme–substrate sequence in cDNA display method to select the peptide based on their functions.

CHAPTER

22

In silico disease model: from simple networks to complex diseases

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Summary

In this chapter we consider in silico modeling of diseases starting from some simple to some complex (and mathematical) concepts. Examples and applications of in silico modeling for some important categories of diseases (such as for cancers, infectious diseases, and neuronal diseases) are also given.

What you can expect to know

Recent advances in bioinformatics and systems biology enable modeling and simulation of subcellular, cellular processes, and disease, using primary methods from dynamical systems theory. In this approach, all interactions among all components in a system are described mathematically and computed models are established. These in silico models encode and test hypotheses about mechanisms underlying the function of cells, the pathogenesis, and pathophysiology of disease and contribute to the identification of new drug targets and drug design. The development of in silico models is facilitated by rapidly advancing experimental and analytical tools that generate information-rich, high-throughput biological data. Bioinformatics providing tools for pattern recognition, machine learning, statistical modeling, and data extraction from databases contribute to in silico modeling. Dynamical systems theory is the natural language to investigate complex biological systems demonstrating nonlinear spatiotemporal behavior. Most of the in silico models aim to complement and not replace experimental research. Experimental data are needed for parameterization, calibration, and validation of in silico models. Typical examples in biology are models for molecular networks, where the behavior of cells is expressed in terms of quantitative changes in the levels of transcripts and gene products as well as models of the cell cycle. In medicine, in silico models of cancer, immunological disease, lung disease, and infectious diseases complement conventional research with in vitro models, animal models, and clinical trials. This chapter presents basic concepts of bioinformatics, systems biology, and their applications in in silico modeling and reviews its applications in biology and disease.

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Bioinformatics in animal biotechnology

Biotechnology is the most promising life science frontiers for the next decade. Together with informatics, biotechnology is leading to revolutionary changes in our society and economy. This genomic revolution is global and is creating new prospects in all biological sciences including medicine, human health, disease, and nutrition, agronomy, and animal biotechnology.

Animal biotechnology is the source of innovation in production and processing, profoundly impacting the animal husbandry sector which seeks to improve animal product quality, health, and well-being. Biotechnological research products, such as vaccines, diagnostics, in vitro fertilization, transgenic animals, stem cells, and a number of other therapeutic recombinant products are now commercially available. In view of the immense potential of biotechnology in the livestock and poultry sectors, the interest in animal biotechnology has increased over the years.

The fundamental requirement to modern biotechnology projects is the ability to gather, store, classify, analyze, and distribute biological information derived from genomics projects. Bioinformatics deals with methods for storing, retrieving, and analyzing biological data and protein sequences, structures, functions, pathways, and networks, and recently in silico disease modeling and simulation using systems biology. Bioinformatics encompasses both conceptual and practical tools for the propagation, generation, processing, and understanding of scientific ideas and biological information (Rhee, 2005).

Genomics is the scientific study of structure, function, and interrelationships of both individual genes and the genome. Recently, genomics research has facilitated the whole-genome mapping of various animals and understanding of the building blocks of biology. This has enabled researchers to decipher the fundamental cellular functions at the DNA level such as gene regulation or protein–protein interactions (PPIs) and thus discover molecular signatures (cluster of genes, proteins, metabolites, etc.), which are characteristic of a biological process or of a specific phenotype. Bioinformatics methods and databases can be developed to provide solutions to the challenges of handling massive amounts of data.

The history of animal biotechnology with bioinformatics is to make a strong research community that will build the resources and support the veterinary and agricultural research. There are some technologies that were used dating back to 5000 BCE. Many of these techniques are still being used today. Hybridizing animals by crossing specific strains of animals so that they can create greater genetic varieties. The offspring of some of these crosses is afterward bred selectively to produce the most desirable traits in those specific animals.

There has been significant interest in the complete analysis of the genome sequence of the farm animals such as chicken, pig, cattle, sheep, fish, and rabbit. The genomes of farm animals have been altered to search for preferred phenotypic traits and then selecting for better-quality animals to continue into the next generation. Access to these sequences has given rise to genome array chips and a number of web-based mapping tools and bioinformatics tools required to make sense of the data. In addition, the organization of gigabytes of sequence data requires an efficient bioinformatics database. Fadiel et al. provide a nice overview of resources related to the farm animal bioinformatics and genome projects (Fadiel et al., 2005).

With farm animals consuming large amounts of genetically modified crops, such as modified corn and soybean crops, it is a good question as to effect this will have on the meats. Some of the benefits of this technology are that what once took many years of trial and error is not completed in just months. The meats that are being produced are coming from animals that are better nourished by the use of biotechnology. Biotechnology and conventional approaches are benefiting both poultry and livestock producers. This will give a more wholesome affordable product that will meet the growing population demands.

Moreover, bioinformatics methods devoted to investigating the genomes of farm animals can bring eventual economic benefits, such as ensuring food safety and better food quality in the case of beef. Recent advances in high-throughput DNA sequencing techniques, microarray technology, and proteomics have led to effective research in bovine muscle physiology, to improve beef quality either by breeding or rearing factors. Bioinformatics is a key tool to analyze the huge data sets obtained from these techniques. The computational analysis of global gene expression profiling at the mRNA or protein level has shown that previously unsuspected genes may be associated either with muscle development or growth and may lead to the development of new molecular indicators of tenderness (Hocquette et al., 2007). Gene expression profiling has been used to document changes in gene expression, for example, following infection by pathological organisms (Meade et al., 2006), during the metabolic changes imposed by lactation in dairy cows (Loor et al., 2005), in cloned bovine embryos (Somers et al., 2006) and in various other models.

Bioinformatics enrichment tools are playing an important role in facilitating the functional analysis of large gene lists from various high-throughput biological studies. Huang et al. discuss 68 bioinformatics enrichment tools which help us understand their algorithms and details of a particular tool (Huang et al., 2009). However in biology, genes do not act

independently, but in a highly coordinated and interdependent manner with each other. In order to understand the biological meaning, one needs to map these genes into gene-ontology (GO) categories or metabolic and regulatory pathways. Different bioinformatics approaches and tools are employed for this task, starting from GO-ranking methods, pathway mappings, and biological network analysis (Werner, 2008). The awareness of these resources and methods is essential to make the best choices for our research interests.

The knowledge of bioinformatics tools will facilitate its wide application in the field of animal biotechnology. Bioinformatics is the computational data management discipline that helps us gather, analyze, and represent this information in order to educate ourselves, understand biological processes in the healthy and disease states, and facilitate the discovery of better animal products. Continued efforts are required to develop cost-effective and efficient computation platforms that can retrieve, integrate, and interpret the knowledge behind the genome sequences. The application of bioinformatics tools for biotechnology research will have significant implications in life sciences and the betterment of human lives. Bioinformatics is being adopted worldwide by academic groups, companies, and national and international research groups, and it should be thought of as an important pillar of current and future biotechnology, without which a rapid progress in the field is not possible. Systems approaches in combination with genomics, proteomics, metabolomics, and kinomics data have tremendous potential in providing insights into various biological mechanisms including the most important human diseases.

Bioinformatics and systems biology

We are witnessing the birth of a new era in biology. The ability to decipher the genetic code of living organisms promises to improve the quality of human life has dramatically changed the landscape of the biological and biomedical sciences and has brought with it new challenges.

One such challenge is that recent and novel technologies produce biological data sets of ever-increasing size, including genomic sequences, RNA, and protein abundances, their interactions with each other, and the identity and abundance of other biological molecules. The storage and compilation of such quantities of biological data is a challenge: the human genome, for example, contains three billion chemical units of DNA, whereas a protozoan genome has 670 billion units of DNA. Data management and interpretation require the development of newly sophisticated computational methods based on research in biology, medicine, pharmacology, and agricultural studies and using methods from computer science and mathematics—in other words, the multidisciplinary subject of bioinformatics.

Bioinformatics enables researchers to store large data sets in a standard computer database format and provides tools and algorithms scientists use to extract integrated information from the databases and use it to create hypotheses and models. Bioinformatics is a growth area because almost every experiment now involves multiple sources of data, requiring the ability to handle those data and to draw out inferences and knowledge. After 15 years of rapid evolution, the subject is now quite ubiquitous.

Due to the tremendous surge in the availability of biological data, the term "big data" is often used to describe the nature, scale, and dimension of these data. Moreover, the upcoming technologies must address and account for new schemas for the complicated and unstructured nature of biological data while designing data repositories. Currently, data repositories like the National Center for Biotechnology (Sayers et al., 2011), GenBank (Benson et al., 2013), Gene Expression Omnibus (Edgar et al., 2002), and Protein Data Bank (Berman et al., 2000) are some of the major bioinformatics resources for research in this domain.

Another challenge lies in deciphering the integrated functions of thousands of genes or systems biology. Systems biology is a term used to describe a number of trends in bioscience research and a movement that draws on those trends. It can be described as a biology-based interdisciplinary field of study that focuses on complex interactions of biological systems. Those in the field claim that it represents a shift in perspective toward holism instead of reduction. Systems biology brings in the aspect of complex systems to cell biology. The fundamental guiding principle of the field of systems biology is that of studying the cell as a whole entity and not merely in parts or isolation. It treats the cell as one functional component with a highly efficient integrated machinery of molecular interactions causing activation of cellular interactions which lead to effects in tissues and organs that subsequently manifest in physiological functions. This integrated understanding makes systems biology truly interdisciplinary combing fields of biology, mathematics, physics, computer science, and electrical engineering.

Systems biology has great potential to facilitate the development of drugs to treat specific diseases. The drugs currently on the market can target only those proteins that are known to cause disease. However with the human genome now completely mapped, we can target the interaction of genes and proteins at a systems biology level. This will enable the pharmaceutical industry to design drugs that will only target those genes that are diseased, improving healthcare in

the United States. Like two organs in one body, systems analysis and bioinformatics are separate but interdependent.

Common computational methods in systems biology

Computational methods take an interdisciplinary approach, involving mathematicians, chemists, biologists, biochemists, and biomedical engineers. The robustness of data sets related to gene interaction and co-operation at the system level requires multifaceted approaches to create a hypothesis that can be tested. Two approaches are used to understand the network interactions in systems biology, namely, Experimental and Theoretical and Modeling techniques (Choi, 2007). Below is a detailed overview of the different computational/ bioinformatics methods in modern systems biology.

Experimental methods in systems biology

Experimental methods utilize real situations to test the hypothesis of mined data sets. As such, living organisms are used whereby various aspects of genome-wide measurements and interactions are monitored. Specific examples on this point include the following.

Protein–protein interactions

PPIs predictions are methods used to predict the outcome of pairs or groups of protein interactions. These predictions are done in vivo and various methods can be used to carry out the predictions. Interaction prediction is important as it helps researchers make inferences of the outcomes of PPI. PPI can be studied by phylogenetic profiling, identifying structural patterns and homologous pairs, intracellular localization, and posttranslational modifications among others (Choi, 2007). A survey of available tools and web servers for analysis of PPIs is provided by Tuncbag et al. (2009).

Transcriptional control networks

Within biological systems, several activities involving the basic units of a gene take place. Such processes as DNA replication, RNA translation, and transcription into proteins must be controlled; otherwise, the systems could yield numerous destructive or useless gene products. Transcriptional control networks, also called gene regulatory networks, are segments within the DNA that govern the rate and product of each gene.

Bioinformatics has devised methods to look for destroyed, dormant, or unresponsive control networks. The discovery of such networks helps in corrective therapy, hence, the ability to control some diseases resulting from such control networks breakdown (Choi, 2007). There has also been rapid progress in the development of computational methods for the genome-wide "reverse engineering" of such networks. ARACNE is an algorithm to identify direct transcriptional interactions in mammalian cellular networks and promises to enhance our ability to use microarray data to elucidate cellular processes and to identify molecular targets of pharmacological drugs in mammalian cellular networks (Margolin et al., 2006). In addition to methods like ARACNE, we need systems biology approaches that incorporate heterogeneous data sources, such as genome sequence and protein–DNA interaction data. The development of such computational modeling techniques to include diverse types of molecular biological information clearly supports the gene regulatory network inference process and enables the modeling of the dynamics of gene regulatory systems (Hecker et al., 2009).

Signal transduction networks

Signal transduction is how cells communicate with each other. Signal transduction pathways involve interactions between proteins, micro and macromolecules and DNA. A breakdown in signal transduction pathways could lead to detrimental consequences within the system due to the lack of integrated communication. Correction of broken signal transduction pathways is a therapeutic approach researched for use in many areas of medicine.

The high-throughput and multiplex techniques for quantifying signaling and cellular responses are being increasingly available and affordable. A high-throughput quantitative multiplex kinase assay (Janes et al., 2003), mass spectrometry-based proteomics (Ong and Mann, 2005), and single-cell proteomics (Irish et al., 2006) are few of the experimental methods to elucidate signal transduction mechanisms of cells. These large-scale experiments are generating large data sets on protein abundance and signaling activity. Data-driven modeling approaches such as clustering, principal components analysis, and partial least squares need to be developed to derive biological hypothesis. The potential of data-driven models to study large-scale data sets quantitatively and

comprehensively will make sure that these methods will emerge as standard tools for understanding signal transduction networks (Janes and Yaffe, 2006).

Mathematical modeling techniques

Modeling biological systems is a major task of systems biology and mathematical biology. Computational systems biology intends to develop and use efficient algorithms, data structures, visualization, and communication tools with the goal of computer modeling of biological systems. A mathematical model can provide new insights into a biological model of interest and help in generating testable predictions.

Modeling or simulation can be viewed as a way of creating an artificial biological system in vitro whose properties can be changed or made dynamic. By externally controlling the model, new data sets can be created and implemented at the system level to create novel insights in treating gene-related problems. In modeling and simulation, sets of differential equations and logic clauses are used to create a dynamic systems environment that can be tested.

Mathematical models of biochemical networks (signal transduction cascades, metabolic pathways, and gene regulatory networks) are a central component of modern systems biology. The development of formal methods adopted from theoretical computing science is essential for the modeling and simulation of these complex networks (de Jong, 2002; Breitling et al., 2008). The computational methods that are being employed in mathematical biology and bioinformatics are (1) directed graphs, (2) Bayesian networks, (3) Boolean networks and their generalizations, (4) ordinary and partial differential equations, (5) qualitative differential equations, (6) stochastic equations, and (7) rule-based formalisms. Below are a few specific examples of the applications of these methods.

Mathematical models can be used to predict drug response or causes of drug resistance under a given set of conditions based on specific tumor properties. This integration can help in the development of tools that aid in the diagnosis, prognosis, and thus improve treatment outcome in patients with cancer. For example, in breast cancer being one of the well-studied diseases over the last decade serves as a model disease. One can thus apply the principles of molecular biology and pathology in designing new predictive mathematical frameworks that can unravel the dynamic nature of the disease. Genetic mutations of BRCA1, BRCA2, TP53, and PTEN significantly affect disease prognosis and increases the likelihood of adverse reactions to certain therapies. These mutations enable normal cells to become self-sufficient in survival in a stepwise process. Enderling et al. (2006) have modeled this mutation and expansion process by assuming that mutations in two tumor suppressor genes are sufficient to give rise to cancer. They modify the earlier model of Enderling et al. which is based on an established partial differential equation model of solid tumor growth and invasion (Anderson et al., 2006). The stepwise mutations from a normal breast stem cell to a tumor cell have been described using a model consisting of four differential equations.

Lauffenburger has applied a novel graphical modeling methodology known as Bayesian network analysis to model discovery and model selection for signaling events that direct mouse embryonic stem, an important preliminary step in hypothesis testing, in protein signaling networks. The model predicts bidirectional dependence between the two molecules ERK and FAK. It is interesting to appreciate that the apparent complexity of these dynamic ERK-FAK interactions is quite likely responsible for the difficulty in determining clear "upstream" versus "downstream" influence relationships by means of standard molecular cell biology methods. Bayesian networks determine the relative probability of statistical dependence models of arbitrary complexity for a given set of data (Woolf et al., 2005). This method offers further clues to apply Bayesian approaches to cancer biology problems.

The cell cycle is a process in which cells proliferate while collectively performing a series of coordinated actions. Cell-cycle models also have an impact on drug discovery. Chassagnole et al. use a mathematical model to simulate and unravel the effect of multitarget kinase inhibitors of cyclin-dependent kinases. They quantitatively predict the cytotoxicity of a set of kinase inhibitors based on the in vitro IC50 measurement values. Finally, they assess the pharmaceutical value of these inhibitors as anticancer therapeutics (Chassagnole et al., 2006).

In cancer, avascular tumor growth is characterized by localized, benign tumor growth where the nearby tissues consume most of the nutrients. Mathematical modeling of avascular tumor growth is important to understand the advanced stages of cancer. Kiran et al. have developed a spatial-temporal mathematical model classified as different zone model for avascular tumor growth based on the diffusion of nutrients, their consumption, and it includes key mechanisms in the tumor. The diffusion and nutrient consumption are represented using partial differential equations. This model predicts that the onset of necrosis occurs when the concentrations of vital nutrients are below critical values and also the overall tumor growth based on the size effects of proliferation zone, quiescent zone, and necrotic zone (Kiran et al., 2009).

The mathematical approaches used to model the three natural scales of interest: subcellular, cellular, and tissue, as discussed above. A challenge is the development of models that predict effects across biological scales. The long-term goal is to build a "virtual human made up of mathematical models with connections at the different biological scales (from genes to tissue to the organ)" (Brook et al., 2011).

Concept of modeling

A model is an optimal mix of hypotheses, evidence, and abstraction to explain a phenomenon. The hypothesis is a tentative explanation for an observation, phenomenon, or scientific problem that can be tested by further investigation. Evidence describes the information, that is, experimental data that help in forming a conclusion or judgment. Abstraction is an act of filtering out the required information to focus on a specific property only. For example, archiving books based on the year of publication, irrespective of the author name, would be an example of abstraction. In this process, we lose some detail and gain some. Through modeling, predictions are made, that may be tested by experiment. A model may be simple, for example, the logistic equation describing how a population of bacteria grows or the model may be complicated. Models may be mathematical or statistical (Coveney and Fowler, 2005).

Mathematical models make predictions, whereas statistical models enable us to draw statistical inferences about the probable properties of a system. In other words, models can be deductive or inductive. If the prediction is necessarily true given that the model is also true, then the model is a deductive model. On the other hand, if the prediction is statistically inferred from observations, then the model is inductive. Deductive models contain a mathematical description, for example, the reaction–diffusion equations that make predictions about reality. If these predictions do not agree with experiment, then the validity of the entire model may be questioned. Mathematical models are commonly applied in physical sciences. On the other hand, inductive models are mostly applied in the biological sciences. In biology, models are used to describe, simulate, analyze, and predict the behavior of biological systems. Modeling in biology provides a framework that enables description and understanding of biological systems through building equations that express biological knowledge. Modeling enables the simulation of the behavior of a biological system by performing in silico experiments, that is, numerically solving the equations/rules that describe the model. The results of these in silico experiments become the input for further analysis, e.g., identification of key parameters or mechanisms, interpretation of data, or comparison of the ability of different mechanisms to generate observed data.

In particular, systems biology employs an integrative approach to characterize biological systems, in which interactions among all components in a system are described mathematically to establish a computable model. These in silico models complement traditional in vivo animal models and can be applied to quantitatively study the behavior of a system of interacting components. The advent of high-throughput experimental tools has allowed for the simultaneous measurement of thousands of biomolecules, opening the way for in silico model construction of increasingly large and diverse biological systems. Integrating heterogeneous dynamic data into quantitative predictive models holds great promise to significantly increase our ability to understand and rationally intervene in disease-perturbed biological systems. This promise—particularly with regard to personalized medicine and medical intervention—has motivated the development of new methods for systems analysis of human biology and disease. Such approaches offer the possibility of gaining new insights into the behavior of biological systems, of providing new frameworks for organizing and storing data and performing statistical analyses, of suggesting new hypotheses and new experiments, and even of offering a "virtual laboratory" to supplement in vivo and in vitro work.

However in silico modeling in the life sciences is far from straightforward and suffers from a number of potential pitfalls. Thus mathematically sophisticated but biologically useless models often arise because of a lack of biological input, leading to models that are biologically unrealistic or address a question of little biological importance. On the other hand, models may be biologically realistic but mathematically intractable. This problem usually arises because biologists unfamiliar with the limitations of mathematical analysis want to include every known biological effect in the model. Even if it were possible to produce such models they would be of little use since their behavior would be as complex to investigate as the experimental situation. These problems can be avoided by formulating clear explicit biological goals before attempting to construct a model. This will ensure that the resulting model is biologically sound, can be experimentally verified, and will generate biological insight or new biological hypotheses. The aim of a model should not simply be to reproduce the biological data, and indeed often the most useful models are those that exhibit discrepancies from the experiment. Such deviations will typically stimulate new experiments or hypotheses. An iterative approach has

been proposed, starting with a biological problem, developing a mathematical model, and then feeding back into the biology. Once established, this collaborative loop can be traversed many times, leading to ever-increasing understanding.

The ultimate goal of in silico modeling in biology is the detailed understanding of the function of molecular networks as they appear in metabolism, gene regulation, or signal transduction. This is achieved by using a level of mathematical abstraction that needs a minimum of biological information to capture all physiologically relevant features of a cellular network. For example, ideally, for in silico modeling of a molecular network, knowledge of the network structure, of all reaction rates, concentrations, and spatial distributions of molecules at any time point is needed. Unfortunately, such information is unavailable even for the best-studied systems. In silico simulations thus always have to use a level of mathematical abstraction, which is dictated by the extent of our biological knowledge, by molecular details of the network, and by the specific questions that are addressed. Understanding the complexity of the diseases and its biological significance in health can be achieved by integrating data from the different functional genomics experiments with medical, physiological, and information on environmental factors and computed mathematically. The advantage of mathematical modeling of disease lies in the fact that such models not only shed insight as to how a complex process works, which could be very difficult to infer an understanding of each component of this process but also predict what may follow as time evolves or as the characteristics of particular system components are modified. Mathematical models have generally been utilized in association with an increased understanding of what models can offer in terms of prediction and insight.

Models have two distinct roles, prediction and understanding, related to the model properties of accuracy, transparency, and flexibility. Prediction of the models should be accurate including all the complexities and population-level heterogeneity having an additional use as a statistical tool. It also provides an understanding of how the disease spreads in the real world and how the complexity affects the dynamics. Understanding the model helps to develop sophisticated predictive models and gather more relevant epidemiological data. A model should be suited for its purpose that is, it should be as simple as possible, but no simpler—having an appropriate balance of accuracy, transparency, and flexibility (Keeling and Rohani, 2008). The model built should be helpful in understanding the behavior of the disease and able to simplify the other disease condition.

In silico models of cells

Several projects are proceeding along these lines such as E-CELL (Tomita, 2001), Virtual Cell (Resasco et al., 2012), and CellDesigner (Funahashi et al., 2003) and simulations of biochemical pathways (Palsson, 2000). Whole-cell modeling integrates information from metabolic pathways, gene regulation, and gene expression. In addition, several software applications model specific aspects of cellular interactions, such as COPASI (Hoops et al., 2006) for simulation and analysis of biochemical networks, PhysioDesigner 1.2 (Asai et al., 2012) for modeling physiological systems, etc. Three elements are needed for constructing a good cell model: precise knowledge of the phenomenon, an accurate mathematical representation, and a good simulation tool (Tomita, 2001).

A cell represents a dynamic environment of interaction among nucleic acids, proteins, carbohydrates, ions, pH, temperature, pressure, and electrical signals. Many cells with similar functionality form tissue. In addition, each type of tissue uses a subset of this cellular inventory to accomplish a particular function. For example, in neurons, electrochemical phenomena take precedence over cell division, in which cell division is a fundamental function of skin, lymphocytes, and bone marrow cells. Thus an ideal virtual cell not only represents all the information but also exhibits the potential to differentiate into neuronal or epithelial cell. The first step in creating a whole-cell model is to divide the entire network into pathways and pathways into individual reactions. Any two reactions belong to a pathway if they share a common intermediate. In silico modeling consists not only of decomposing events into manageable units but also of assembling these units into a unified framework. In other words, mathematical modeling is an art of converting biology into numbers.

For whole-cell modeling, a checklist of biological phenomena that call for mathematical representation is needed. Biological phenomena taken into account for in silico modeling of whole cells are the following:

- DNA replication and repair
- translation
- transcription and regulation of transcription
- energy metabolism
- cell division
- chromatin modeling
- signaling pathways
- membrane transport (ion channels, pump, nutrients)
- intracellular molecular trafficking
- cell membrane dynamics
- metabolic pathways.

The whole-cell metabolism includes enzymatic and nonenzymatic processes. Enzymatic processes cover most of the metabolic events, while nonenzymatic processes include gene expression and regulation, signal transduction, and diffusion.

In silico modeling of whole cells not only requires precise qualitative and quantitative data but also an appropriate mathematical representation of each event. For metabolic modeling, the data input consists of kinetics of individual reactions and also effects of cofactors, pH, and ions on the model. The key step in modeling is to choose an appropriate assumption. For example, a metabolic pathway may be a mix of forward and reverse reactions. Furthermore inhibitors that are part of the pathway may influence some reactions. At every step, enzymatic equations are needed that best describe the process. In silico models are built because they are easy to understand, controllable, and can store and analyze large amounts of information. A well-built model has diagnostic and predictive abilities. A cell by itself is a complete biochemical reactor that contains all the information one needs to understand life. Whole-cell modeling enables investigation of the cell cycle, physiology, spatial organization, and cell–cell communication. Sequential actions in whole-cell modeling are the following:

- catalog all the substances that make up a cell
- make a list of all the reactions, enzymes, and effectors
- map the entire cellular pathways: gene regulation, expression, metabolism, etc.
- build a stoichiometric matrix of all the reactions versus substances (for qualitative modeling)
- add rate constants, concentration of substances, strength of inhibition
- assume appropriate mathematical representations for individual reactions
- simulate reactions with suitable simulation software
- diagnose the system with system analysis software
- perturb the system and correlate its behavior to an underlying genetic and/or biochemical
- phenomenon using a hypothesis generator.

In silico metabolic modeling

Metabolic modeling is important to simulate the cell's function and phenotype. In recent years, metabolic modeling is frequently used in the field of drug discovery, clinical trial, and precision medicine. Computational and mathematical techniques are essential for metabolic modeling and several approaches and computational tools are currently available for this purpose. Essential features of metabolic modeling include metabolic systems biology, genome-scale metabolic models, constraint-based modeling and flux balance analysis, and multiomic flux balance analysis among others. Apart from these applications, metabolic modeling is also important for developing various disease models for tissue- and patient-specific insights into human diseases such as diabetes, neurodegenerative diseases, and cancers (Angione, 2019). Among the several approaches, machine and deep learning is applied for genome-scale metabolic modeling and Python-based models are available for cellular metabolic functional and phenotype or disease analysis (Cardoso et al., 2018; Zampieri et al., 2019). Several tools are nowadays available for metabolic modeling and analysis such as "Cameo" for in silico design of cell factories (Cardoso et al., 2018), "Escher-FBA" for flux balance analysis (Rowe et al., 2018), and "COBRAme" for genome-scale models of metabolism and gene expression (Lloyd et al., 2018). The area is evolving and several new approaches and tools are emerging.

In silico modeling of disease: in practice

In silico modeling has been applied in cancer, systemic inflammatory response syndrome (SIRS), immune disease, neuronal disease, and infectious disease. In silico models of disease can contribute to a better understanding of the pathophysiology of the disease, suggest new treatment strategies, and provide insight into the design of experimental and clinical trials for the investigation of new treatment modalities.

In silico modeling of disease combines the advantages of both in vivo and in vitro experimentation, without subjecting itself to the ethical considerations and lack of control associated with in vivo experiments. Unlike in vitro experiments, which exist in isolation, in silico models allow the researcher to include a virtually unlimited array of parameters, which render the results more applicable to the organism as a whole (Colquitt et al., 2011).

In silico modeling of disease is quite challenging. Attempting to incorporate every single known interaction rapidly leads to an unmanageable model. Further parameter determination in such models can be a frightening experience. Estimates come from diverse experiments, which may be elegantly designed and well executed but can still give rise to widely differing values for parameters. Data can come from both in vivo and in vitro experiments and results that hold in one medium may not always hold in the other. Further despite the many similarities between mammalian systems, significant differences do exist and so results obtained from experiments using animal and human tissue may not always be consistent.

In silico models of cancer

In silico modeling of cancer has become an interesting alternative approach to traditional cancer research. In silico models of cancer are expected to predict the complexity of cancer at multiple temporal and spatial resolutions, with the aim of supplementing diagnosis and treatment by helping plan more focused and effective therapy via surgical resection, standard chemotherapy, novel treatments. In silico models of cancer include (1) statistical models of cancer, such as molecular signatures of perturbed genes and molecular pathways, and statistically inferred reaction networks; (2) models that represent biochemical, metabolic, and signaling reaction networks important in oncogenesis, including constraint-based and dynamic approaches for the reconstruction of such networks; and (3) models of the tumor microenvironment and tissue-level interactions for microenvironment-tissue level (Edelman et al., 2010; Araujo and McElwain, 2004; Byrne et al., 2006; Bellomo et al., 2008; van Riel, 2006; Rejniak and Anderson, 2011).

Statistical models of cancer can be broadly divided into those that employ unbiased statistical inference and those that also incorporate a priori constraints of specific biological interactions from data. Statistical models of cancer biology at the genetic, chromosomal, transcriptomic, and pathway levels provide insight about molecular etiology and the consequences of malignant transformation, despite incomplete knowledge of underlying biological interactions. These models are able to identify molecular signatures that can inform diagnosis and treatment selection, for example, with molecularly targeted therapies such as Imatinib (Gleevec) (Edelman et al., 2010).

However in order to characterize specific biomolecular mechanisms that drive oncogenesis, genetic, and transcriptional activity must be considered in the context of cellular networks that ultimately drive cellular behavior. In microbial cells, network inference tools have been developed and applied for the modeling of diverse biochemical, signaling, and gene expression networks. However due to the much larger size of the human genome compared with microbes, and the substantially increased complexity of eukaryotic genetic regulation, the inference of transcriptional regulatory networks in cancer presents increased practical and theoretical challenges (Edelman et al., 2010).

Biochemical reaction networks are constructed to represent explicitly the mechanistic relationships between genes, proteins, and the chemical interconversion of metabolites within a biological system. In these models, network links are based on preestablished biomolecular interactions rather than statistical associations; significant experimental characterization is thus needed to reconstruct biochemical reaction networks in human cells. These biochemical reaction networks require, at a minimum, knowledge of the stoichiometry of the participating reactions. Additional information such as thermodynamics, enzyme capacity constraints, time-series concentration profiles, and kinetic rate constants can be incorporated to compose more detailed dynamic models (van Riel, 2006; Edelman et al., 2010).

Microenvironment-tissue level models of cancer apply an "engineering" approach that views tumor lesions as complex micro-structured materials, where three-dimensional tissue architecture ("morphology") and dynamics are coupled in complex ways to cell phenotype, which in turn is influenced by factors in the microenvironment. Computational approaches of in silico cancer research include continuum models, discrete models, and hybrid models.

In continuum models, extracellular parameters can be represented as continuously distributed variables to mathematically model cell−cell or cell−environment interactions in the context of cancers and the tumor microenvironment. Systems of partial differential equations have been used to simulate the magnitude of interaction between these factors. Continuum models are suitable for describing the individual cell migration, change of cancer cell density, the diffusion of chemoattractants, heat transfer in hyperthermia treatment for skin cancer, cell adhesion, and the molecular network of a cancer cell as an entire entity. However this type of in silico models has limited ability in investigating single-cell behavior and cell−cell interaction.

On the other hand, "discrete" models, that is, cellular automata models represent cancer cells as discrete entities of defined location and scale, interacting with one another and external factors in discrete time intervals according to predefined rules. Agent-based models expand the cellular automata paradigm to include entities of divergent functionalities interacting together in a single spatial representation, including different cell types, genetic elements, and environmental factors. Agent-based models have been used for modeling three-dimensional tumor cell patterning, immune system surveillance, angiogenesis, and the kinetics of cell motility.

Hybrid models have been created which incorporate both continuum and agent-based variables in a modular approach. Hybrid models are ideal for examining direct interactions between individual cells and between the cells and their microenvironment, but they also allow us to analyze the emergent properties of complex multicellular systems (such as cancer). Hybrid models are often multiscale by definition integrating processes on different temporal and spatial scales, such as gene expression, intracellular pathways, intercellular signaling, cell growth, or migration. There are two

general classes of hybrid models, those that are defined upon a lattice and those that are off lattice.

The classification of hybrid models on these two classes depends on the number of cells these models can handle and the included details of each individual cell structure, that is, models dealing with large-cell populations but with simplified cell geometry, and those that model small colonies of fully deformable cells. Interested readers can find details on hybrid models of cancer in the interesting recent review of Rejniak and Anderson (2011).

For example, a hybrid model investigated the invasion of healthy tissue by a solid tumor. The model focused on four key parameters implicated in the invasion process: tumor cells, host tissue (extracellular matrix), matrix-degradative enzymes, and oxygen. The model was considered to be hybrid since the latter three variables were continuous (i.e., concentrations) and the tumor cells were discrete (i.e., individuals). This hybrid model aimed to investigate how individual-based cell interactions (with one another and the matrix) can affect the tumor shape (Anderson, 2005). Another model incorporated a continuous model of a receptor signaling pathway, an intracellular transcriptional regulatory network, cell-cycle kinetics, and three-dimensional cell migration in an integrated, agent-based simulation of solid brain tumor development (Zhang et al., 2007). The interactions between cellular and microenvironment states have also been considered in a multiscale model that predicts tumor morphology and phenotypic evolution in response to such extracellular pressures (Anderson et al., 2006).

In silico models of tumor microenvironment integrate information about the biological context in which cancers develop. Multiple factors involved in the development of an intrinsically complex tumor microenvironment have been studied including extracellular biomolecules, vasculature, and the immune system. However rarely these methods have been integrated with a large cell–cell communication network in a complex tumor microenvironment. Recently, an interesting effort of in silico modeling was described, in which the investigators integrated all the intercellular signaling pathways known to date for human glioma and generated a dynamic cell–cell communication network associated with the glioma microenvironment. Then, they applied evolutionary population dynamics and the Hill functions to interrogate this intercellular signaling network and execute an in silico tumor microenvironment development. The observed results revealed a profound influence of the microenvironmental factors on tumor initiation and growth and suggested new options for glioma treatment by targeting cells or soluble mediators in the tumor microenvironment (Wu et al., 2012).

In silico models and inflammatory response syndrome in trauma and infection

Trauma and infection elicit an acute inflammatory response. In certain circumstances, the degree of the acute inflammatory response may result in pathologic manifestations, namely, SIRS, sepsis, and multiple organ failure. Further research is needed for the appropriate management of these states. Despite longstanding efforts, there has been uniform difficulty in translating the results of basic science research into effective therapeutic regimes. It has been suggested that this difficulty is due in part to a failure to account for the complex, nonlinear nature of the inflammatory process of which SIRS/MOF represents a disordered state. In silico modeling seems to be a promising research approach in this field. Indeed, in silico modeling of inflammation has been applied in an effort to bridge the gap between basic science and clinical trials. Specifically, both agent-based modeling and equation-based modeling have been utilized (Vodovotz et al., 2008; Geris et al., 2010). Equation-based modeling encompasses primarily ordinary differential equations (ODEs) and partial differential equations (PDEs). Initial modeling studies were focused on the pathophysiology of the acute inflammatory response to stress, and these studies suggested common underlying processes generated in response to infection, injury, and shock. Later, mathematical models included the recovery phase of injury and gave insight into the link between the initial inflammatory response and the subsequent healing process. The first mathematical model of wound healing dates back to the 1980s and early 1990s. These models and others developed in the 1990s investigated epidermal healing, repair of the dermal extracellular matrix, wound contraction, and wound angiogenesis. Most of these models were deterministic and formulated using differential equations. In addition, recent models have been formulated using differential equations to analyze different strategies for improved healing, including wound VACs, commercially engineered skin substitutes, and hyperbaric oxygen. In addition, agent-based models have been used in wound healing research. For example, Mi et al. (2007) have developed an agent-based model to analyze different treatment strategies with wound debridement and topical administration of growth factors. Their model produced the expected results of healing when analyzing for different treatment strategies including debridement, release of PDGF, reduction in tumor necrosis factor-α, and increase of TGF-β1. The investigators suggested that a drug company should use a mathematical model to test a new drug before going through the expensive process of basic science testing, toxicology, and clinical

trials (Mi et al., 2007). Indeed, clinical trial design can be improved by prior in silico modeling. For example, in silico modeling has led to the knowledge that patients who suffered from the immune-suppressed phenotype of late-stage multiple organ failure and were susceptible to usually trivial nosocomial infections demonstrated sustained elevated markers of tissue damage and inflammation through 2 weeks of simulated time. However anticytokine drug trials with treatment protocols of only one dose or 1 day had not incorporated this knowledge into their design, with subsequent failure of candidate treatments.

Applications of in silico disease modeling

The application of mathematical modeling has been successfully deployed in representing several pathophysiological disorders and is known as disease modeling. Several dynamics of pathophysiological and cell-signaling pathways have been studied using disease modeling. Disease modeling has also been used to study the spread of diseases and epidemics (Dezső and Barabási, 2002; Wang et al., 2016).

Infectious diseases

By now the reader is expected to be familiar with the meaning and the basics of in silico model (ing). In this section, we discuss the application of in silico modeling in the understanding of infectious diseases and in the proposition/development of better treatments for infectious diseases. In fact, it is worthy of note that the applications of in silico modeling can help far beyond just the understanding of the dynamics (and sometimes, statics) of infectious diseases, and far beyond the proposition/development of better treatments for infectious diseases. In silico modeling can be helpful even in the understanding of better prevention of infectious diseases.

The process of infection is defined as the level of a pathogen within the host which in turn is determined by the growth rate of the pathogen and its interaction with the host's immune response. Initially, no pathogen is present but just a low-level nonspecific immunity within the host. On infection, the parasite grows abundantly over time with the potential to transmit the infection to other susceptible individuals.

Triad of infectious diseases as the source of parameters for in silico modeling of infectious diseases

To comprehensively understand in silico modeling in the domains of infectious diseases, one should first understand the "triad of infectious diseases," and the characteristics of "infectious agent," "host," and "environment" on which the models and always based. In fact, modeling of infectious diseases is just impossible without this triad, after all, the model would be built on some parameters (also called variables, in a more general language), and those parameters always have their origin from the so-called "triad of infectious diseases." At this point, a good question would be: What is "triad of infectious diseases?"

By "triad of infectious diseases," we mean the interactions between (1) agent, which is the disease-causing organism—the pathogen; (2) host, which is the infected organism, or in the case of preinfection, the organism to be infected is the host—thus in this case host is the animal the agent infects; and (3) environment, which is a kind of the link between the agent and the host—essentially, the environment is an umbrella word for the entirety of the possible media through which the agent reaches the host (Park, 2009).

Now that we have set the direction, and we (now) know what in silico modeling of infectious diseases are fundamentally based on, let us proceed gradually and get a better understanding of the parameters on which most in silico infectious disease models are based. To discuss the parameters in an orderly manner, we just categorize them under each of the three components of the "triad of infectious diseases" and summarize them in the next subsection. It must be emphasized at this point that (1) even though all the possible parameters for in silico modeling of infectious diseases can be successfully categorized under the characteristics of one of any of the three components of the "triad of infectious diseases"—agent, host, and environment, (2) the parameters discussed in the next subsection are by no mean the entirety of all the possible parameters that can be included in in silico modeling of infectious diseases—in fact, several parameters exist that this section cannot possibly enumerate them all, and that is why we have discussed the parameters using categorical approach.

Parameters for in silico modeling of infectious diseases

Parameters derived from characteristics of agent

Some of the parameters for in silico modeling of infectious diseases are essentially a measure of infectivity (ability to enter the host), pathogenicity (ability to cause divergence from homeostasis/disease), virulence (degree of divergence from homeostasis caused/ability to cause death), antigenicity (ability to bind to mediators of host's adaptive immune system), and immunogenicity (ability to trigger adaptive immune response) (Scott and De Groot, 2010) of the concerned infectious agent. The exact measure (and thus the

units) used can vary markedly depending on the intentions for which the in silico infectious diseases model is built, as well as the assumptions on which the in silico disease model is based. From the knowledge of agent's characteristics, one should know that unlike parameters related to the other characteristics of agent, the parameters related to infectivity find their most important use only in the modeling of preinfection stage in infectious disease modeling.

Finally, some of the agent-related parameters of great importance in in silico modeling of infectious diseases are the concentration of agent's antigen–host's antibody complex, case fatality rate, the strain of the agent, other genetic information of the agent, etc.

Parameters derived from characteristics of host

The parameters originating from characteristics of host can also be so diversified and be based on the intentions for which the in silico infectious diseases model is built and the assumptions on which the in silico disease model is based, but then the parameters could be grouped and explained under host's genotype (the allele at host's specified genetic locus), immunity/health status (biological defenses to avoid infection), nutritional status (feeding habits/food intake characteristics), gender (often categorized as male, or female), age, and behavior (host's behaviors that affect its resistance to homeostasis disruptors).

Typical examples of host-related parameters are the alleles at some specifically targeted genetic loci; the total white blood cell counts; differential white blood cell counts, and/or much more sophisticated counts of specific blood cell types; blood levels of some specific cytokines, hormone, and/or neurotransmitters; daily calories, protein, and/or fat intake; daily amount of energy expended and/or duration of exercise; etc.

Parameters derived from characteristics of environment

At first, parameters originating from the environment might seem irrelevant to the in silico modeling of infectious diseases, but they are. Even after the preinfection stage, the environment still modulates the host–agent intersections. For example, the ability (and thus the related parameters) of the agent to multiply and/or harm the host are continually influenced by the host's environmental conditions, and in a similar way, the host's defense against the adverse effects of the agents are modulated by host's environmental conditions. But somehow, not so many of these parameters have been included in in silico infectious disease models in the recent past. A few examples of these parameters are host's ambient temperature, host's ambient atmospheric humidity, altitude, host's light–dark cycle, etc.

Infectious diseases in silico model proper, a typical approach/scenario

Now that we know the parameters for in silico infectious disease modeling, the next reasonable question would be "What form does a typical in silico infectious disease model take?" So, this subsection attempts to answer this very important question.

To answer this question, we start by employing the reader to view in silico model as a system of well-integrated functional equations/formulae. Then, these integrated functional equations/formulae should be seen as been so highly integrated that, ultimately, they could altogether be seen as a single gigantic functional equation/formula. From this big single functional equation, it is then possible (at least through the aid of a computer) to make any of the contained variables the subject of the equation depending on what one wants to obtain from the model. This is essentially an in silico model, and it is basically characterized by the possibility of computationally working with any (and sometimes, close to infinite) number of possible data points within the reasonable limits one set.

So the equations behind a typical infectious disease in silico model could take the form

$$H = \beta(link\,function)\, f(A)(link\,function)\, g(E) + \varepsilon\ldots \quad (22.1)$$

where H is the output from a smaller equation that is based on host parameters, β is a constant, f and g are link functions which may be same as or different from each other and other link functions in this system of equations, A is the output from a smaller equation that is based on agent parameters, g is a link function which may be same or different from other link functions in this system of equations, E is the output from a smaller equation that is based on environment parameters, and $\mathcal{E}$ is a random error parameter.

Readers should know that we use the term "link function" to refer to any of the various possible forms of mathematical operations or functions. Which means that based on the complexity of the model, a particular "link function" might be as simple as a mere addition or as complex as several combinations of an operator with high-degree polynomials.

H in Eq. (22.1) could have resulted from a smaller model/function of the form

$$H = \beta_h\ (\text{link function})\ f_{h1}(h_1)\ (\text{link function})\ f_{h2}(h_2)\ (\text{link function})\ldots f_{hx}(h_x)\ (\text{link function}) + \varepsilon\ldots \quad (22.2)$$

where β_h is a constant; f_{h1}, f_{h2}, … f_{hx} are link functions that may be the same or different (individually) from (every) other link functions in this system of equations; h_1, h_2, … h_x are a set of host's parameters (e.g., age, gender, white blood cell count, cytokine level, etc.); and $\mathcal{E}$ is a random error parameter.

A in Eq. (22.1) could have resulted from a smaller model/function of the form

$$A = \beta_a(\text{link function})\, f_{a1}(a_1)\,(\text{link function})\, f_{a2}(a_2)\,(\text{link function}) \ldots f_{ax}(a_x)\,(\text{link function}) + \varepsilon \ldots \quad (22.3)$$

where β_a is a constant; f_{a1}, f_{a2}, … f_{ax} are link functions which may be the same or different (individually) from (every) other link functions in this system of equations; a_1, a_2, … a_x are a set of agent's parameters (e.g., case fatality rate, agent's genotype, etc.); and $\mathcal{E}$ is a random error parameter.

In a similar way, E in Eq. (22.1) could have resulted from a smaller model/function of the form

$$E = \beta_e\,(\text{link function})\, f_{e1}(e_1)\,(\text{link function})\, f_{e2}(e_2)\,(\text{link function}) \ldots f_{ex}(e_x)\,(\text{link function}) + \varepsilon \ldots \quad (22.4)$$

where β_e is a constant; f_{e1}, f_{e2}, … f_{ex} are link functions which may be the same or different (individually) from (every) other link functions in this system of equations; e_1, e_2, … e_x are a set of environmental parameters (e.g., host's ambient temperature, host's ambient atmospheric humidity, etc.); and $\mathcal{E}$ is a random error parameter.

Specific examples of infectious diseases in silico model

Muñoz-Elías et al. (2005) have documented (through their paper Replication dynamics of *Mycobacterium tuberculosis* in chronically infected mice) a successful in silico modeling of infectious diseases (specifically, tuberculosis). In their in silico modeling of tuberculosis in mice, the researchers investigated both the static and dynamic of host–pathogen/agent equilibrium (i.e., mice-mycobacterium tuberculosis static and dynamic equilibrium). The rationale behind their study was that a better understanding of host–pathogen/agent interactions would make possible the development of better antimicrobial drugs for the treatment of tuberculosis (as well as provide similar a understanding for the cases of other chronic infectious diseases). They modeled different types of host–pathogen/agent equilibriums (ranging from completely static equilibrium, all the way through semidynamic, down to completely dynamic scenarios) by varying the rate of multiplication/growth and the rate of death of the pathogen/agent (*M. tuberculosis*) during the infection's chronic phase. Through their in silico study (which was also verified experimentally), Muñoz-Elías et al. (2005) documented a number of remarkable findings. For example, they established that "viable bacterial counts and total bacterial counts in the lungs of chronically infected mice do not diverge over time" and explained that "rapid degradation of dead bacteria is unlikely to account for the stability of total counts in the lungs over time, because treatment of mice with isoniazid for 8 weeks led to a marked reduction in the viable counts without reducing the total count."

Readers who are interested in the further details on the generation of this in silico model for the dynamics of *M. tuberculosis* infection, as well as the complete details of the parameters/variables considered, and the comprehensive findings of the study should refer to the article of Muñoz-Elías et al. published in infection and immunity (Muñoz-Elías et al., 2005).

Another one of the many other notable works in the domain of infectious diseases in silico modeling is the study by Navratil et al. (2011). Using PPIs data that authors obtained from available literature and public databases, the authors (after first curating and validating the data) computationally (in silico) re-examined virus–human protein interactome. Interestingly, the authors were able to show that onset and the pathogenesis of some disease conditions (most especially, chronic disease conditions) that are often believed to be of genetic, lifestyle, or environmental origin, are in fact modulated by infectious agents.

The reader may also be interested in a few other studies or reviews such as Chavali et al. (2008) and Watterson and Ghazal (2010) which involve applications of in silico techniques in the better understanding of infectious agents. But we warn that these two articles do not deal completely/exactly with in silico modeling of infectious disease(s).

Model of bacterial and viral dynamics

Models have been constructed to simulate bacterial dynamics, such as growth under various nutritional and chemical conditions (Brookmeyer et al., 2005), chemotactic response (Andrews et al., 2006), and interaction with host immunity (Ben-David et al., 2005). Clinically important models of bacterial dynamics relating to peritoneal dialysis (Hotchkiss et al., 2004), pulmonary infections (Henson, 2003), and particularly of antibiotic treatment and bacterial resistance (Hupert et al., 2002) have also been developed.

Baccam et al. (2006) utilized a series of mathematical models of increasing complexity, which incorporate target cell limitation and the innate interferon response, the model is applied to examine influenza A virus kinetics in the upper respiratory tracts of experimentally infected adults showing the models to be applicable to improve the understanding of influenza A virus infection and estimated that during an upper respiratory tract infection, influenza virus initially spreads rapidly with one cell, on average, infecting ~20 others (Daun and Clermont, 2007).

Model parameter and spread of disease: model parameters are one of the main challenges in mathematical modeling since all models do not have a physiological meaning. Sensitivity analysis and bifurcation analysis give us the opportunity to understand how model outcome and model parameters are correlated, how the sensitivity of the system is with respect to certain parameters and the uncertainty in the model outcome yielded by the uncertainties in the parameter values (Bernard et al., 2004). Uncertainty and sensitivity analysis was used to evaluate the input parameters play on the basic productive rate (*Ro*) of the severe acute respiratory syndrome (Chowell et al., 2006) and tuberculosis (Sanchez and Blower, 1997). The control of the outbreak depends on identifying the disease parameters that are likely to lead to a reduction in *R*.

Challenges in in silico modeling of infectious diseases

Difficulty in finding the most appropriate set of parameters for the in silico modeling of infectious diseases is often a challenge. But this challenge hopes to subside with the advancement in infectonomics and high-throughput technology. However another important challenge lies in the understanding (and the provision of reasonable interpretations for) the results from all the complex interaction of parameters considered.

Neuronal diseases

In this subsection, we focus on the application of in silico modeling to improve knowledge of neuronal diseases and thus improve the applications of neurological knowledge in the solving of neuronal health problems. It is not an overstatement to say that one of the many aspects of life sciences where in silico disease modeling would have the biggest applications is in the better understanding of the pathophysiology of nervous system (neuronal) diseases. This is basically because of the inherently delicate nature of the nervous system and the usual extra need to be much sure of how to proceed prior to attempting to treat neuronal disease conditions. By these, we mean that the need to first model neuronal disease conditions in silico prior to deciding on or suggesting (for example) a treatment plan is, in fact, rising. This is not unexpected, after all, it is better to much sure of what would work (say through in silico modeling) than to try what would not work.

Pathophysiology of neuronal diseases as the source of parameters for in silico modeling of neuronal diseases

Obtaining appropriate parameters for the in silico modeling of a nervous system (neuronal) disease is rooted in a good understanding of the pathophysiology of such neuronal disease. But since comprehensive details of pathophysiology of neuronal diseases are beyond the scope of this book, we only present the basic idea that would allow the reader to understand how in silico modeling of a nervous system (neuronal) disease can be done. We encourage readers who are interested in more details of the pathophysiology of neuronal disease to proceed to available textbooks and articles (de la Torre, 2008).

To give a generalized explanation and still concisely present the basic ideas underlying the pathophysiology of neuronal diseases, we proceed by systematically categorizing the mediators of neuronal diseases pathophysiology under (1) nervous cell characteristics, (2) signaling chemicals and body electrolytes, (3) host/organism factors, and (4) environmental factors. Readers need to see all these categories as been highly integrated pathophysiologically rather than see them as been spate entities—and that we have only grouped them this way to make simple the explanation of how the parameters for in silico modeling of neuronal diseases are generated.

When something goes wrong with (or there is a marked deviation from equilibrium in) a component of any of the four categories above, the other components (within and/or outside the same category) try hard to make adjustments so as to annul/compensate for the undesired change. For example, if the secretion of a chemical signal suddenly becomes abnormally low, the target cells for the chemical signal may develop mechanisms to use the signaling chemical more efficiently and the degradation rate of the signaling chemical may be reduced considerably. Through these, the potentially detrimental effects of reduced secretion of the chemical signal are annulled, through the compensation from the other components. This is just a very simple example—much complex regulatory and homeostasis mechanisms exist in the neuronal system. But despite the robustness of those mechanisms, things still go out of hand sometimes, and disease conditions result. The exploration of what happens in/to each and all of the components of this giant system in disease conditions is called the pathophysiology of the neuronal disease, and it this pathophysiology that "provides" parameters for the in silico modeling of neuronal diseases.

Parameters for in silico modeling of neuronal diseases

Parameters derived from characteristics of nervous cell

Some of the important parameters (that are of nervous cell characteristics origin) for a typical in silico

modeling of a neuronal disease [e.g., Alzheimer's disease (AD)] are the population (or relative population) of specific neuronal cells (such as glial cells—microglia, astrocytes, etc.), motion of specific neuronal cells (e.g., microglia), amyloid production, aggregation, and removal of amyloid (Edelstein-Keshet and Spiros, 2002), morphology of specific neuronal cells, status of neuronal cell receptors, generation/regeneration/degeneration rate of neuronal cells, status of ion neuronal cells' channels, etc. Based on their relevance to the pathophysiology of the neuronal disease being studied, many of these parameters are often considered in the in silico modeling of the neuronal disease. And more importantly, their spatiotemporal dynamics are often seriously considered.

Parameters derived from characteristics of signaling chemicals and body electrolytes

The importance of signaling chemicals and electrolytes in the nervous system makes parameters related to them very important. The secretion, uptake, degradation (Edelstein-Keshet and Spiros, 2002), and diffusion rates, of various neurotransmitters and cytokines are often very important parameters in the in silico modeling of neurodiseases. Other very important parameters are the concentration gradients of the various neurotransmitters and cytokines, the availability and concentration of second messengers, and the cells'/system's electrolyte status/balance. The spatiotemporal dynamics of all of these are also often seriously considered.

Parameters derived from host/organism factors

The parameters under host/organism factors can be highly varied depending on the intentions and the assumptions governing the in silico disease modeling. Nonetheless, one could basically group and list the parameters collectively under genotype (based on allele at specified genetic locus), nutritional status (feeding habits/food intake characteristics, e.g., daily calories, protein, etc., intake), gender (male or female), age, and behavior (host's behaviors/lifestyle that influences homeostasis and/or responses to stimuli).

Parameters derived from environmental factors

A few examples of these parameters are ambient temperature, altitude, light–dark cycle, social network, type of influences from people in network, etc.

Neuronal disease in silico model proper, a typical approach/scenario

Just like other in silico models, the neuronal disease in silico model is also based on what could be viewed as a single giant functional equation, which is composed of highly integrated simpler functional equations.

So, the equations behind a typical neuronal disease in silico model could take the form

$$N = \beta\ (\text{link function})\ f(C)\ (\text{link function})\ g(S)\ (\text{link function})\ j(H)\ (\text{link function})\ k(E) + \varepsilon \ldots \tag{22.5}$$

where N could be a parameter that is directly a measure of the disease manifestation; β is a constant; f, g, j, and k are link functions which may be the same or different from other link functions in this system of equations; C, S, H, and E are the outputs from smaller equations that are based on parameters from neuronal cell characteristics, signaling molecules and electrolyte parameters, host parameters, and environment parameters, respectively; and $\mathcal{E}$ is a random error parameter.

Reader should know that each of N, C, S, H, and E could have resulted from smaller equations that could take forms similar to those (Eqs. (22.2)–(22.4)) described under in silico modeling of infectious diseases (previous subsection).

Specific examples of neuronal disease in silico model

In their work, Edelstein-Keshet and Spiros (2002) used in silico modeling to study the mechanism and/formation of AD. The target of their in silico modeling was to explore and demystify how various parts implicated in the etiology and pathophysiology of AD work together as a whole. Employing the strength of in silico modeling, the researchers were able to transcend the difficulty of identifying detailed disease progression scenarios, and they were able to test a wide variety of hypothetical mechanisms, at various levels of detail.

Readers who may be interested in the complete details of the assumptions that govern in silico modeling of AD, the various other aspects of the model, and more detailed account of the findings would love to look at the article of Edelstein-Keshet and Spiros.

Several other interesting studies have applied in silico modeling techniques to investigate various neuronal diseases. A few examples include the work of Altmann and Boyton (2004) who investigated multiple sclerosis (a very common disease resulting from demyelination in the central nervous system) using in silico modeling techniques; Lewis et al. (2010) who used in silico modeling to study the metabolic interactions between multiple cell types in AD condition; and Raichura et al. (2006) who applied in silico modeling techniques to dynamically model alpha-synuclein processing (in normal and) in Parkinson's disease state(s).

A more specific example of a molecular level in silico AD model can be found in Ghosh et al. (2010, 2012). Among the amyloid proteins, amyloid-β (Aβ) peptides

(Aβ42 and Aβ40) are known to form aggregates that deposit as senile plaques in the brains of AD patients. The process of Aβ aggregation is strongly nucleation-dependent and is inferred by the occurrence of a "lag-phase" prior to fibril growth that shows a sigmoidal pattern. Ghosh et al. (2010) dissected the growth curve into three biophysically distinct sections to simplify the modeling and to allow the data to be experimentally verifiable. Stage I is where the prenucleation events occur whose mechanism is largely unknown. The prenucleation stage is extremely important in dictating the overall aggregation process where critical events such as conformation change and concomitant aggregation take place, and it is also the most experimentally challenging to decipher. In addition to mechanistic reasons, this stage is also physiologically important as low-molecular weight species are implicated in AD pathology. The rate-limiting step of nucleation is followed by growth (stage II, Fig. 22.1). The overall growth kinetics and structure and shape of the fibrils are mainly determined by the structure of nucleating species. An important intermediate along the aggregation pathway, called "protofibrils" have been isolated and characterized that have propensities to both elongate (by monomer addition) as well as to laterally associate (protofibril−protofibril association) to grow into mature fibrils (stage III in the growth curve).

Simulation of the fibril growth process in Aβ42 aggregation. Ghosh et al. (2010) generated an ODE-based molecular simulation (using mass-kinetics methodology) of this fibril growth process to estimate the rate constants involved in the entire pathway. The dynamics involved in the protofibril elongation stage of the aggregation (stage III of the process) were estimated and validated by in vitro biophysical analysis.

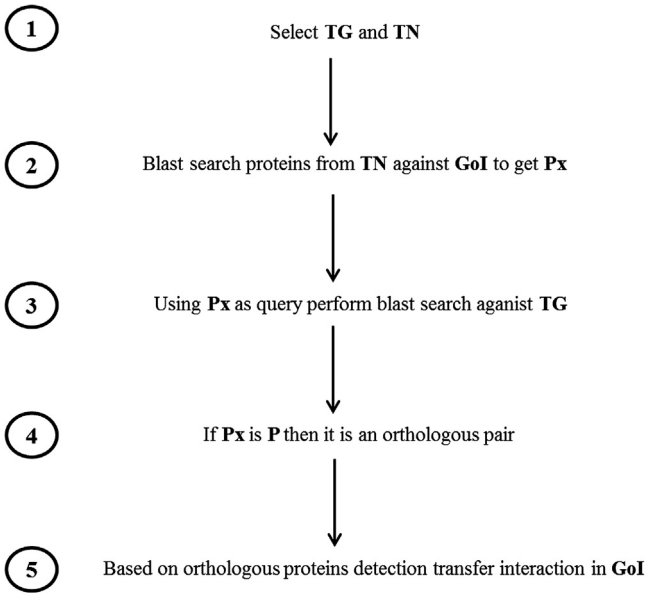

FIGURE 22.1 Flow Chart showing use of bio-informatics in disease modeling.

Preliminary identification of nucleation mass: Ghosh et al. (2012) next used the rate constants identified from stage III to create a complete aggregation pathway simulation (combining stages I, II, and III) to approximately identify the nucleation mass involved in Aβ aggregation.

In order to model the Aβ system, one needs to estimate the rate constants involved in the complete pathway and the nucleation mass itself. It is difficult to iterate through different values for each of these variables to get close to the experimental plots (fibril growth curves measured via fluorescence measurements with time) due to the large solution space and finding the nucleation phase cannot be done independently without estimating the rate constants alongside. However having separately estimated the postnucleation stage rate constants as mentioned above reduces the overall parameter estimation complexity.

The complete pathway simulation in Ghosh et al. (2012) was used to study the lag times associated with the aggregation pathway and hence predict possible estimates of the nucleation mass. The following strategy was used: estimate the prenucleation rate constants that give the *maximum lag times* for each possible estimate of the nucleation mass. This led to four distinctly different regimes of possible nucleation masses corresponding to four different pairs of rate constants for the prenucleation phase (regime 1: $n = 7,8,9,10,11$; regime 2: $n = 12,13,14$; regime 3: $n = 15,16,17$; regime 4: $n = 18,19,20,21$). However it was experimentally observed that the semilog plot of the lag times against the initial concentration of Aβ is linear and this characteristic was used to figure out what values of nucleation mass are most feasible for the Aβ42 aggregation pathway. The simulated plots show a more stable relationship between the lag times and the initial concentrations, and the best predictions for the nucleation mass was reported to be in the range 10,11,. . .,16.

Such molecular pathway level studies are extremely useful in understanding the pathogenesis of AD in general and can motivate drug development exercises in the future. For example, the characterization of the nucleation mass is important as it has been observed that various fatty acid interfaces can arrest the fibril growth process (by stopping the reactions beyond the prenucleation stage). Such in-depth modeling of the aggregation pathway can suggest what concentrations of fatty acid interfaces should be used (under a given Aβ concentration in the brain) to arrest the fibril formation process leading to direct drug dosage and interval prediction for AD patients.

Possible limitations of in silico modeling of neuronal diseases

Despite that we have mentioned several possible parameters for in silico modeling of neurodiseases, it is worthy of note that finding a set of the most reasonable set of parameters for the modeling is, in fact, a big challenge. On the other hand, understanding (and thus finding reasonable biological interpretations for) the results from the complex interaction of all parameters considered is also a big challenge. In addition, a number of assumptions that models are sometimes based on still have controversial issues. Accurately modeling the spatiotemporal dynamics of neurons and neurotransmitters transmitters (and other chemicals/ligands) also constitutes a huge challenge (Edelstein-Keshet and Spiros, 2002).

Conclusion

Understanding the complex systems involved in a disease will make it possible to develop smarter therapeutic strategies. Treatments for existing tumors will use multiple drugs to target the pathways or perturbed networks that show an altered state of activity. In addition, models can effectively form the basis for translational research and personalized medicine.

Biological function arises as the result of processes interacting across a range of spatiotemporal scales. The ultimate goal of the applications of bioinformatics in systems biology is to aid in the development of individualized therapy protocols to minimize patient suffering while maximizing treatment effectiveness. It is now being increasingly recognized that multiscale mathematical and computational tools are necessary if we are going to be able to fully understand these complex interactions, for example, in cancer (Anderson and Quaranta, 2008) and heart diseases (Liang et al., 2009).

With these bioinformatics tools, computational theories, and mathematical models introduced in this article, readers should be able to dive into the exhilarating area of formal computational systems biology. Investigating these models and confirming their findings by experimental and clinical observations is a way to bring together molecular reductionist with quantitative holistic approaches and create an integrative mathematical view of disease progression. We hope to have shown that there are many interesting challenges yet to be solved and that a structured principled approach will be essential for tackling them.

Systems biology is an emerging field that aims in understanding the biological system at the system level with a high aspect of mathematical and statistical modeling methods. In silico modeling of infectious disease is a rich and growing field focused on modeling spread and containment infection with designs being a flexible enabling adaptation to new data types.

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Long answer questions

1. Explain the role of bioinformatics in animal biotechnology.
2. Explain the common computational methods in systems biology.
3. Explain the concept of in silico modeling.
4. Discuss the advantages, disadvantages, and ethical issues of in silico modeling.
5. What are the different application areas of in silico modeling? Discuss in detail how in silico modeling is applied in one application area.

Short answer questions

1. Describe the template-based methods to reconstruct transcriptional regulatory networks.
2. What is the goal of in silico modeling?
3. What are the challenges in in silico modeling of infectious diseases?
4. What are the three types of cancer models discussed in the chapter?
5. Discuss the parameters considered for in silico modeling of infectious diseases.

Answers to short answer questions

1. The template-based transcriptional control network reconstruction method exploits the principle that orthologous proteins regulate orthologous target genes. In this approach, regulatory interactions are transferred from a genome (such as a genome of a model organism or well-studied organism) to the new genome.
2. The ultimate goal of in silico modeling in biology is the detailed understanding of the function of molecular networks as they appear in metabolism, gene regulation, or signal transduction.
3. There are two major challenges in modeling infectious diseases:
   a. Difficulty in finding the most appropriate set of parameters for the in silico modeling of infectious diseases is often a challenge.
   b. Understanding the results from all the complex interactions of parameters considered.
4. There are three types of cancer models. *Continuum models:* In these models, extracellular parameters can be represented as continuously distributed variables to mathematically model cell–cell or cell–environment interactions in the context of cancers and the tumor microenvironment. *Discrete models:* These models represent cancer cells as discrete entities of defined location and scale, interacting with one another and external factors in discrete time intervals according to predefined rules. *Hybrid models:* These models incorporate both continuum and discrete variables in a modular approach.
5. There are three types of parameters considered for in silico modeling of infectious diseases:

a. *Parameters derived from characteristics of agent:* Examples: concentration of the agent's antigen–host antibody complex; case fatality rate; strain of the agent; other genetic information of the agent; etc.
b. *Parameters derived from characteristics of host:* Examples: the total white blood cell counts; differential white blood cell counts, and/or much more sophisticated counts of specific blood cell types; blood levels of some specific cytokines, hormones, and/or neurotransmitters; daily calories, protein, and/or fat intake; daily amount of energy expended and/or duration of exercise; etc.
c. *Parameters derived from characteristics of environment:* Examples: host's ambient temperature; host's ambient atmospheric humidity; altitude; host's light–dark cycle; etc.

Yes/no type questions

1. Does bioinformatics play a role in animal biotechnology?
2. Does systems biology require computational approaches?
3. Does network analysis is important in disease modeling?
4. Can we develop e-cell for cellular phenotype simulation?
5. Does computational modeling or organ require mathematics?
6. Does metabolic modeling play a role in disease modeling?
7. Is E-CELL a computational model of a cell and help in analysis of biological systems in a cell?
8. Is a specific cancer can be modeled in silico?
9. Is it true that in silico disease modeling is not helpful in drug discovery and clinical trial.
10. Is metabolic pathway modeling possible using genomic data?

Answers to yes/no type questions

1. Yes—Bioinformatics is now essential for molecular aspects to phenotype analysis and prediction in animal biotechnology
2. Yes—Computational and mathematical modeling is essential in systems biology.
3. No—Network analysis is important for identification of pathways and key molecules in a disease.
4. Yes—Using various in silico modeling approach of cell, we can develop e-cell and study the phenotype.
5. Yes—Mathematical approach is essential in computational modeling of organ and disease for fixing various parameters of the organ or disease.
6. Yes—Without metabolic modeling approach, in silico disease modeling is not possible.
7. Yes—It helps in the analysis of biochemical pathways and simulation.
8. Yes.
9. No—Computational modeling of a disease in commonly practiced in drug discovery studies to understand efficacy and toxicity of the drug.
10. Yes.

SECTION III

Animal biotechnology: applications and concerns

CHAPTER

23

Transgenic animals in research and industry

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Summary

Transgenic animals are animals that have been genetically transformed by splicing and inserting foreign (animal or human) gene(s) into their chromosomes. The gene, if successfully integrated, enables an animal to produce the desired protein in milk, urine, blood, sperm, or eggs or to grow rejection-resistant organs for transplant. These commonly known recombinant proteins have therapeutic potentials, for example, in the treatment of cystic fibrosis, hemophilia, osteoporosis, arthritis, and parasitic or infectious diseases such as malaria, AIDS, and hepatitis-B. Transgenic animals can also produce monoclonal antibodies specifically targeted toward disease proteins that are used in vaccine development to meet global demand. Rapidly growing technologies in genomic knowledgebase have tremendously fastened the process of transgenesis. The clustered regularly interspaced short palindromic repeat system eases the positional integration of the gene of interest in animal models. Alteration of transgenic animals' genome whether to understand diseases or sufficing human needs must be conducted under ethically restricted guidelines.

What you expect to know

This chapter has been framed in a manner to educate the reader about transgenic animals, methods of transgenesis, their effects on the environment, and the socioethical concerns associated with the creation and use of these animals. Readers will also have a glimpse of the history of transgenic animal production and their implications in pharmaceutical research and industry. The overall purpose of selection and production of animal models required for human welfare has been described. In medical research, animal models are required to understand various cellular and molecular pathways that are explained here with examples, along with disease models used to study physiology of the host leading to a disease or disorder. This chapter also includes the engineering of animals as food sources or to produce human tissues to be used in histocompatible transplantations. Finally, we discuss ethical issues related to genetic engineering and the US Food and Drug Administration's (FDA) ethical guidelines regarding the production of transgenic animals.

Introduction

The practice of carefully selecting and reproducing animals with new combinations of genes is not new. In nature, however, new gene combinations are found only in the same or similar species. Transgenic or genetically engineered animals are the result of novel gene combinations thoughtfully manipulated and implemented by scientists. Because DNA contains the universal genetic code for all living organisms, it can be easily altered and transferred between two completely unrelated organisms to produce a combination of characteristics that would not otherwise be possible.

The term "transgenic" was coined in 1981 by Gordon and Ruddle to describe an animal in which an exogenous gene has been introduced into the genome (Gordon and Ruddle, 1981). A genetically engineered or "transgenic" animal carries a known sequence of

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recombinant DNA in its cells and passes the recombinant DNA onto its offspring. In the late 1980s, the term "transgenic" was extended to gene-targeting experimentation and the production of chimeric or "knockout" mice in which particular gene has been selectively removed from the host genome. Recombinant DNA refers to fragments of DNA sequences that have been joined together in a molecular biology laboratory. To express a desired protein, recombinant DNA is constructed in such a manner that it can express the functional protein when inserted into the nucleus of a prospective transgenic animal. The gene encoded in the resultant DNA construct is capable of producing the same kind of protein no matter which animal or microbe or even plant is producing it. However, the nucleotides coding for signal peptide that leads to release of the protein at a particular location is added along with the gene of interest. Most of the recombinant proteins are exact copy of their natural counterpart unless otherwise certain modifications are required to make the protein biologically active or identical in behavior and appearance. Various recombinant proteins particularly for therapeutic purposes have been produced in transgenic animals. Some of these engineered proteins provide the animals with better disease resistance than their wild-type counterparts (Richt et al., 2007). Other examples are proteins that provide healthier milk, meat, or eggs.

Rapidly growing knowledge about DNA and its functions in this era of next-generation sequencing has drastically changed the quickness of generating desired animal models. Especially by using the new robust techniques such as clustered regularly interspaced short palindromic repeat (CRISPR) systems or CRISPR/Cas9, creation of transgenic animals has become easy and quick. Transgenic animals can be produced through various methods, but in all procedures, first requirement is to generate a transgene, that is, the DNA sequence that encodes for a particular protein along with other necessary sequences. Generally, three parts are required to construct a transgene: first, a promoter sequence that determines which tissue should express the recombinant protein; second, the sequence of the gene coding for amino acids of the desired recombinant protein; and third, the sequence responsible for the termination of the expressed protein. The most common method of producing transgenic animals is microinjection in which the constructed transgene is inserted into the male pronucleus of a freshly fertilized egg. Generally, several copies of the transgene are inserted into the male pronucleus, which is larger than the female pronucleus. The other common method for producing transgenic animals is embryonic stem cell (ESC) manipulation. In this method, a transgene is inserted into the stem cells of the blastocyst with the help of microinjection, chemicals, or viral transduction. The ease of screening of ESCs carrying transgene is the main advantage leading to the high efficiency of this technique. Polymerase chain reaction (PCR) and Sanger sequencing are two main techniques for screening the animals carrying the desired transgene.

Different categories of transgenic animals include those produced as disease models, xenotransplanters, transpharmers, food sources, and scientific research models. Through genetic engineering, various disease models have been developed to mimic the human symptoms of disease. Examples of such models include the oncomouse (mouse model for cancer study), the AIDS mouse, the Alzheimer's mouse, and the HLA-A2.1/DTR mouse to study the presentation of antigens that are normally not presented by mouse antigen-presenting cells' surface (Sagar et al., 2014) and Parkinson's fly. Animals that are engineered to express a desired protein in their milk are known as transpharmers, for example, mice, sheep, goats, and cows have been engineered by this method. Also there are animals that have been engineered to produce histocompatible organs that can be implanted in humans without fear of rejection by the human body. This technique has been used for producing pigs as xenotransplanters, but the use of those organs has not been approved yet. Similarly, to meet the daily increasing demand of food, animals have been engineered that grow larger than their wild-type counterparts without requiring extra food. Two examples of transgenic food sources are superfish and superpig. Superfish is a promising food source, but superpig proved futile because the animals developed multiple health issues.

Transgenic animal models for scientific research are generally produced by inserting a transgene in their DNA to study the effect of overexpression of that particular gene on the animal's physiological processes. Sometimes the gene under investigation is knocked out to determine its effect on normal body metabolism. Well-known examples of scientific research models are ANDi, the transgenic monkey; smart mouse; youth mouse; supermouse; and influenza-resistant mouse.

Many ethical issues are associated with the production of transgenic animals. First, is it ethical to generate transgenic animals? It seems clear that altering the genome of an animal to create artistic effects (e.g., the green fluorescent rabbits) is unnecessary and cannot be recommended. If the creation of an animal would increase scientific knowledge and possibly help understand a disease condition, then alteration of the genome can be accepted in common. Although some animals die in most transgenic experiments, a number of human lives are saved as a result may be much greater than the animals' suffering although it is recommended that the suffering of the transgenic animals should be reduced as much as possible. We cannot justify the production of all transgenic animals but each experimental case should be judged on a case-to-case basis. For example, Alzheimer's mouse feels no

pain due to its transgene, and its creation is justified because studying this mouse model might lead to new Alzheimer's treatments. Conversely, the Beltsville pig or so-called superpig experienced much suffering for a remote possibility of helping meet world food demand, so this experiment was rightly terminated. Another important example is oncomouse, a model that straddles this ethical edge because the mouse does suffer tremendously and eventually dies of cancer, but the knowledge gained from this transgenic model is highly valuable in the fight against cancer in human beings. Therefore, the creation of oncomouse seems to be justified, but measures must be applied to reduce the animals' pain, or they can be sacrificed before advanced tumor development that might cause them unbearable suffering. Although animal physiology is not identical to human physiology, the knowledge obtained from living disease models is much more important and useful, thus justifying the creation of transgenic animals as disease models.

Another concern is that transgenic animals will escape and outbreed with their wild-type natural cousins, but despite this concern, transgenic animals can be reared and used with restrictive security measures in place. Established regulatory authorities such as universities' Institutional Animal Care and Use Committees (IACUC) monitor scientific research and require research scientists to justify animal use for each experiment. The creation of transgenic animals per se is opposed by many religious groups and by some voluntary and nongovernmental organizations that feel the practice interferes with nature. Most, however, have no problem if the suffering of the animals is minimized and the experiment is important scientifically.

Creating transgenic animals

Transgenic animals can be created using various techniques as outlined earlier by microinjection of a transgene into the male pronucleus of a freshly fertilized egg in vitro or ESC transfer with the help of a chemical or virus, homologous recombination, or gene knockdown.

Construction of a transgene

Although the genetic code is almost the same for all organisms, the fine details of gene control are different; therefore, codon optimization is required before inserting the gene specifically into a different category of organism. For example, a gene from a bacterium will often not work correctly if it is introduced unmodified into an animal cell. Therefore, genetic engineers first construct a transgene containing the gene of interest with optimized codon plus extra DNA sequences that correctly controls the function of the gene in the new animal. When constructing a transgene, scientists generally substitute the donor's promoter sequence with one that is specially designed to ensure that the gene will function in the correct tissues of the recipient animal; for example, if the gene needs to be expressed in the milk of a mammal, the promoter specific for mammary tissues is used in the transgene (Murphy et al., 1993).

Microinjection

Microinjection is the most popular technique for creating transgenic animals. In this technique, recombinant DNA (transgene) is inserted into the male pronucleus. First, eggs are collected from superovulating female animals and then fertilized in vitro. The freshly fertilized egg is held stable by a microtube suction device (Fig. 23.1), and a solution containing 200–300 copies of the recombinant DNA is injected using a micropipette. The recombinant DNA is injected into the male pronucleus because it is larger than the female pronucleus (Wong et al., 2000). Ethical concerns arise because a very small percentage of animals are born with the desired transgene, while a huge number of eggs are wasted in the experiment. Therefore, optimized experiment condition must be followed.

Embryonic stem cell transfer

Another commonly used technique of creating transgenic animals is by transferring the transgene to ESCs. This technique is generally used when a particular gene must be altered in a particular tissue. A transgene is constructed to target a specific site in the genome of the animal to be created. For ESCs, a blastocyst is harvested from a female animal or by in vitro fertilization, and the inner cell mass is collected (Jacenko, 1997). Sometimes chemicals or steroids can be given to an animal to prevent implantation of the embryo. The transgene can be introduced into the ESCs with the help of a chemical or by a virus. Embryonic cells are used because they are capable of differentiating into any of the three germ layers. Therefore, if the ESCs carrying the recombinant gene are injected into an embryo, then that embryo when implanted into the uterus of a host animal will develop into an animal carrying that particular transgene (Fig. 23.2). Once the transgene is inside the ESCs, with the division of the cells, the transgene incorporates itself by homologous recombination. This natural

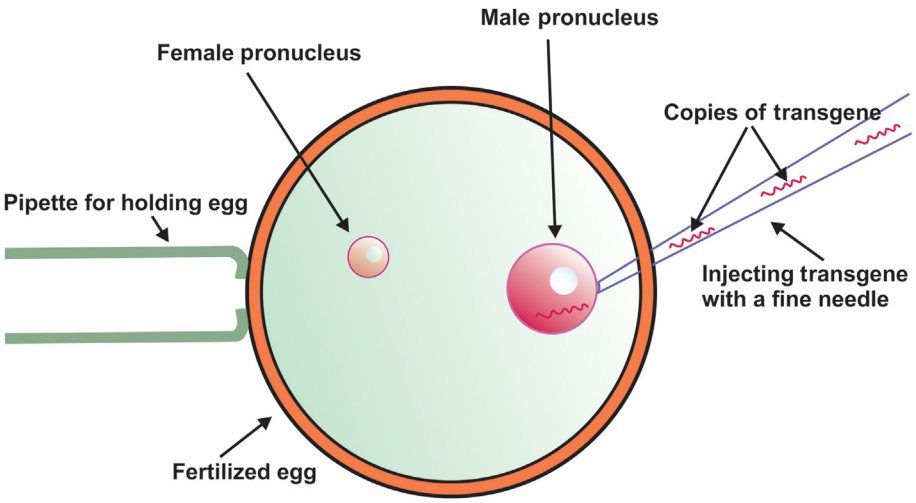

FIGURE 23.1 **Microinjection.** After fertilization, the egg is held stable by a microtube suction device, while a solution containing many copies of the transgene is injected using a micropipette into the male pronucleus (because it is larger than the female pronucleus) (Wong et al., 2000).

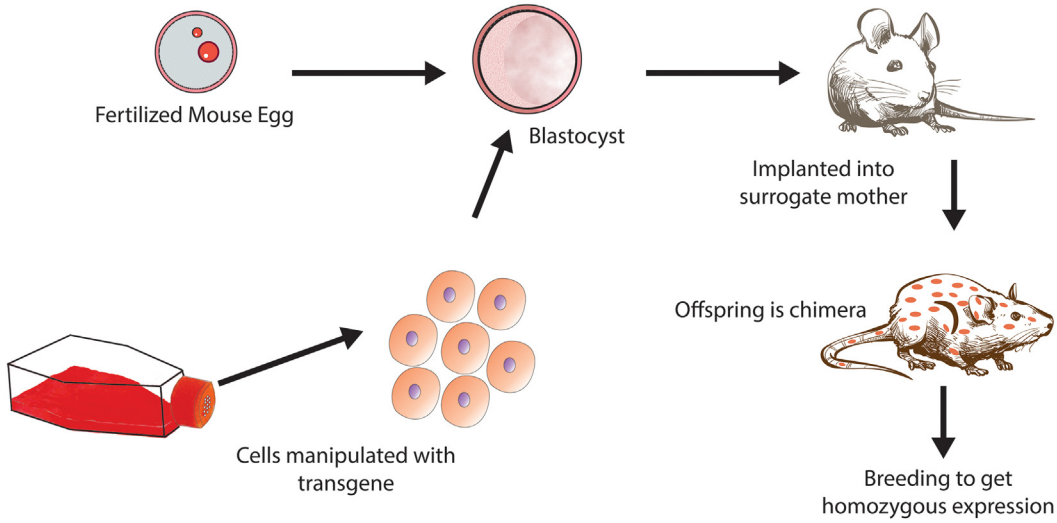

FIGURE 23.2 **Embryonic stem cell transfer.** Once a the transgene is incorporated into the embryonic stem cells, those cells can be left to divide in vitro, or they can be injected into a blastocyst and implanted into a host's uterus to grow normally (Jacenko, 1997).

technique involves crossing over between paired-sister-chromatids, which can lead to a new recombinant sister chromatid. These homologous sequences are considered when the transgene is designed to determine where to integrate the desired gene. The flanking sequences of that particular area are chosen and added to both flanking sides of the transgene. Thus, via this technique, a transgene can be integrated at a particular position, which is not possible using general techniques of viral transduction, chemicals such as calcium phosphate or rubidium chloride, or even with microinjection; as all of these techniques insert the transgene randomly into the genome.

It has also been seen that haploid ESCs can be generated successfully from mice, rats, monkeys, and humans and carries the ability to generate germline-modified animals via intracytoplasmic haploid androgenetic/parthenogenetic ESC injection directly into oocytes. Haploid ESC method is more convenient and ideal gene transfer technique for generating genetically modified animal models. The advantages of haploid ESCs are that these possess just one copy of each gene, helping in easy editing and can also be used as substitutes for sperm to generate healthy mice by direct injection into oocytes (Bai et al., 2016).

Retrovirus-mediated gene transfer

Retroviral vectors have also been used to transfer genes of interest into animal genomes (van der Putten et al., 1985). Although embryos can be used up to

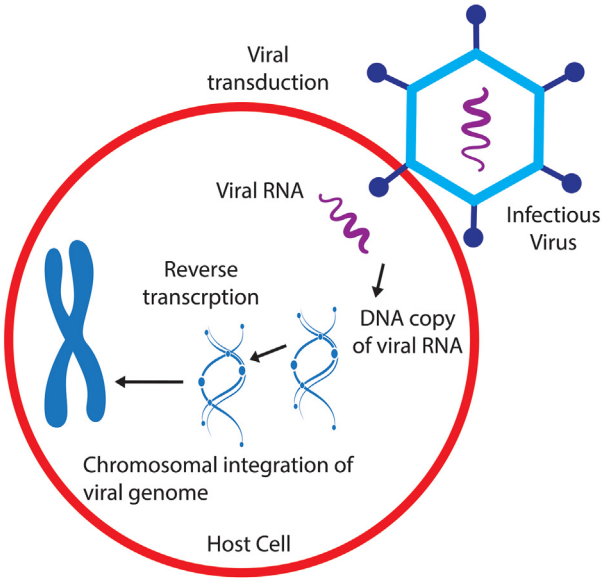

FIGURE 23.3 **Retrovirus-mediated gene transfer via transduction.** After viral transduction of host cell, viral RNA genome is reverse transcribed into DNA by reverse transcriptase (delivered into the cell along with viral RNA), and this cDNA is then integrated into the host cell genome.

midgestation, 4- to 16-cell stage embryos are generally used for infection with one or more retroviruses carrying recombinant DNA (effectively transducing only mitotically active cells). Immediately following infection, the retrovirus produces a DNA copy of its RNA genome using the viral enzyme reverse transcriptase (Fig. 23.3). Usually without any deletions or rearrangements, the DNA copy of the viral genome, or provirus, integrates randomly into the host cell genome. Very high rates of gene transfer, approaching 100% efficiency, have been achieved with the use of retroviruses. However, as with gene transfer by microinjection, integration events are random. For safety reasons, retroviruses are frequently modified by removing structural genes, such as *gag*, *pol*, and *env*, which support viral particle formation. However, most retroviral lines used in transgenic animal experiments are ecotropic, meaning that they infect only the model systems (e.g., mice or rats); hence, rodent cell lines, rather than humans, could be at risk of contamination if correct precautions are not met. Disadvantages of retrovirus-mediated gene transfer include low copy number integration; the additional steps required to produce retroviruses in comparison with microinjection or ESC-based techniques are as follows: a general limitation on the length of the foreign DNA insert (usually <15 kb), a potential for undesired genetic recombination that might alter the replicative characteristics of the retrovirus, a high frequency of mosaicism, and finally, possible interference of retroviral sequences on recombinant gene expression.

Gene knockdown and RNA interference

Efforts at gain-of-function and loss-of-function modeling have usually concentrated on introducing specific mutations into the nuclear genome. RNA interference (RNAi) technology has broadened the possibilities for creating loss-of-function animal models. Short interfering RNA (siRNA) exists in a double-stranded state and inhibits endogenous genes (and/or exogenous sequences, as in viral genes) as the result of complementary sequence homology. From an evolutionary point of view, RNAi appears to protect the cell against foreign (e.g., viral) RNA invasion. The mechanism of RNAi is thought to involve a double-stranded (sometimes hairpin) RNA molecule that is cleaved into small fragments of $\sim$22 nucleotides in length and assembled into a ribonucleoprotein complex referred to as the RNA-inducing silencing complex. The RISC then binds to homologous mRNA and performs its endonucleolytic cleavage. Recently, it has been shown that alterations in the complementary oligonucleotide (i.e., length and nucleotide composition) can have a significant effect on both the degree and the duration of "gene silencing." These oligonucleotides that silence gene expression (i.e., protein synthesis) are referred to as siRNAs. Small interfering RNA has been shown to be a potent inhibitor of gene function in vivo. Mouse and rat models harboring small hairpin RNA (shRNA) transgenes, following shRNA transcription, produced lower levels of the homologous protein when compared with controls (Hasuwa et al., 2002). Gene silencing and knockdown technology has potential medical and agricultural applications, including the inhibition of viral gene transcription and of endogenous genes coding for deleterious gene products. In small animals such as the mouse, RNAi has several advantages over homologous recombination and ESC-mediated gene knockout methods. RNAi can be chemically synthesized directly, thus avoiding laborious cloning steps. Therefore, this methodology is significantly advanced since nuclear transfer for efficient loss-of-function modeling in mammalian species (particularly for non-murine species) in which ESC transgenesis has not been successful.

CRISPR/Cas9 systems

With the help of advanced sequencing technologies, important progress has been achieved for the generation of transgenic mice based on CRISPR systems. The CRISPR is a part of DNA containing short repetitive sequences (20—50 bp) and was discovered in 1987 (Ishino et al., 1987) and was later on redefined in 2002 (Jansen et al., 2002). More than 40% of bacteria and nearly 90% of archea carries CRISPR locus in their genome. The *cas* genes code for helicase proteins or

nuclease enzymes used to unwind and cut DNA and were recently found to be associated with CRISPR. *Streptococcus pyogenes* Cas9 is widely used in genome editing. The type II CRISPR/Cas is important for genome editing applications, and this include three components: target-specific CRISPR-derived RNA (crRNA), target-independent transactivating RNA (tracrRNA), and the putative nuclease Cas9 (Fig. 23.4). The tracrRNA binds to crRNA and with Cas9 forms a ribonucleoprotein complex and crRNA guides the complex to cleave the chromosomal DNA at target position, which can be repaired by homologous or nonhomologous end-joining system, where former leads to precise correction and the latter creates insertion/deletion (indel) mutations. Important part of the crRNA and tracrRNA can be joined to form a single-chain guide RNA (sgRNA), which can be easily programmed to target 18—25 bp DNA segment of interest. This sgRNA binding site must be followed by a protospacer adjacent motif, a small DNA segment, NGG which can be found every 8 bp in human genome. These new platforms are helping tremendously for analysis of gene function, human disease modeling, and gene therapy. Techniques involving site-specific nucleases has opened new era for rapid generation of transgenic models via nucleases, specifically, zinc finger nucleases, transcription activator-like effector nucleases, and CRISPR systems. Of these techniques CRISPR can be applied more easily and flexibly to functionally inactivate and activate genes in cells (Fig. 23.5). Even the direct injection of the CRISPR system into zygotes is an efficient

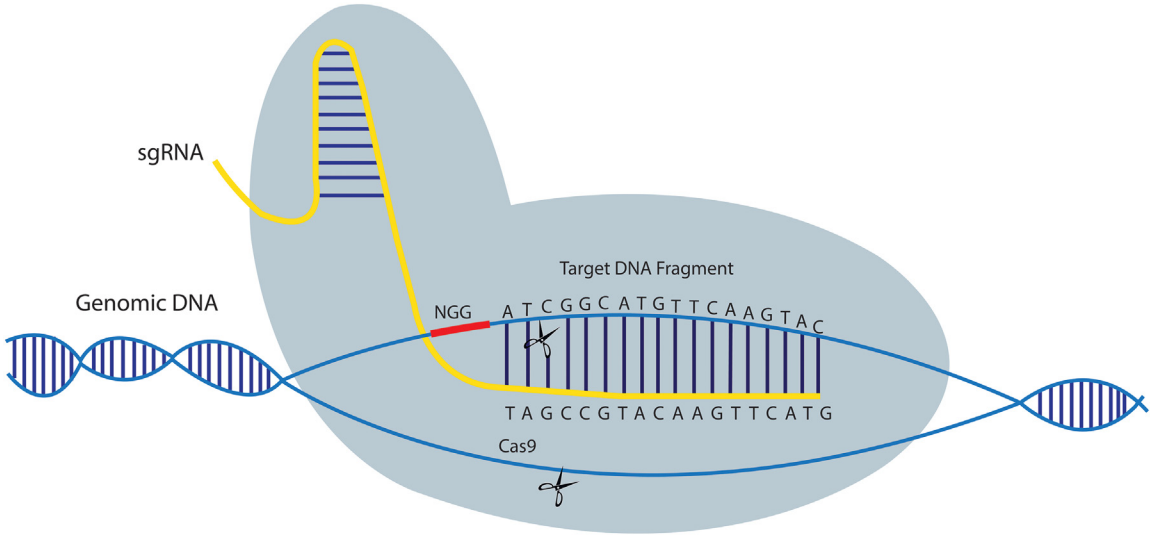

FIGURE 23.4 **CRISPR/Cas9 complex used for genetic modifications in animals.** The CRISPR/Cas9 is the latest technology for generation of genetically modified animals. This technique is easy and more precise to target the DNA sequence to be mutated (Jansen et al., 2002).

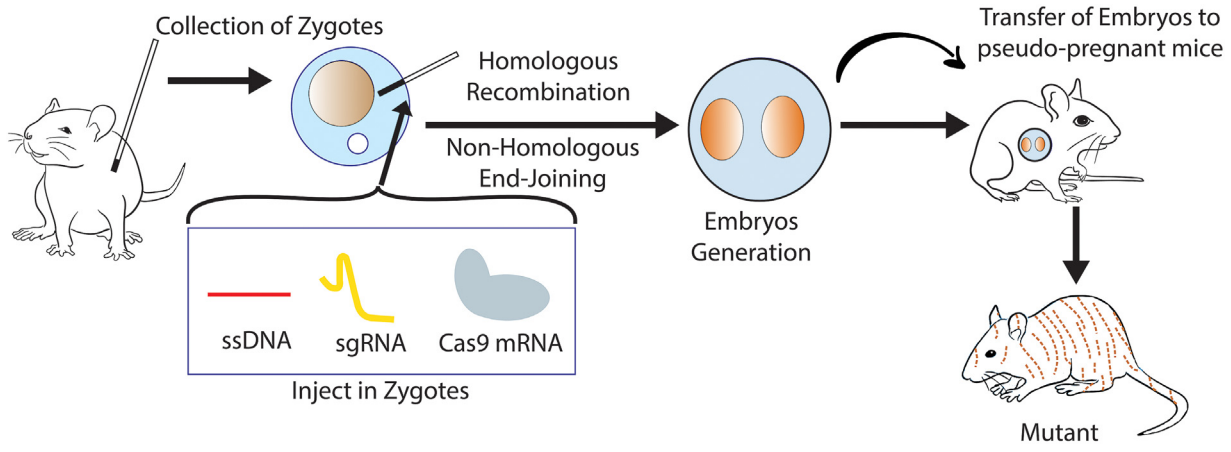

FIGURE 23.5 **CRISPR/Cas9 technology procedure for genetic modifications in animals.** The Cas9-mRNA along with ssDNA and sgRNA is injected into the zygotes collected from mice. During DNA repair using homologous recombination or nonhomologous end-joining system, desired change is created and embryos are generated. These embryos are then transferred to pseudopregnant mice, which ultimately give birth to mutated mice.

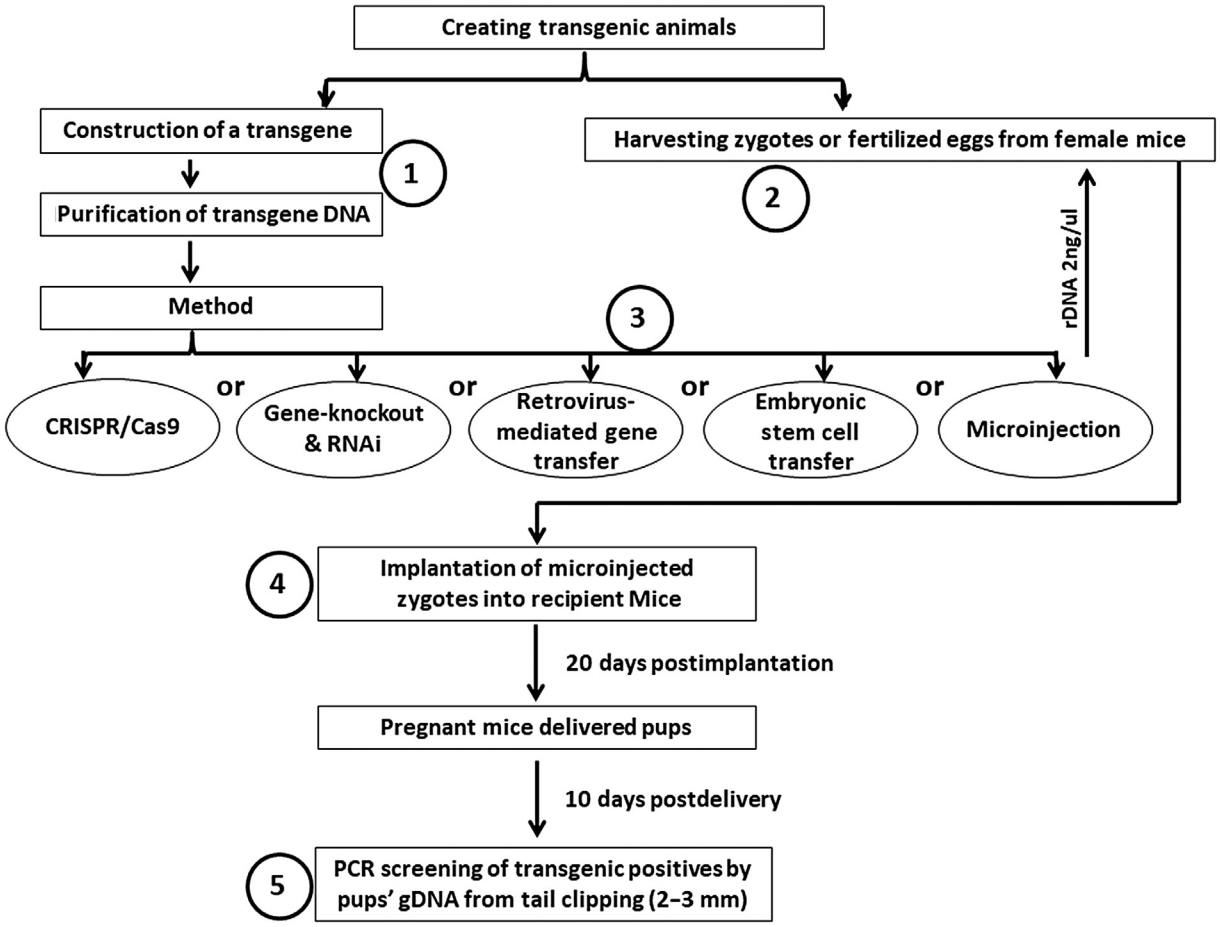

FIGURE 23.6 Flowchart of methodology discussed in this chapter.

technique to produce transgenic animals (Li et al., 2013). Recent study has shown that a dual single-guide RNA (sgRNA)-guided Cas9 nuclease when delivered as a ribonucleoprotein complex through electroporation can efficiently targeted deletion of faulty COL7A1 exons in polyclonal patient keratinocytes and therefore holds prospective translation as therapeutic strategy to the clinic for treating recessive dystrophic epidermolysis bullosa (Bonafont et al., 2019).

Screening for transgenic positives

After the transgenic animals are born, they are screened either by PCR or by Sanger sequencing. The expression of transgene depends on the site of integration of the transgene; some transgenes may not be expressed if they are integrated into a site that is transcriptionally inactive. Therefore, a common practice is to breed the animals further to obtain the optimal expression of the desired protein (Fig. 23.6).

Transgenesis versus cloning

Transgenesis should not be confused with cloning. Cloning refers to the reproduction of an exact replica of a living organism using the DNA (without manipulation) of that organism, whereas transgenesis (genetic engineering) refers to the human manipulation of genetic material in a manner that does not occur in nature. In 1997, Dolly the sheep was born as a result of cloning experiment at Roslin Institute (Midlothian, Scotland, United Kingdom) using a somatic cell from an adult sheep.

Transgenic animals as disease models

Many animal models have been created and various are on researchers' lists to be generated for studying the mechanisms of disease or changes in physiology that characterize some disorders, and a few important examples are discussed here. Small animal models such as

mouse models are favored because of its relatively short gestation time, easy manipulation, and housing. However, many researchers like pigs as disease models for their anatomical and physiological similarities to humans in studying diseases related to inflammation, heart, skin, eye, Lou Gehrig's, sickle cell, and so on.

Oncomouse

This is an important mouse model created by researchers at Harvard University to study cancer. Cancers develop in different ways from various causes; therefore, there are many ways to create a model for studying cancer. One way is to create mice lacking the *p53* allele, which is the tumor suppressor gene that is involved in most cancers and that is crucial in checking the uncontrolled growth and characteristic of cancer. Removal of this allele leaves mice susceptible to many types of cancer, most frequently lymphoma. Oncomouse was created in 1984 by replacement of the normal mouse *myc* gene with a virus tumor promoter/*myc* fusion recombinant gene. The mice and their offspring developed carcinomas. This first mouse was created at Harvard Medical School in Boston for DuPont. The researchers applied for and received a patent in 1988 for the process of creating the animal (Leder and Stewart, 1984), and for the mouse itself, making oncomouse the world's first patented animal. This caused considerable stir in the scientific community (as will be discussed further in "Ethics in Transgenesis" section), because to study this cancer model or to create a new model using Harvard's techniques of producing the oncomouse, a license was required to be obtained. DuPont argued that the patent covered any transgenic animal predisposed to cancer. Since then, the company has allowed researchers working with the US National Institutes of Health to work on the mouse for nonprofit research. Various experiments continue on oncomouse that may lead to preventing and curing multiple forms of cancer.

AIDS mouse

AIDS mouse is another good example of a disease model. AIDS mouse was created in 2001 at the University of Maryland by microinjecting the genome of HIV-1 into fertilized mouse eggs. The recombinant DNA of HIV-1 (the transgenic genome) was altered by deletion of the two genes that cause the virus to become infectious. Thus, HIV-1 mouse cannot transmit the virus to humans, making handling of the animal relatively safe while still allowing study of the HIV biology. This model allows researchers to study early-stage symptoms to better diagnose the disease in humans. It also allows researchers to track chronic conditions associated with AIDS and to test numerous treatments in the search for a cure for HIV disease. Previous reports showed that chimpanzees were capable of supporting HIV replication, but no small animal developed the virus, and use of chimpanzees as a disease model is very expensive. An original female mouse that possessed the modified viral gene was bred with a healthy male mouse to produce HIV gene-bearing pups. Skin lesions also seen in the AIDS mouse were similar to those seen in Kaposi's sarcoma, often occurring in AIDS patients. These observations indicated that HIV may indeed be a cause of cancer (Vogel et al., 1988). Thus, AIDS mouse was a big step toward finding treatments to prevent or cure the disease.

After integration into the host genome, gene expression of HIV is regulated by the long terminal repeat (LTR) promoter. It can be assumed that gene editing of the HIV promoter, for example, creating short nucleotide mutations or fragment excision may deactivate viral transcription. Before recent breakthroughs in gene-editing techniques using CRISPR/Cas9, gene therapy for HIV was progressing very slowly. By the use of shorter version of the Cas9 endonuclease carried by an adenoviral vector, critical segments spanning between the LTR and gag regions of the viral DNA genome were removed successfully in an HIV transgenic mice model. All tissues as well as circulating lymphocytes were examined and confirmed for the removal of the region, which caused in a significant reduction of HIV-RNA. This was also observed that CRISPR/Cas9 carried by lentiviral precisely removed the entire HIV genome within 50 and 30 LTRs of integrated HIV proviral DNA in latently infected CD4 + T lymphocytes collected from HIV-infected persons, proving that there is a huge potential of these genome-editing methods. However, these technologies like the CRISPR/Cas9 must face several major issues especially in the HIV cases such as keeping a check on resistant-viral generation. Also, there may be some mutations occurring during the process in the adjacent regions of the targeted site and could result into the selection of those strains of the virus, which might not be recognized and cleaved by CRISPR/Cas9 (Badia et al., 2017).

Alzheimer's mouse

Alzheimer's mouse is another important disease model. Alzheimer's disease has been linked to the formation of beta-amyloid plaques in the brain, places where fibers have developed tangles that can block and degrade neurons during the progression of the disease. Similar to humans with the disease, Alzheimer's mouse overproduces a protein that forms these amyloid plaques, and it displays both the symptoms and diagnostic

characteristics of Alzheimer's disease. Alzheimer's mouse was generated in 1995 by a collaborative experiment between Worcester Polytechnic Institute and Transgenic Sciences, Inc. (which became Athena, then Exemplar Corp., then Elan Pharmaceuticals). This mouse line was generated by overexpressing a mutation that causes an aggressive early-onset form of Alzheimer's disease. Scientists studying Alzheimer's disease were desirous of an animal model for some time before this breakthrough occurred. The first Alzheimer's vaccine, which almost entirely prevented the creation of amyloid plaques in young mice and even reduced the damage of the plaques already allowed to develop in older mice, was developed from research using Alzheimer's mouse (Schenk et al., 1999). This vaccine moved to human clinical trials in 2000 but was cancelled in 2001 because a minority of patients developed brain inflammation; the trial for a second-generation vaccine by the same company was also terminated. Thus far, no vaccine has been approved by the FDA for the treatment of Alzheimer's disease in humans, but on the basis of the scientific credibility established using the mouse model, researchers may be on track to preventing and curing Alzheimer's disease entirely.

Parkinson's fly

Studying neurological diseases without a model is very difficult, but animal models produced through transgenic approaches have yielded significant progress. *Drosophila* fly was created at Harvard Medical School in 2000 as a model for Parkinson's disease. This fly has a mutation of the *α-synuclein* gene linked to inheritable Parkinson's disease. Parkinson's fly shows disease-specific characteristics that are seen in humans during the progression of the disease such as loss of motion control and loss of dopamine. This fly carries a much simpler genome and serves as an excellent model for studying Parkinson's disease at the genetic level. Many previously unobservable characteristics in the progression of the disease have been studied with the help of this fly. The symptoms of Parkinson's generally do not become visible in humans until an estimated 60%–80% of dopamine nerve cells have already died (Vatalaro, 2000). Parkinson's fly allows scientists to understand early-onset symptoms, which could lead to earlier diagnosis and ultimately to treatment or cure of the disease in humans.

Transgenic animals as biological models

Transgenic animals are also created as biological models with the aim of increasing knowledge about genetics and expression of genes during certain natural and physiological conditions. Some of the important biological models that have been produced through transgenesis are discussed here.

ANDi (monkey)

The first transgenic monkey, ANDi ("inserted DNA" spelled backward), was born in 2000. ANDi is one of most important biological models (Chan et al., 2001). A harmless gene for green fluorescence protein (GFP) was inserted into ANDi's rhesus genome using an engineered virus. The eyes and fingernails of two other monkeys in the project, stillborn twins, glowed under ultraviolet light, although ANDi himself did not. The *GFP* gene was chosen for two reasons: first, it would have very little effect on the monkey, and second, detecting whether the transgene had been transmitted properly would be easy. ANDi is the only monkey of 40 fertilized eggs to be born alive and to express the gene for GFP. Therefore, ANDi proved that transgenic primates could be created and could express a foreign gene if delivered into their genome. ANDi opened the door for the creation of other primate biological models for the study of primates' natural physiology and behavior.

Doogie (the smart mouse)

In 1999, another important biological model was created at Princeton University. The transgenic mouse "Doogie," the smart mouse, was engineered to overexpress NR2B receptors in synaptic pathways. Overexpression of these receptors makes the mice learn faster, like juveniles, throughout their lives. When tested for learning and memory, "Doogie" mice performed better than their wild-type counterparts (Tang et al., 1999). To test the memory of a mouse, two objects are presented to the mouse in a cage for exploration. Then, one object is replaced with another, and the mouse is again allowed to explore the objects. If the mouse spends more time paying attention to the new object, it means a good sign that it remembers the old one. If the mouse explores each object equally, the mouse has probably forgotten the old object that it explored previously. Doogie mice do consistently better on these tests as they grow old. In the near future, this research may lead to improving learning and memory in humans and other animals. The fact that overexpression of this gene improves memory confirms an old theory about how mammals think and learn. Further research on Doogie mice can provide valuable information on human development, learning, and memory.

Supermouse

Supermouse is a transgenic biological model developed in 1982 in which the gene of a rat growth hormone is microinjected into fertilized eggs. These mice grew noticeably larger than their wild-type littermates and became the world's first expressing transgenic animals and the first ones with a noticeable phenotypical response to the transgene. Researchers hoped to use these mice to study the effects of growth hormone, accelerated animal growth, gigantism, and as a means of correcting genetic defects related to the growth pattern of animals and humans (Palmiter et al., 1982). This mouse model was also used in research aiming to create food-producing animals with the accelerated growth. Correction of dwarfism is the most obvious application of these animals.

Youth mouse

In 1997, the Weizmann Institute of Science in Rehovot, Israel, created a mouse model called "youth mouse." These mice characteristically overexpress urokinase-type plasminogen activator, primarily thought to be helpful in dissolving blood clots. These mice are smaller, eat less, and live about 20% longer than normal wild-type mice (Miskin and Masos, 1997). Overexpression of the clot dissolver extends life by preventing atherosclerosis, a process in which plaques develop in the arteries of an animal as it ages and which can lead to clots, hemorrhages, and heart attacks. Of the four lines of transgenic mice attempted, only one kind autonomously ate less and lived longer, but it also displayed infrequent muscle tremors. This transgenic mouse line, dubbed Alpha MUPA, shows the same characteristics as normal wild-type counterparts on restricted diets. Therefore, "youth mouse" promises to be a useful biological model for studying development processes and aging, especially in relation to diet.

Influenza-resistant mouse

Influenza-resistant mice are a biological model that was created to study the use of genetic alteration affecting disease resistance against, for example, influenza. These mice overproduce Mx protein, which is known to act as an antiviral agent. These mice are significantly more resistant to influenza and other orthomyxoviruses compared with their normal wild-type littermates. If these findings were applied to farm animals such as pigs and ducks, the animals' chances of infection with avian strains of influenza and other viruses, and also their chances of passing these infections to humans, might be lowered. The rate of evolution of these viruses in the animal hosts might also be lowered, thus helping humans retain our immunoprotective response against future viral outbreaks.

Transgenic animals as xenotransplanters

Organ transplantation is necessary in those cases when the whole self-organ fails to function (e.g., liver or kidney). However, organ transplantation can be performed only if a donor's organ is histocompatible with the recipient (patient) to take over the function of the diseased or failed organ. Generally, a small percentage of donated organs are found to be histocompatible with any given patient, and such matched organs are in extremely short supply. Currently, in the United States alone, about 10 patients die each day just on the waiting list to receive vital organ transplants. Thus, immunosuppressive drugs are frequently given to lower the patient's immune response against the transplanted organ to avoid graft rejection. However, this forced decrease response of the immune system leaves the patient vulnerable to opportunistic infections. Therefore, to address this issue of organ shortage and histocompatibility problem, xenotransplanters are being engineered to provide animal organs that are histocompatible with humans. Generally, the recipient rejects organs that come from other tissue types, even from the same species. The well-known cause of this rejection is the repertoire of antigens present on the surface of the organ those inform the host that the organ is nonself. Thus, the host's own immune system attacks the transplanted organ, causing a cascade of reactions (most notably blood clotting) that are dangerous to the already weakened patient. Xenotransplanters are animals engineered not to express those antigens that are recognized by the host immune system. The only animal currently chosen for xenotransplant research is the pig because its physiology closely matches that of humans, and pigs are much less expensive to study than monkeys or other primates. In the pig, a sugar called alpha-1,3 galactosyltransferase present on the surface of the cells needed to be knocked out. In 2002, at the University of Missouri, four pigs were produced whose transferase genes had been knocked out (Lai et al., 2002). A null gene was introduced into the pig embryos using the nuclear transfer technique. A second copy of the blank gene was introduced by nuclear transfer into the embryos from these adult pigs, resulting in piglets with both copies of the gene that encoded the antigen knocked out. Previously, the organs from transgenic pigs designed in this way were transplanted into baboons, and no rejection was seen. Human trials have not yet been approved for organs from transgenic xenotransplanters, from fear that the pig organs will allow the crossover of viruses from animals to humans (zoonotic

infection), especially to humans with weakened immune systems like patients waiting for a transplant. The most clear-cut example of zoonotic infection is influenza, which is often transmitted from pigs to humans even without organ transplantation. Human trials may begin soon, but debate is ongoing about the issue of transmission of infections.

Transgenic animals as food source

Another purpose of producing transgenic animals is to create food sources to meet increasing global food requirements. Following are examples of the transgenic animals that have been created to be used as food sources.

Superpig

Growing animals more efficiently and with less food would be very beneficial to society. "Superpig," one of the food strains created, was a pig engineered to grow bigger and faster, thus producing a more efficient food source. Many of the transgenic superpigs were created by microinjection of the transgene for a growth hormone, whether porcine, ovine, bovine, or even rat (Pursel et al., 1997). The popular Beltsville pig was created in Beltsville, Maryland, under the supervision of the US Department of Agriculture (USDA). Human or bovine growth hormone and higher levels of growth factors were expressed in these pigs. Unfortunately, the Beltsville pigs harbored many health problems, most commonly arthritis. Animal rights groups even claimed that the pig was impotent and had ulcers along with heart problems, lameness, kidney disease, and pneumonia. Therefore these pigs were euthanized, and biologists imposed a voluntary moratorium on performing further studies on mammals involving alteration in the expression of growth hormone.

Superfish

The creation of "Superfish" was another attempt at creating a more efficient food source. Tilapia, a species of engineered fish, was created at the Centro de Ingenieria Genética y Biotecnologia in Havana, Cuba, to overexpress its own growth hormone, which was microinjected. This animal was not transgenic as it was not created by using another animal's gene, but it was genetically engineered. Superfish showed increased growth, but it reached an adult size that was not larger than normal tilapia. Similarly, a transgenic salmon was engineered that produced growth hormone continuously, irrespective of the season (which otherwise produced seasonally). In this experiment, the eggs of a species of usually slow-growing trout were used for microinjection with the gene of a salmon that grew very fast after many generations of selective breeding. There was a concern that these fish might escape into the environment, and therefore, tight control was kept over transgenic fish farms. The biggest fear is that these fishes will breed with native wild-type fishes and outcompete them for food, although one of the companies involved in the farming of transgenic salmon claimed that the salmon were raised on fish pellets and therefore would not know how to forage for themselves in the wild natural environment. There is still considerable opposition to the generation and farming of these "superfish" though this transgenic fish look like a much more likely source of food than any other transgenic animal species.

Transgenic animals for drug and industrial production

Transgenic animals may better serve as important models in translating basic science breakthroughs to potential clinical applications. Moreover, the use of transgenic animals as bioreactors in the pharmaceutical industry has far-reaching implications, from protein production in various forms (e.g., milk, blood, urine, and other tissues) to the modification of tissues and organs for transplantation. Thus, the applications of transgenic technologies, although potentially of great significance, have yet to be fully recognized.

Transgenic animal research has been used mostly in the field of human medicine. Various therapeutic proteins or peptides for the treatment of human diseases require animal cell–specific modifications to be effective and are generally produced in mammalian cell–based bioreactors. To start a new cell culture–based manufacturing facility for a single therapeutic protein or peptide may cost more than US $600 million, and the resultant drug may not be affordable to most patients. It is difficult for the therapeutic protein manufacturing industry to keep pace with the rapid increase in drug discovery and development, resulting in unmet patient needs and dramatically rising medicine costs. Genetically engineered animals may provide an important source of these protein/peptide drugs in future because the production of recombinant proteins in the animal secretive products, for example, milk, blood, or eggs of transgenic animals presents much less-expensive approach than producing therapeutic proteins in animal cells. The first human therapeutic protein, antithrombin III (ATryn, GTC Biotherapeutics, Framingham, MA), was derived in 2006 from the milk of

genetically engineered goats and was approved by the European Commission for the treatment of patients with hereditary antithrombin deficiency. Serum biopharmaceutical products are also obtained from transgenic animals, such as antibodies that can be used for the treatment of infectious diseases, cancer, organ transplant rejection, and autoimmune diseases such as rheumatoid arthritis (Patel et al., 2007). Currently, the main production system for such blood products is donated human blood, a system that is limited by disease concerns (e.g., HIV/AIDS/hepatitis-B/hepatitis-C), lack of qualified donors, and regulatory issues. Genetically engineered animals, such as cattle carrying human antibody genes, provide a steady supply of polyclonal antibodies for the treatment of a various infections and other diseases. Much information about human diseases has also been obtained from transgenic mice models, which have become important in biological and biomedical research.

A transgenic sheep has been produced for the production of α1-proteinase inhibitor (α1-PI) protein, which when released in blood serum and binds to the elastase secreted by neutrophils in response to certain spores, bacteria, and other antigens. Elastase released in large amounts can damage elastin in the walls of lung alveoli, leading to severe emphysema. People who have dysfunctional gene controls for α1-antitrypsin production (emphysema or cystic fibrosis) can be supported in two ways: by gene therapy using a functional α1-antitrypsin gene or by administration of high doses of α1-PI as an aerosol. Gene therapy is still debated; therefore, the only way of producing α1-PI in large quantities is by creating a transgenic animal with α1-PI-producing gene. Pharmaceutical Protein Ltd. of Midlothian, Scotland, tried to produce this enzyme in sheep's milk. There are potential advantages of using sheep, as being mammals they can produce same kind of α1-PI as in human. Sheep are less expensive than cows, and they mature more quickly. The enzyme is formed only in the milk, which can be collected easily, and the sheep remain fit and healthy for a long time. Furthermore, large quantities of enzyme can be produced because flocks of these sheep can be easily bred, and the purified enzyme from milk thus will be inexpensive. Once the purified enzyme is produced, it must undergo clinical trials and receive approval from the regulatory authorities before it becomes available in the market.

Several biomedical research models are being produced from transgenic animals, including livestock species produced specifically for various human afflictions such as Alzheimer's disease and ophthalmic disease and for the possible xenotransplantation of cells, tissues, and organs. Transgenic animals are also helpful in studying animal diseases such as "mad cow" disease (bovine spongiform encephalopathy) (Richt et al., 2007) and infection of the udder (mastitis). Although scientists have now developed transgenic livestock for agricultural purposes, including some with enhanced production traits and disease resistance traits (Richt et al., 2007), no company other than Aqua Bounty (Boston, MA), which produced the growth-enhanced salmon, has announced its intent to pursue the commercialization of these agricultural applications. Economic profits are higher with the production of genetically engineered/transgenic animals for human medicine than for agricultural applications. Commercialization of agricultural applications of transgenic animals is hindered by concerns about the cost and timelines associated with the regulatory process and by consumer acceptance issues. Also, potential investors are reluctant because public acceptance of agricultural applications of genetic engineering has generally been lower than that of medical applications (e.g., recombinant insulin).

Transgenic animals' impact on the environment

The possible environmental impacts of transgenic animals will initially depend on their phenotype rather than on the fact that they are transgenic per se. Thus, if the animal in question has a phenotype giving it absolutely no chance of even short-term survival in the wild, then the fact that it is transgenic is of little to no concern as an environmental issue. If, conversely, the animal's phenotype does not limit or perhaps increases its chances for survival and dispersal in the wild, and if this animal is also capable of breeding in the wild, then this raises serious environmental concerns. The only way to ensure that transgenic animals can have no environmental impact is to make their escape or intentional release into the wild impossible, something that can be never assured. Although the long-term environmental impact of transgenic animals is hard to predict with certainty, it is generally accepted that if such impacts arise, they will be difficult or impossible to reverse.

Currently, the production and the evaluation of transgenic salmon emphasize accelerating growth rates through engineered changes in growth hormone (Zhu et al., 2005) and its expression. The rapid growth of these fishes clearly suggests a competitive advantage over wild fishes, indicating a possibility of transgenic individuals escape. Given that absolute body size is often a deciding factor in the outcome of competition for access to resources and/or mates, any animal that more rapidly attains a certain size is likely to displace or prey upon smaller, slow-growing individuals of the same species. Such animals may also exhibit interspecific competitive/predatory advantages due to changes

in spatial and temporal distributions as a result of their faster growth. Similar outcomes are possible from fish engineered for traits such as freeze resistance, salinity tolerance, disease resistance, or other economically valuable characteristics. Based on what is currently known about the phenotype of growth hormone-transgenic salmon, it is impossible to adequately predict the environmental outcomes should these fishes escape or be released into the wild. Beyond these near-term ecological effects, the greatest concerns with transgenic fish relate to the genetic effects of interbreeding with wild populations. Even if they are not well adapted for survival in the wild, transgenic animals may have detrimental impacts on the genetic structure of wild populations by allowing the introgression of "exotic" genes into natural gene pools. Changes in the genetic make-up of a well-adapted wild population may ultimately affect the animals' abilities to withstand environmental change. Of particular concern in this regard is the so-called Trojan gene effect, whereby transgenic animals that are poorly adapted for survival in the wild but exhibit mating advantages, for example, through faster growth and/or larger size, drive populations to extinction by successfully breeding with wild individuals and thereby reduce the fitness of their progeny (Muir and Howard, 2002). Nontransgenic farmed salmon exhibit characteristics that predispose them to such Trojan gene effects, such as reduced survival of progeny from mating between farmed and wild salmon.

An additional environmental concern with transgenic fish is their capacity for transferring diseases and/or parasites to wild population. This is a general concern with aquaculture, not so much because farmed fish is an initial source of pathogens or parasites, but because their confinement allows the amplification of pathogen and parasite loads for subsequent transmission back to wild population. Transgenic fish that are engineered for disease resistance would potentially increase the risk of becoming vectors for transferring pathogens or parasites to wild population either through direct contact or through the release of their feces or contaminated rearing water.

The use of transgenic salmon also offers several possible environmental benefits to aquaculture. For instance, the improved food conversion efficiency of growth hormone-transgenic salmon should reduce the amount of fishmeal required per unit body mass produced. Although not yet realized, the engineering of transgenic fish that can use plant meal as a protein source would have tremendous environmental benefit in this regard. Engineering disease- and parasite-resistant fish could potentially reduce the use of antibiotics and pesticides in aquaculture. Finally, if the use of transgenic fish makes on-shore, fully contained fish farming an economically viable enterprise, then this will allow the concentration and better management of fish waste products. These include organic wastes in the form of uneaten food and feces, as well as inorganic wastes such as phosphates and nitrates. The accumulation of these wastes from aquaculture can cause increased biochemical oxygen demand, eutrophication, and sedimentation problems, all of which can be eliminated in on-shore systems with recirculated water supplies.

Patenting transgenic animals

In general, consumers have expressed the support for the principle of patenting, including the patenting of genes and gene sequences. When the Canadian Court of Appeals agreed (decision overturned by the Supreme Court of Canada) with the patentability of the Harvard oncomouse, only about 50% of Canadians said they were not comfortable with the Appeals Court decision. When asked, "Is it okay for someone to have a patent on a new plant modified through the use of transgenesis?" 66% were in agreed. However, only 30% agreed that "granting a patent on an animal modified through the use of transgenesis is no different than granting a patent on a consumer product." Furthermore, 66% agreed that "we should not grant patents on a new species of guinea pig that includes human genes" and the same majority were in agreement that patents should not be granted on "a new species of chimpanzee that includes human genes."

Harvard's oncomouse raised general ethical issues regarding transgenic technology. It also raised two key issues for the patent system: (1) Should patents be granted at all for animals or animal varieties, particularly for higher-order animals such as mammals, even if they do otherwise meet patentability criteria (e.g., novelty, industrial applicability/usefulness, inventive step)? (2) How should moral implications be addressed in relation to specific cases, for example, the question of suffering caused to the transgenic animal? These issues have been resolved differently by the patent authorities of different countries as the following examples illustrate.

The United States Patent Office in 1988 granted patent #4,736,866 to Harvard College claiming the creation of "a transgenic nonhuman mammal whose germ cells and somatic cells contain a recombinant activated oncogene sequence introduced into said mammal..." The claim explicitly excluded humans, apparently reflecting moral and legal issues regarding patents on human beings or modification of the human genome.

The European Patent Office (EPO) considered the oncomouse case at length and at several levels. The EPO applies the patent standards of the European

Patent Convention, which contains two key relevant provisions: Article 53(a) excludes patents for inventions "the publication or exploitation of which would be contrary to *ordre public* or morality," and article 53(b) excludes patents on "animal varieties or essentially biological processes for the production of . . .animals." The EPO applied the utilitarian test and decided that the prohibition on patenting animal varieties did not constitute a ban on patenting animals as such. It concluded further that the oncomouse was not an animal variety and so did not fall within that exclusion.

To address the *ordre public* or morality exception, the EPO developed a utilitarian balancing test. This aimed to assess the potential benefits of a claimed invention against negative aspects, in this case weighing the suffering of the oncomouse against the expected medical benefits to humanity. Other considerations could also be taken into account in the balancing test, such as environmental risks (neutral in this case) or public unease (there was no evidence in European culture for moral disapproval of the use of mice in cancer research, thus no moral disapproval of the proposed exploitation of the invention in this case). The EPO concluded that the usefulness of the oncomouse in furthering cancer research satisfied the likelihood of substantial medical benefit and outweighed moral concerns about suffering caused to the animal. In the original application, the claims referred to animals in general, but in the course of the proceedings, the patent was amended and finally maintained with claims limited to mice.

Ethics in transgenesis

The production of transgenic animals remains a hot topic of debate, as some people are ethically uncomfortable with the idea of genetically engineering animals. Two central ethical concerns are associated with the creation of transgenic animals. The first relates to breaching species barriers or "playing God." According to this view, life should not be regarded solely as if it were a chemical product subject to willful genetic changes and patentable for economic benefit or commercialization. The second major ethical issue is the belief that the transgenesis of animals interferes with the integrity or telos of the animal. "Telos" can be defined as "the set of needs and interests which are genetically based, and environmentally expressed, and which collectively constitute or define the form of life or way of living exhibited by that animal, and whose fulfillment or thwarting matter to that animal." Such concerns are not unique to genetic engineering, and traditional breeding and selection practices can also change animals in similar ways. Cows from the Belgian Blue cattle breed, for example, require cesarean delivery of their calves because they have been selected for increased birth weight resulting from the naturally occurring "double-muscle" trait and the narrowness of the cow's pelvic passageway.

The success rate in creating transgenic animals is still low. There are more failures than successes, and the general assumption is that the higher the species of transgenic animal, the greater the cloning failure. The failures in transgenesis are animals that die before they are born or animals that are born without the transgene and are of no use for fulfilling the desired purpose. However, the number of failures is decreasing as cloning techniques improve. Comparing the prenatal deaths of a few animals against saving the lives of potentially thousands of humans suggests that the reward is very high, and improvements in the process are continuously decreasing the costs.

A very good example of a transgenic animal that does not suffer is Alzheimer's mouse. Alzheimer's mouse does poorly on maze tests, but does not feel any pain related to its condition by any standards used in laboratories. Because Alzheimer's mouse spends its days in a laboratory setting, any survival skills that would be hampered in the wild by its diminished memory do not come into play. Also, Alzheimer's mouse continues to provide significant information on Alzheimer's disease that could lead to a cure. The Alzheimer's mouse that was created in part at Worcester Polytechnic Institute (Worcester, MA) was used to develop a vaccine that lowers senile plaque burden in mice. The benefits are tremendous, and the animal suffering is almost nonexistent; thus, creation of Alzheimer's mouse was a brilliant idea.

The oncomouse is more complicated ethically. In 2018, an estimated 9.6 million people died of cancer worldwide (WHO, 2018). Any transgenic or genetically engineered animal/mouse that can help increase knowledge about this deadly disease would have enormous medical benefits. However, the problem with the oncomouse is that as it grows, it begins to suffer from the tumors just as humans do with this disease. Therefore, in this complex case, although the animal has enormous medical benefits, its use is associated with strong ethical contraindications. However, because most of the work on oncomouse focuses on early development of the tumors, the mouse can be euthanized before its suffering increases unbearably. If advanced oncogenesis needs to be studied, university/institutional animal care committees could require that painkillers be used. Therefore, it is difficult to say that the creation of the oncomouse was completely a good idea, because the mice do suffer like humans and die

in pain. The need to prevent cancer and palliate its symptoms is overwhelming, however, and to save millions of human lives, most scientists believe that oncomouse is the best hope.

FDA guidelines on genetically engineered animals

The US FDA is the lead agency responsible for the control and regulation of genetically engineered animals to be used for food, and it plans to regulate transgenic animals under the "new animal drug" provisions of the Food, Drug, and Cosmetic Act. The new animal drug provisions ask (1) if the new drug is safe for the animal? (2) If the new drug is effective? (3) If the drug for an animal that is used for food, is the resulting food safe to eat? Although premarket regulatory review of genetically engineered animals is mandatory, the FDA has not yet issued general guidelines explaining what information will be required for this regulatory review. Also, the regulatory path to commercialization of genetically engineered animals remains ill defined (Pew Initiative on Food and Biotechnology, 2005). However, transgenic animal research is subject to existing FDA regulations governing animal research. All organizations or institutions receiving or applying for federal funding to carry out research using animals are required by the Federal Animal Welfare Act of 1966 to have the IACUC review research protocols involving dogs, cats, rabbits, guinea pigs, hamsters, gerbils, nonhuman primates, marine mammals, captive wildlife, and domestic livestock species used in nonagricultural research and learning. The Animal Welfare Act also requires the following: (1) research institutions must have a veterinary care program in place, (2) all personnel using or caring for live animals must be well qualified to do so, and (3) a mechanism must be in place at the organization for reporting of issues regarding animal care and unethical use. The Animal Welfare Act is administered through the USDA and is enforced through unannounced and random inspections by a veterinary medical officer designated by the USDA. On an international level, the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) oversees the voluntary accreditation and assessment of research organizations committed to responsible animal care and use.

Translational significance

Transgenic animals are an important part of today's consumer health world, and we may expect even more dependency in near future as most of the recombinant proteins or monoclonal antibodies are derived from these animals. Thus, it becomes relevant to know how transgenic animals are produced and can be used for fulfilling our needs. For example, it is not possible to use human subjects to study the role of a particular gene that can be easily studied if inserted into a fertilized egg or embryo, which will then eventually grow into a transgenic human. Similarly, a particular disease condition can be generated in transgenic animals to study the effect of a specific drug that cannot be directly applied to human beings. No doubt, animals do feel pain and sufferings during laboratory experiments, but this suffering is still less if we compare relieve of pain or cure from a disease for millions of people worldwide. At the same time, this is very important to consider the relevance of the end result, and any kind of fun science must not be practiced on transgenic animals. Thus, one should not blindly follow the misconceptions of allowing or denying the use of transgenic animals and instead should be aware of the actual benefits and ethical issues associated with these animals.

Clinical correlations

Creation of transgenic animals either for disease or research model has tremendously increased the knowledge toward improvement of diagnostic methodology or treatment regimes. Detection of cancer at early stage is still a challenging task, but animal models has helped the development of noninvasive or blood-based tests for detecting circulating tumor biomarkers in the form of either proteins or miRNA or circulating cell-free DNA. Also, critical interactions of new therapeutic drugs have been furthered on the basis of preclinical trials on disease models (Chang et al., 2018).

Turning point

Transgenic animal production remains an ethical debatable issue, as there are both advantages such as use in studies for research and disease mechanism exploration, at the same time suffering to living beings and fear of escape of transgenic animal are considerable issues. A transgenic model such as Alzheimer's mouse if created and used under strict ethical regulations can definitely help improve disease management in humans without putting the animal for any unbearable sufferings. Therefore, a controlled and thoughtful research work is needed before creating a transgenic animal for any purpose.

World Wide Web resources

Following web resources are being visited for the content of the chapter along with scientific publications. Reader if interested can search these websites to get more information, although searching may take time and some time distract the reader. Therefore, web resources can be visited after reading the full chapter.

http://www.nih.gov
http://www.pubmed.com
http://www.princeton.edu
http://www.nature.com
http://www.sciencemag.com
http://www.sciencedaily.com
http://www.pewtrusts.org
http://www.harvard.edu
http://www.wustl.edu
http://www.transgenelifesciences.com
http://www.med.umich.edu
http://www.hhmi.org
http://www.rnainterference.org
http://www.mit.edu
http://www.fda.gov
http://www.stanford.edu
http://www.medicalnewstoday.com
http://www.who.int
http://www.uspto.gov
http://www.wisc.edu
http://www.dnalc.org

Box

Nobel Prize in the field

Drs Mario R. Capecchi, Martin J. Evans, and Oliver Smithies were awarded the 2007 Nobel Prize in physiology or medicine. Martin J. Evan's principles for introducing specific gene modifications in mice by the use of modified ESCs in foster mother and Capecchi's and Smithies' independent research on homologous recombination for modification of genes that led to generation of knockout mouse together earned them this prize.

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Glossary

Embryonic stem cells Embryonic stem cells are the pluripotent cells derived from inner mass of early stage embryo, for example, cells from 4 to 5 days postfertilization embryo in case of human.

Patent Patent is a form of intellectual property right granted by a government or a sovereign state to an inventor or their assignee for a limited period of time in exchange for the public disclosure of an invention, for example, patent granted on Harvard oncomouse.

RNA interference RNA interference (RNAi) is a natural process that cells use to turn down, or silence, the activity of specific genes. It was first discovered in Petunia in 1998. RNAi destroys the messenger RNA that carries information coded in genes to the cell's protein producing factories.

Transgene A transgene is an artificial gene, manipulated in the molecular biology lab that incorporate all appropriate elements critical for gene expression generally derived from a different species, for example, production of α1-proteinase inhibitor protein in transgenic sheep carrying transgene of human origin.

Xenotransplantation Xenotransplantation is a procedure that involves the transplantation, implantation, or infusion into a human subject (recipient) of either (a) live cells, tissues, or organs from a nonhuman animal source, or (b) human body fluids, cells, tissues, or organs that have had been produced from live nonhuman animal cells, tissues, or organs.

Long answer questions

1. How are transgenic animals useful for medical research? Describe with examples.
2. What are the different methods of creating transgenic animals?
3. How can transgenic animals affect the natural environment? Explain with suitable examples.
4. How do you justify the use of transgenic animals over cell-based bioreactors? Do you think transgenic animals can meet world food demands?
5. Explain CRISPR/Cas9 method and discuss the guidelines on the creation of transgenic animals.

Short answer questions

1. What was the first animal for which a patent was issued?
2. What is the "Trojan gene" effect?
3. What do you understand by RNAi?
4. What is the difference between cloning and transgenesis?
5. Name the agency responsible for regulation of animal experimentation in various organizations.

Answers to short answer questions

1. Oncomouse was the first animal to be patented in 1988.
2. Transgenically produced animal, for example, transgenic salmon overexpressing growth hormone gene if escaped in the environment may wipe out their wild-type counterparts by growing faster competitively.
3. RNA interference is a technique used to inhibit the expression of protein by degrading its mRNA. This technique was first discovered in plants.
4. Cloning is the reproduction of an exact copy of a living organism using the DNA (without manipulation) of that organism, whereas transgenesis (genetic engineering) refers to the human manipulation of genetic material in a manner that does not occur in nature.
5. Institutional Animal Care and Use Committees (IACUC) is the agency responsible for regulation of animal experimentation in most of the institutions and organizations.

Yes/no type of questions

1. Was creation of oncomouse a good idea?
2. Is generation of improved animal varieties possible by transgenesis?
3. Did Alzheimer's mouse suffered pain while disease becoming symptoms?
4. Do various therapeutical recombinant proteins are produced by transgenic animals?
5. Has CRISPR/Cas9 technology fastened the process of targeted gene modification?
6. Do all experiments related to transgenesis using embryonic stem cell transfer are highly successful?
7. Is there any problem if transgenic animal breeds with wild-type animals?
8. Can transgenic animals be produced in any lab without any restrictions?
9. Do USFDA play any role in transgenic animal production and usage?
10. Was the oncomouse the first transgenic animal to be patented?

Answers to yes/no type questions

1. Yes—It helped in understanding various cancer-related pathways.
2. Yes—Desired gene of interested can be selected, amplified, or cloned and inserted into prospective animal.
3. No—Alzheimer's mouse does not feel suffering as it is grown in an artificially controlled condition.
4. Yes—Various recombinant proteins such as tPA, insulin, etc. has been produced in animals.
5. Yes—CRISPR/Cas9 technology provide precise deletion or insertion of the gene at targeted region.
6. No—Not all experiments related to embryonic stem cell transfer are highly successful although the rate is higher compared to other methods.
7. No—There is a big concern for environmental escape of transgenic animals as they can grow fast and can replace their competitive wild-type species and can disturb the gene pool.
8. No—Transgenic animals are produced in high stringent conditions, working under controlled regulatory acts.
9. No—USFDA has a big role in preparing guidelines for production and usage of transgenic animals.
10. Yes—Oncomouse was the first transgenic animals to be patented.

CHAPTER

24

Role of cytogenetics and molecular genetics in human health and medicine

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Summary

In this chapter, the authors have tried to explore the basics of Genetics starting from chromosomes to genes. This chapter also elucidates the different techniques in Cytogenetics and Molecular genetics and their application in the diagnosis of genetic disorders. Over the last 25 years, the conventional cytogenetics has matured largely due to the advances in molecular biology and major technological innovations. So, the present form of molecular cytogenetics is more of a multidisciplinary science that has bridged the gap between conventional cytogenetics and molecular genetics with the addition of another discipline, that is, bioinformatics. One of the significant contributions in the field of clinical genetics is the prenatal diagnosis, which is being discussed in this new edition.

What you can expect to know

In the "Cytogenetics: an overview" section, a description of chromosomes, their structure, and their role in inheritance, along with their abnormalities, both numerical and structural leading to common genetic disorders like Down syndrome and Turner syndrome have been provided. The "Molecular genetics: an overview" section describes the structure of deoxyribonucleic nucleic acid (DNA) and gene and how changes in the DNA sequences lead to the manifestation of genetic disorders. Both the sections outline the basics and give an overview of how this basic information is applied in the clinical perspective in the field of medical genetics. It also gives an overview of the laboratory procedures or different tools applied for the detection and diagnosis of different genetic disorders. The main categories of genetic disorders—single-gene, chromosomal, multigenic, mitochondrial and epigenetic disorders—have been briefly explained to expose students to different patterns of inheritance and their genetic mechanism, with examples of common diseases. Detection of genetic disorders before birth in the unborn child using different methods has also been discussed.

Introduction

To understand the role of genetics in medicine, one must first know how it plays a significant role in cell division, growth, and cell differentiation, as the developmental program is implemented gradually from a zygotic stage to an adult human being. This entire process of development involves a vast array of cellular, biochemical, and molecular interactions.

The basic and fundamental laws of genetics—which revolutionized our understanding of genetics—were illustrated by a set of simple experiments in 1865 by an Austrian monk named Gregor Mendel. His breeding experiments on garden peas helped us to understand the inheritance pattern, and the way different characteristics are passed on from one generation to the next. However, his work was not recognized until 16 years after his death. For his major contributions, he is known as the "Father of Genetics."

With the development of knowledge of classical and molecular genetics, the need to explore the medical

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aspects of human genetics was felt. In 1949, Neel recognized that sickle cell anemia is a hereditary disease. In the same year, Singer and Wells identified that sickle cell anemia is caused by an alteration in the normal hemoglobin (Pauling et al., 1949). The American Society of Human Genetics and the first Journal of Human Genetics (i.e., American Journal of Human Genetics) were established in the same year, and even the first textbook of human genetics appeared in 1949 (Curt Stern, Principles of Human Genetics). In 1959, 47 chromosomes were observed for the first time in a patient with Down Syndrome. Following this, molecular techniques were developed to identify genes and disease-causing mutations. Great leaps in this field have not only changed the diagnosis and management of single-gene disorders but also provided new molecular approaches to the genetic diseases. The field of medical genetics began to develop at the end of 19th century, and the entire human genome was sequenced in 2000. Knowledge of the genome and genetics has increased tremendously in the last few decades. This revolution in knowledge helped us to understand the importance of genetics in almost every area of medicine. Genetics is now not restricted to single-gene disorders but has made remarkable contributions to other common multifactorial diseases such as hypertension, psychiatric illness, and cardiovascular diseases. Thus, it is essential to introduce to students the basic concepts of cytogenetic and molecular genetics to understand the latest developments in medicine.

Most diseases have a probable genetic and environmental basis. The genetic component may be the major one, but it is notable that in up to 50% of all cases, no clear explanation can be established. The integration of cytogenetics and molecular technologies has thus opened up an exciting field in modern biology.

One of the most significant advances in medical genetics is a prenatal diagnosis that aims to attain genetic information about embryo or fetus. Although effective treatment is available for some genetic disorders but for most there are none. So, management and prevention are an integral part of clinical genetics.

Cytogenetics: an overview

Cytogenetics, the study of chromosomes, was originated more than a century ago. However, it is in the last few decades that studies on human chromosomes have become a major field in the biomedical sciences. Flemming published the first drawn illustration of a chromosome in 1882, and Waldeyer used the term chromosome (Greek word for "stained body") for the first time in 1888. It took many years to identify the exact number of chromosomes in a normal diploid human cell (Gartler, 2006). In 1912, Hans von Winiwarter reported 47 chromosomes in spermatogonia and 48 in oogonia, and this count remained as 48 chromosomes until Joe Hin Tjio and Albert Levan discovered the correct human chromosome count. This revolutionary finding of Tjio's was published (with Levan as his coauthor) in the Scandinavian journal *Hereditas* on January 26, 1956 (Tjio and Levan, 1956). Chromosome banding methods, for example, are today's vital tools in clinical genetics. In 1956 researchers in Sweden used acetoorcein dye for direct chromosome staining and could count 46 chromosomes in human cells, but could only distinguish chromosomes according to their sizes and centromere positions (Trask, 2002). Based on these two criteria, human chromosomes were classified into seven groups: A through G. In the late 1960s, when quinacrine mustard was used to stain human chromosomes, accurate chromosome identification could be done under a fluorescence microscope into bright and dark regions called Q-bands. Since then, numerous banding techniques have been developed, of which G-banding (Giemsa banding) is the most widely used technique for chromosome analysis. Banding techniques are extremely useful for the detection of structural changes associated with chromosomal disorders. The microscopic analysis of chromosome structure and behavior in mitosis and meiosis revealed changes in the chromosome sets of plants, animals, and man. These changes were referred as chromosomal aberrations, and research was carried out on corn by the American cytogeneticist B. McClintock in the period 1929–1938.

Chromosome morphology and classification

There are two kinds of cell division: mitosis and meiosis. Mitosis is a somatic cell division responsible for the growth, proliferation, and tissue differentiation of the body, whereas meiosis is responsible for the production of gametes. Because mitotic cells are easy to obtain, morphological studies are generally based on mitotic metaphase chromosomes. Chromosomes are not visible under a light microscope in non-dividing (interphase) cells. As the cell begins to divide, the thread-like chromatin material in the nucleus begins to condense; in the metaphase stage, the chromosomes are best recognizable.

A chromosome consists of two arms separated by a primary constriction called a *centromere*. The short chromosome arm is designated as p (petite) and the long arm as q (one letter after p) (according to Paris nomenclature). A centromere consists of several hundred kilobases of repetitive DNA and is responsible for the separation of chromosomes during cell division.

Each chromosome consists of two identical strands known as *chromatids* or *sister chromatids*, which are visible after the S (synthetic) phase of the cell cycle. Each of the two sister-chromatids contains a highly coiled double helix of DNA and is joined at the centromere.

The most distal end of each chromosome is called telomere that plays a vital role in facilitating complete chromosome replication. Telomeres are highly conserved and consist of repeated DNA sequence TTAGGG. Telomeres maintain the structural integrity of chromosomes and help to distinguish the chromosome ends from broken DNA by sealing the ends of chromosomes, just like caps. Telomeres get shorter with each cell division and when they get too short, the cell no longer can divide and dies. This process has been associated with aging and cancers.

Chromosomes are arranged and are numbered according to their size and the position of their centromeres. A chromosome with the centromere at or near the middle is known as *metacentric*. A *submetacentric* chromosome has a centromere somewhat displaced from the middle point. *Acrocentric* chromosomes have centromeres very near to one end. *Telocentric* chromosomes, which are absent in human cells, have their centromeres at the very tip of one end (Fig. 24.1).

The number of chromosomes in the somatic cell is diploid and is designated by the symbol 2 N. The gametes have the haploid number N. In humans, the diploid number is 46, inheriting 23 from each parent through the sperm or egg. Homologous chromosomes form a pair with one constituent from each parent. Thus, there are 23 pairs of chromosomes in human cells. Of these, 22 pairs are known as *autosomes*, and the remaining chromosome pair consists of the *sex chromosomes* and is directly involved in sex determination. In females, the two sex chromosomes are identical (XX), whereas in males, the two sex chromosomes are not identical (XY). The Y chromosome is smaller than the X chromosome (Verma and Babu, 2002a).

The term *karyotype* refers to a display of the chromosomes of a cell by lining them up, beginning with the largest chromosome and with the short-arm oriented toward the top (Fig. 24.2). In humans, seven (A–G) groups of autosomes are recognized. Sex chromosomes (X,Y) are placed at the end. A diagram of the karyotype based on chromosome measurements in many cells is called an *ideogram*. Chromosome numbers 1–3 (A group) are metacentric, numbers 4–5 (B group) and 6–12 (C group) are submetacentric, 13–15 (D group) and 21–22 (G group) are acrocentric and have satellites, and chromosomes 16–18 (E group) are again metacentric (Table 24.1).

Nomenclature

Human chromosome nomenclature systems were developed to prevent confusion in reporting cytogenetic results. In 1995, the International System for Human Cytogenetic Nomenclature was recommended by the International Standing Committee on Human Cytogenetics Nomenclature.

A karyotype description is written from left to right without leaving any space, separating each item with a comma unless otherwise specified. The karyotype formula begins with the total number of chromosomes in the cell followed by the notation of the sex chromosomes (first the Xs and then the Ys). A normal male is designated as"46, XY" and a normal female as "46,XX". An extra or a missing chromosome is designated with a " + " or " − " sign, respectively, before the number of chromosomes. Thus, a male with trisomy (extra chromosome) for chromosome 21 is 47,XY, + 21, and a female with a monosomy for 22 is 45, XX, − 22. The addition or deletion of a chromosome segment is denoted with a plus " + " or minus " − " sign after the symbol of the chromosome arm, respectively.

For example, a female having a deletion of the short arm of chromosome 5 (cri du chat syndrome) is written as 46,XX,5p − . The formula for a male with a translocation between chromosome 14 and 21 is 46,XY, t(14;21) (Southern and Mellor, 1975; Watson and Crick, 1953).

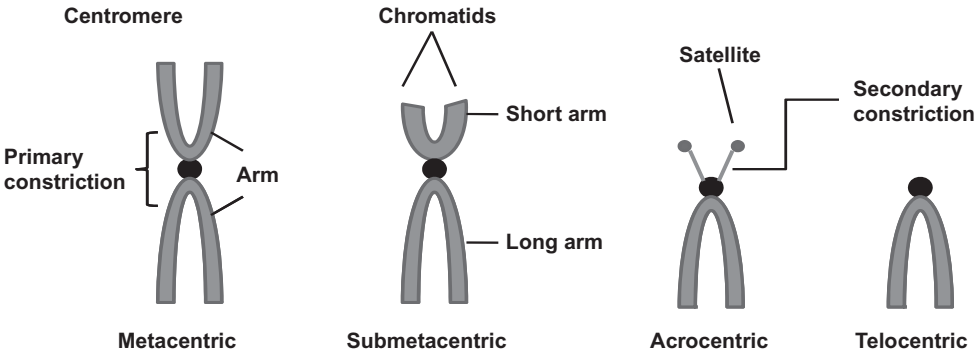


FIGURE 24.1 Diagrammatic representation showing chromosome classification according to centromere position and size.

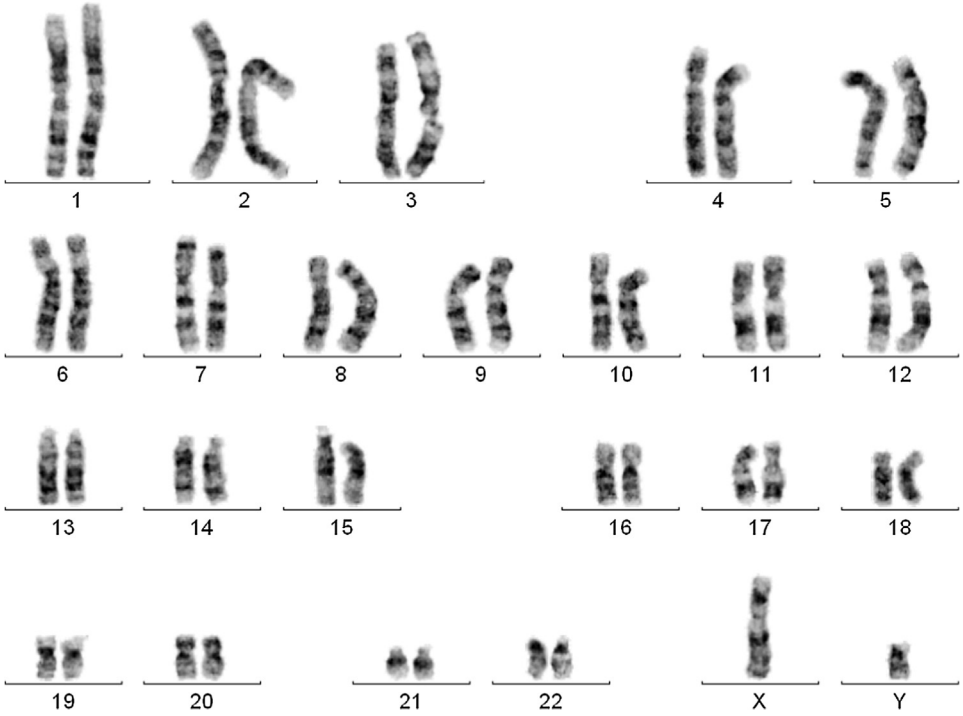


FIGURE 24.2 Karyotype showing a normal male chromosomal constitution.(Courtesy: Cytogenetic Lab Genetic Unit, All India Institute of Medical Sciences (AIIMS), New Delhi, India).

TABLE 24.1 Grouping of chromosomes based on descending order of size and position of the centromere.

Sr. No	Group	Chromosome	Description
1	Group A	1–3	Large metacentric chromosomes easily identified based on their size and centromere position.
2	Group B	4–5	Large submetacentric chromosomes.
3	Group C	6–12, X	Medium-sized submetacentric chromosomes.
4	Group D	13–15	Medium-sized acrocentric chromosomes with satellites.
5	Group E	16–18	Moderately short metacentric or submetacentric chromosomes.
6	Group F	19–20	Short metacentric chromosomes.
7	Group G	21–22, Y	Short acrocentric chromosomes with satellites, Y chromosome does not bear satellites.

While describing a human chromosome complement and its abnormalities /aberrations, sex chromosome aberrations are specified first (X chromosome abnormalities are presented before those involving Y), followed by abnormalities of the autosomes listed in numerical order, irrespective of aberration type. Table 24.2 lists some of the commonly used symbols for chromosome nomenclature.

Chromosomal disorders

Chromosomal disorders account for approximately 6% of all recognized congenital abnormalities, and these can be divided into numerical and structural aneuploidy, with a third category consisting of different chromosome constitutions in two or more cell lines. Aneuploidy is a term that is used when the chromosome number in the cells is not the typical number that it should be for a particular species. This gives rise to chromosome abnormalities such as an extra chromosome or loss of one or more chromosomes and leads to a particular chromosomal disorder.

The presence of an extra chromosome is referred to as trisomy. Of the 22 autosomes in man, only three occur regularly as trisomies in live-born infants: trisomy 21 (Down syndrome), trisomy 18 (Edward syndrome), and trisomy 13 (Patau syndrome). Other trisomies are not observed in live-born infants because they are lethal in

TABLE 24.2 List of some of the commonly used symbols for chromosome nomenclature with descriptions.

Sr. No.	Chromosome nomenclature	Symbols	Examples	Description
1	The sex chromosomes	X,Y	46,XX/46,XY	Normal female/normal male.
2	Deletion	del	46,XX, del (6)(p2)	Deletion of p arm of 6 at band level 2.
3	Duplication	dup	46,XY dup (6)(q21−q24)	Duplication in the long arm of chromosome 6 involving breakpoint from 6q21−6q24.
4	Inversion	inv	46,XY, inv(6)(q21−q24)	Inversion in the long arm of chromosome 6 involving breakpoint from 6q21−6q24.
5	Short arm of chromosome	p	p10	Short arm of chromosome 10.
6	Long arm of chromosome	q	q10	Long arm of chromosome 10.
7	Satellite	s	15 ps	Satellites on the short arm of chromosome 15.
8	Translocation	t	46,XY,t(14;21)	Translocation involving chromosome 14 and 21.
9	Addition of the whole chromosome	(+)	47,XY, + 21	Trisomy 21 (three copies of chromosome 21).
10	Deletion of the whole chromosome	(−)	45,XX, − 22	Loss of chromosome 22.

early embryonic life and not compatible with life at birth. The presence of an additional sex chromosome (X or Y) has only mild phenotypic effects. Trisomy 21 is usually caused by the failure of separation of one of the pairs of homologous chromosomes during anaphase of maternal meiosis I. This failure of the bivalent to separate is called "nondisjunction." Nondisjunction also occurs during meiosis II when a pair of sister chromatids fail to separate and thus leading to trisomy.

Down syndrome is the most common chromosomal disorder having an incidence of 1 in 800−1000 live births. It is also one of the most common causes of mental retardation. The absence of a single chromosome is referred to as monosomy. Monosomy for an autosome is almost always incompatible with survival to term. Monosomy X (karyotype 45,XO) is another chromosomal disorder, also known as Turner syndrome, representing about 5% of conceptions. It affects about 1 in every 2500 females.

Polyploid cells contain multiples of the haploid number of chromosomes such as 69 (triploidy) or 92 (tetraploidy). It has been observed that triploidy is often the cause of spontaneous miscarriages.

Structural abnormalities

Structural chromosome rearrangements result from chromosome breakage with subsequent re-union in a different configuration. They can be balanced or unbalanced. In balanced rearrangements, the chromosome complement is complete with no loss or gain of genetic material. Thus, such rearrangements are generally harmless with the exception of rare cases in which one of the breakpoints damages an important functional gene. However, carriers of balanced rearrangements are often at risk of having children with an unbalanced chromosomal complement. In an unbalanced rearrangement, there is either loss or gain of chromosomal material, and the clinical effects are usually very serious. A *translocation* refers to the transfer of genetic material from one chromosome to another (Fig. 24.3A). In a reciprocal translocation, two non-homologous chromosomes break and exchange fragments. Since they still have a balanced complement of chromosomes, they generally have a normal phenotype. A Robertsonian translocation occurs in acrocentric chromosomes, namely 13, 14, 15, 21, and 22. During a Robertsonian translocation, any two acrocentric chromosomes break at their centromeres and the long arms fuse to form a single chromosome with a single centromere. The short arms also fuse together and are usually lost within a few cell divisions due to the absence of centromeres. Since short-arm regions do not have any essential genes, a Robertsonian translocation carrier will have no health problems but will have 45 chromosomes.

A *deletion* involves the loss of part of a chromosome. A deletion can happen in every chromosome and be of any size (Fig. 24.3B). The consequences of a deletion depend on the size of the missing segment and the genes located on it.

Ring chromosomes usually occur when a chromosome breaks in two places and the ends of the chromosome arms fuse together to form a circular structure (Fig. 24.3C) and the deleted genetic material gets lost during cell division.

An *isochromosome* is an abnormal chromosome with two identical arms, either two short (p) arms or two long (q) arms. This is sometimes seen in some females with Turner syndrome or in tumor cells (Fig. 24.3D).

An *inversion* is a chromosome rearrangement where a single chromosome undergoes breakage and is then

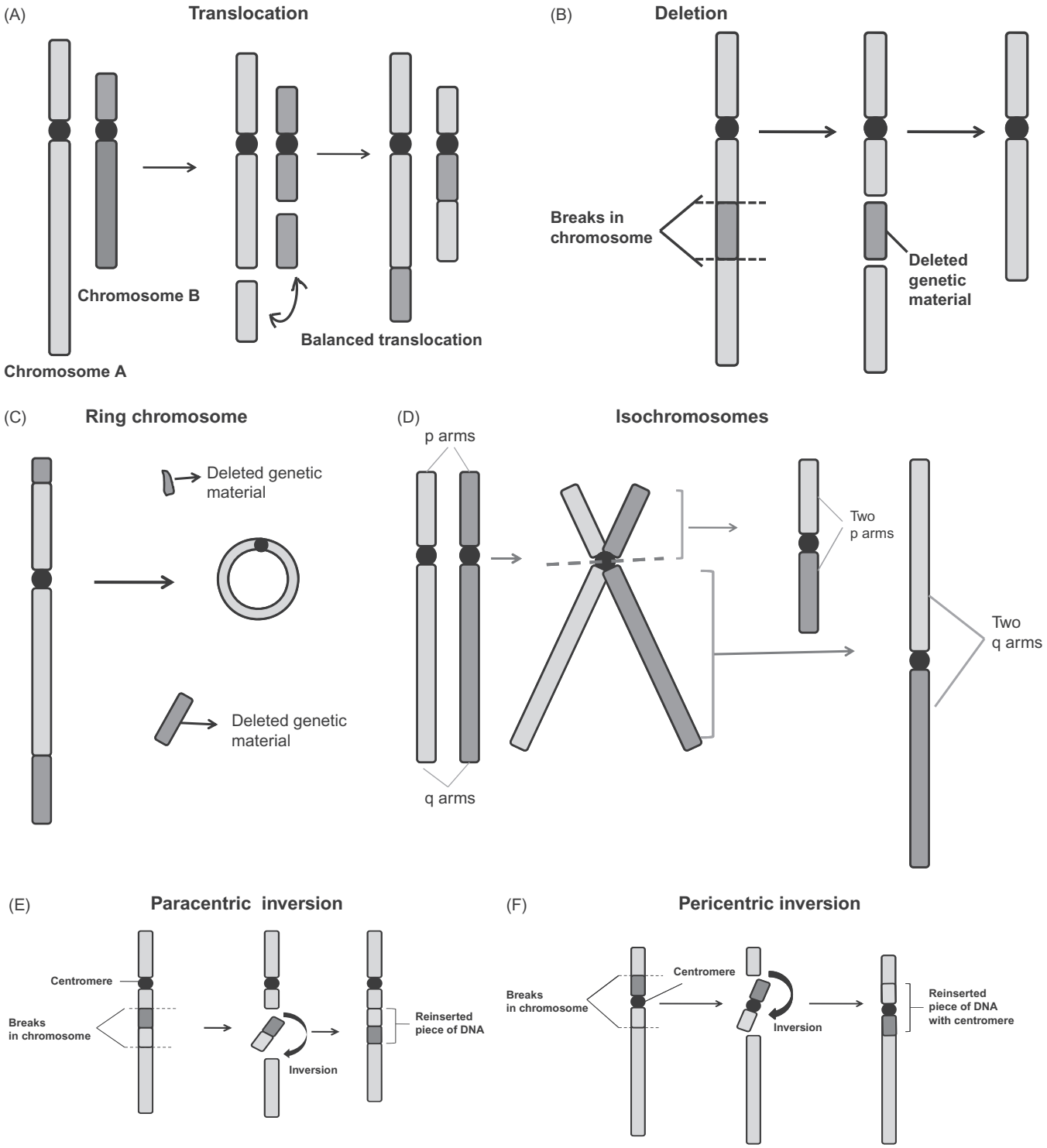


FIGURE 24.3 A: Diagrammatic representation of a balanced translocation. B: Diagrammatic representation showing deletion of part of a chromosome. C: Diagrammatic representation of a ring chromosome. D: Diagrammatic representation of an isochromosome. E & F: Diagrammatic representation showing paracentric and pericentric inversion.

reversed and rearranged within itself. Inversions are of two types: paracentric and pericentric. *Paracentric inversions* do not include the centromere and both breaks occur on the same arm of the chromosome (Fig. 24.3E). *Pericentric inversions* (Fig. 24.3F) include the centromere, and there is a breakpoint in each arm (p and q arm)

The terms "chimera" and "mosaic" are both used to describe people with two sets of DNA in their cells. *Chimerism* is caused by the fusing of more than one zygote and results in a person with more than one genetic identity. *Mosaicism* refers to DNA differences that arise from only one zygote. Sometimes genetic disorders affect some cells and not others. For example, patients with Down syndrome may have three copies of chromosome 21 in all cells. However, in some cases, only some cells have the extra chromosome 21, while other cells have two copies of chromosome 21; this condition is known as mosaic Down syndrome.

Mosaic disorders sometimes manifest with milder features than disorders affecting all cells, depending on the percentage of mosaicism, which varies in different patients. This is because the unaffected cells are still able to produce proteins normally and are therefore able to compensate.

Chromosome breakage and fragile sites

There is another group of genetic disorders known as chromosomal breakage syndromes. The characteristic features of these disorders are increased frequency of breaks and interchanges that occur either spontaneously or when exposed to various DNA-damaging agents. The cultured cells from affected individuals exhibit elevated rates of chromosomal breakage or instability, leading to chromosomal rearrangements. Defects in the repair system lead to permanent DNA damage and thus the affected individuals are more prone to cancers. A few examples of chromosomal breakage disorders are ataxia telangiectasia, Fanconi's anemia, and Bloom's syndrome.

Chromosomal fragile sites are specific genomic regions exhibiting gaps and breaks on metaphase chromosomes. Common fragile sites are presumably present in all individuals and in all populations consisting of adenine/thymidine (AT)-rich regions and are prone to chromosomal rearrangements. These common fragile sites are of interest in cancer genetics because they are frequently affected in cancer, and they can be found in healthy individuals. A well-known example is FRA3B, which is located at chromosome region 3p14.2. The FHIT gene, encompassing this FRA3B region, is a tumor suppressor gene and allelic losses at FRA3B have been observed in many types of cancer. FRA16D is another common chromosomal fragile site, located at 16q23.2. Homozygous deletion of the FRA16D locus has been observed in adenocarcinomas of stomach, colon, lung, and ovary. In about 5% of the population, rare fragile sites are found which are either inherited or apparently *de-novo* and are characterized by repeat expansion composed of two or three nucleotide repeats. Fragile X syndrome is one of the most common examples of rare fragile sites present in the X chromosome at Xq27.3 and causes mental impairment.

Methodology: application of different cytogenetic techniques in the diagnosis of genetic disorders

Cytogenetic and molecular techniques have wide applications in various kinds of cancers and in the screening of various congenital anomalies. Cytogenetic approaches to study chromosomes and their association with human disease have improved greatly over the past several decades. Traditionally, cytogenetics relied on cell culture, which is labor-intensive. Moreover, deletions and duplications smaller than 4 million bases (4 MB or 4×10^6 base pairs) cannot be detected by routine cytogenetics. Such limitations were overcome by the advancement in technology when fluorescence in situ hybridization (FISH) was introduced. From 1980 onwards, a combination of cytogenetic and molecular genetic techniques started becoming popular, and over the years considerable progress has been made from FISH to comparitive genome hybridization (CGH) and array-comparative genomic hybridization (aCGH) (Fan, 2002).

Identification of chromosomes and karyotyping

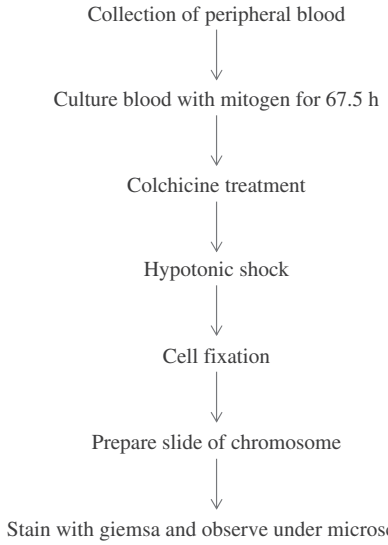
Cells in culture or in vitro are a useful model for studying the activity of cells in the whole organism or in vivo. Peripheral blood lymphocyte cultures are easy to obtain and generate abundant metaphases, and the simplicity of the cell culture technique makes this the most convenient approach to study human chromosomes for both clinical and research purposes. After initiation of culture in a growth medium containing supplements, antibiotics, fetal calf serum, and mitogen-like phytohemagglutinin, the addition of a spindle inhibitor like colchicine or colcemid after 72 hours arrest the cells in the metaphase stage. A hypotonic solution (0.075 M KCl) is added, causing cells to swell and ensuring that the chromosomes are adequately dispersed within the lymphocytes. The cells are fixed before spreading on a glass slide, using methanol and acetic acid in the ratio of 3:1 (Rooney and Czepulkowski, 1997). Giemsa banding is one of the most commonly used techniques to identify chromosomes, both normal and abnormal. Giemsa reagent is a DNA stain that consists of a mixture of dyes including the basic aminophenothiazine dyes (azure A, azure B, azure C, thionin, and methylene blue) and the acidic dye eosin. Prior to staining, an enzyme treatment with trypsin is needed to digest the proteins. This technique produces patterns of light-staining (G-light) regions and dark-staining (G-dark) regions. The pattern is unique to each chromosome and, therefore,

serves as a landmark for chromosome identification. The lightly stained regions are called *euchromatin* and contain actively expressed genes, and darkly stained regions are called *heterochromatin* and contain inactive unexpressed DNA (Swansbury, 2003). A flowchart describes the procedure of lymphocyte culture (Flowchart 24.1).

Simultaneous to these advances in cytogenetics, the field of molecular biology was also making significant progress. Cytogenetics took advantage of this additional knowledge and technologies and a new area called molecular cytogenetics emerged, which is mainly represented by the techniques of FISH and other, more advanced, methods such as spectral karyotyping, CGH and a-CGH, as discussed below.

Fluorescence in situ hybridization

FISH is based on the principle of hybridization (i.e., the ability of a single-stranded DNA to hybridize with its complementary sequence to form a double-stranded DNA). In FISH, the DNA probes which are short sequences of single-stranded DNA are tagged with a fluorescent dye and applied to cell preparations on a slide under conditions that allow for the probe to attach itself to the complementary sequence in the specimen if it is present. The site of hybridization is then visualized under a fluorescence microscope. It is a powerful technique used to detect chromosomal aneuploidies such as loss of a chromosomal region, a whole chromosome, or trisomies (e.g., Down Syndrome). This technique is also useful in the identification of genetic abberration, such as the fusion of BCR and ABL genes in breast cancer, and to monitor the progression of an aberration that can help in both the diagnosis and suggesting prognostic outcomes in cancer genetics. These techniques have further evolved, and using this same principle of hybridization, the whole genome can be screened simultaneously through comparative genomic hybridization (CGH), in which a test and a control are differentially labeled and competitively hybridized to metaphase chromosomes. But a limitation of the CGH technique is that it can only detect copy losses/gains which are at least 5–10 Mb in length. To overcome this limitation, another technique was developed, which combines the traditional CGH and microarray techniques, and can detect copy number changes at a level of 5–10 kilobases of DNA sequences known as array CGH or chromosomal microarray (CMA) (Speicher and Carter, 2006).



FLOWCHART 24.1 Flow chart showing human lymphocyte culture.

Array-comparative genomic hybridization

In the array-CGH (aCGH) technique, instead of using metaphase chromosomes, thousands of short sequences of DNA are used as targets for analysis (also known as probes), which are printed on a glass slide called a chip. DNA of the test sample and a control sample are then differentially labeled with fluorescent dyes; commonly used colors are red and green which are mixed together and hybridized on to the glass slide. The fragments of DNA hybridize with their complementary strand on the array and are then scanned in a machine called a microarray scanner, which measures the amount of red and green fluorescence on each probe. The ratios of the red to green fluorescent dyes are then calculated by the software to identify the copy number variation in the genome (Mei et al., 2000). Array CGH or CMA is becoming the technique of choice to screen chromosome abnormalities such as aneuploidy, chromosomal rearrangements, and microdeletion/duplication disorders and in cancer genetics.

Principle

The basic principle of all cytogenetic techniques is to minutely examine and scrutinize all 46 chromosomes to look for any kind of chromosomal change, either numerical or structural. Karyotyping is still considered to be the gold standard technique the world over. Later techniques started combining principles of molecular genetics with better image analysis and current bioinformatics tools. This was done in an effort to overcome some of the limitations of common methods used in the study of chromosomes.

Molecular genetics: an overview

Molecular genetics is the field of biology and genetics that studies the structure and function of genes at a

molecular level and how they are inherited from one generation to the next. Molecular information is used to determine the patterns of descent and to understand the genetic errors or mutations that can cause certain types of diseases (Epstein, 2003; Strachan and Read, 2004).

The history of molecular genetics dates back to 1869, when Friedrich Miescher extracted a viscous white substance from the nucleus of a cell, which was slightly acidic and rich in phosphorous and nitrogen. He named it as "nuclein" because it was found in the nucleus, but he did not know its true nature. Phoebus Levene, a Russian-American biochemist identified components inside this material (i.e., ribose in 1909 and deoxyribose in 1929). He also showed that these components were linked together in the order phosphate–sugar–base to form units and coined the term "nucleotide" for these units. He also discovered the ribose sugar in RNA, the deoxyribose sugar in DNA. Oswald Avery in 1944 discovered that genetic information is stored in DNA and is transmitted from one generation to the next. This was a revolutionary concept, as it was opposing the prevailing concept that proteins were the hereditary material.

In the 1950s, Erwin Chargaff studied DNA in many different organisms and found that the proportion of Adenine in a DNA molecule is equal to that of thymine, and the proportion of Guanine is equal to that of Cytosine. This is known as Chargaff's rule which was further used by Crick and Watson to unravel the structure of DNA (Turnpenny and Ellard, 2011).

Hereditary material

A cell is the basic fundamental unit of life. Each cell of the body has a darkly stained nucleus surrounded by cytoplasm. The nucleus contains the hereditary material in the form of chromosomes. These chromosomes are made up of tightly coiled very long DNA molecules that contain a series of genes. A gene in a simplest term can be defined as a segment of a DNA molecule formed by millions of nucleotides or nitrogenous bases joined together in a long chain held in position by hydrogen bonds between them and joined to each other by sugar and phosphate molecule. A gene determines the cell properties, both structure and functions, which are unique to each cell by the information contained in these sequences of DNA.

The structure of DNA

DNA is a highly complex macro- or rather a megabiomolecule that is essential for all known forms of life. The long-chain molecule is formed of repeating units of Adenine (A), Thymine (T), Guanine (G), and Cytosine (C) called nucleotides or bases. Thus, DNA is also known as the polynucleotide molecule. It consists of two polynucleotide antiparallel strands, which are spirally coiled around each other along their lengths. The model for the structure of the DNA molecule was proposed by Watson and Crick in 1953, who won the Nobel Prize for Medicine in 1962.

Chemical components of DNA: the highly complex DNA molecule is composed of only three types of chemical components. These are (1) deoxyribose sugar, (2) a phosphate, and (3) nitrogen-containing organic bases.

The DNA molecule is a double helix structure. The molecule is formed by two antiparallel polynucleotide strands that are spirally coiled round each other in a right-handed helix. The two strands are held together by hydrogen bonds. Each strand is a long polynucleotide of deoxyribonucleotides, and the backbone of the strand is formed by alternately arranged deoxyribose sugar and phosphate molecules which are joined by the phosphodiester linkages.

A chromosome consists of a single, very long DNA molecule that contains genetic information, and a complete set of chromosomal DNA constitutes the genome. Genomes vary widely in size: the smallest known genome (of a bacterium) contains about 600,000 DNA base pairs, while human and mouse genomes have some 3 billion base pairs.

A gene is divided into coding regions known as *exons*, and noncoding regions called *introns*. In eukaryotes, DNA is mostly noncoding and consists of repetitive sequences.

There are two classes of genes that can have an effect on how other genes function; they are called modifying genes and regulator genes. *Modifying genes* are those that alter the expression of another gene present at a different locus. Different modifier genes can produce a variety of clinical effects. For example in cystic fibrosis which is an autosomal recessive disorder caused by mutations in the CF transmembrane conductance regulator (CFTR) gene displays a wide variability in clinical features and survival. Studies have revealed that for some of the traits such as lung function, neonatal intestinal obstruction, and diabetes, genetic modifiers play a significant role.

Regulator genes can either initiate or block the expression of other genes. Some genes are incompletely penetrant (i.e., their effect does not normally occur unless certain environmental factors are present). For instance, one may inherit the genes that are responsible for type 2 diabetes but never get the disease unless one becomes greatly overweight or persistently stressed psychologically.

Mutation: A mutation is defined as any change in the nucleotide sequence of DNA. Mutations are broadly categorized into two types, fixed mutations and dynamic mutations. Fixed mutations are again subdivided into

two main groups, synonymous and nonsynonymous mutations (Haldane, 1935).

Synonymous mutation: A mutation is known as synonymous if it does not alter the amino acid in the polypeptide chain. It is also known as silent mutation (i.e. it does not affect the phenotype).

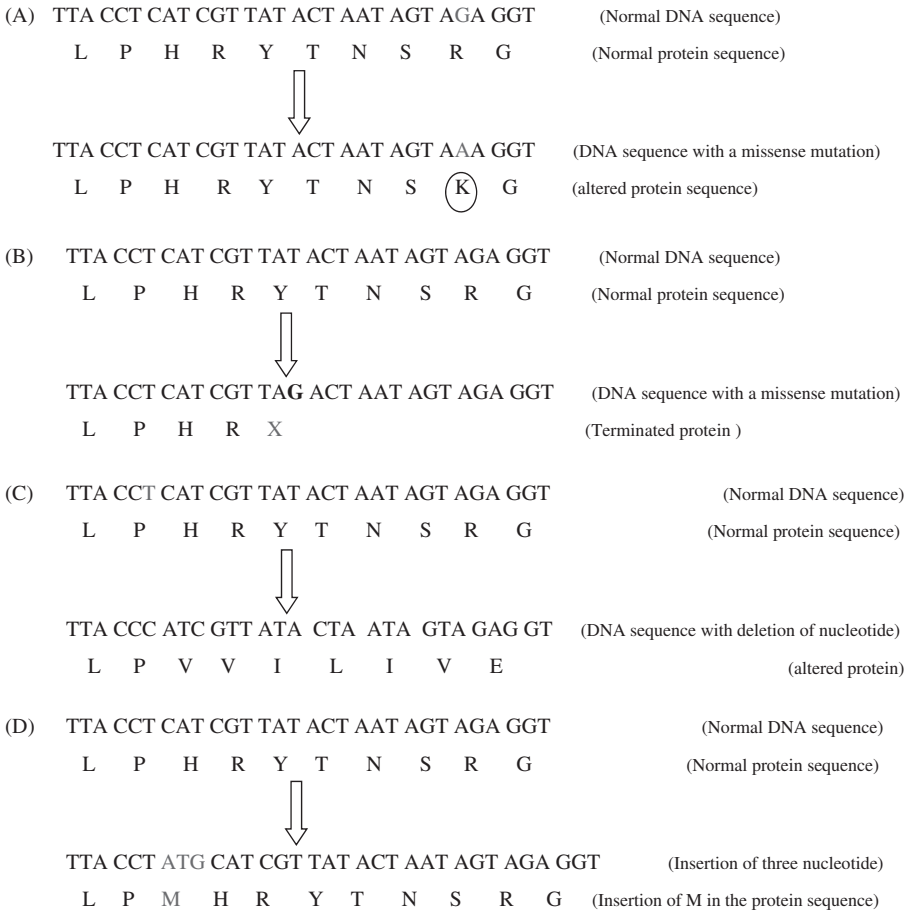
Nonsynonymous mutation: Any change in the nucleotide sequence that results in the alteration of amino acids in the polypeptide chain is termed as nonsynonymous mutation. It affects the phenotype, depending on the importance of amino acids in the functioning of the protein. When there is a replacement of purine to purine or pyrimidine to pyrimidine (A-to-G or C-to-T), it is called *transition*, and when the change is from purine to pyrimidine or pyrimidine to purine (A-to-C or G-to-T), it is known as *transversion*. Nonsynonymous mutations are less frequently observed than the synonymous mutations. Nonsynonymous mutations are of three types: missense, nonsense, and frameshift mutation.

Missense mutation: This occurs when there is a single base-pair substitution, which results in different amino acids, leading to an altered protein.

For example, in Scheme 24.1A, the upper panel shows a DNA sequence that codes for a segment of protein with a sequence as LPHRYTNSRG. Substitution of G to A in 9th codon AGA that codes for R (Arginine) will result in AAA codon that codes for K (Lysine), thereby altering the sequence of amino acids, resulting in an altered protein as shown in the lower panel.

Non-sense mutation: Mutations resulting in replacement or substitution that causes premature termination of translation of a peptide chain lead to loss of function/activity. For example, Scheme 24.1B shows that substitution of T to G at fifth codon TAT that codes for Y (tryptophan), results in codon TAG, which does not code for any amino acid (and therefore terminates the peptide chain).

Frameshift mutation: A mutation that causes an insertion or deletion of nucleotides (not multiple of three) results in disruption of the reading frame of a polypeptide chain. This leads to the inactivation of polypeptide chain/protein. When there is a deletion or insertion of one or two nucleotides, it results in the interruption of the reading frame of a protein. But if the insertion or deletion is of three or multiples of three nucleotides, then the reading frame of the protein is not changed. For example, Scheme 24.1C shows that the deletion of T in the second codon CCT that codes for P (proline) results



SCHEME 24.1 Different types of mutations leading to either altered protein or protein termination.

in disruption of the reading frame, leading to an altered protein with different amino acids. The addition of one or two nucleotides will also lead to similar disruption of the protein, thus losing its significance/activity.

Scheme 24.1D shows that the insertion of the three nucleotides ATG after the second codon in the DNA sequence results in the addition of M (methionine) at the third position in the protein sequence. Although there is a change in the polypeptide chain, the reading frame is not changed. Thus, when there is an insertion of three or multiples of three nucleotides, the reading frame is not changed. Similarly, deletion of three or multiples of three nucleotides will also not change the reading frame of a protein.

Dynamic mutations: Triplet repeats are typically short sequences that are repeated many times and are commonly found in normal DNA sequences. These are highly polymorphic in nature; in other words, they are found in varying sizes in individuals. These repeats transmit normally below a certain level and become unstable above a critical level, and this expansion can occur either during mitosis or meiosis, leading to triplet repeat disorders. Common examples are of triplet disorders—Fragile X mental retardation (caused by CCG repeats), Huntington's disease (caused by CAG repeats), and Myotonic dystrophy (caused by CTG repeats).

For example, normal individuals have less than 45 CCG repeats in the 5′ untranslated region of the *FMR1* gene while in an affected individual with Fragile X syndrome, this number may exceed over 200 repeats, leading to silencing of the *FMR1* gene, and thus stopping the formation of FMRP protein.

Numerous diseases are clinically similar (i.e., having similar phenotypes) but may have different causes. This phenomenon is called *genetic heterogeneity*. *Allelic heterogeneity* is used when different mutant alleles are present at one gene locus, while in *locus heterogeneity*, different gene loci are responsible for a single disorder. Genetic heterogeneity and locus heterogeneity are often used interchangeably in practice, but locus heterogeneity is only used for the involvement of different loci in the causation of a disease/phenotype individually. The term genetic heterogeneity may also be used for a combined effect of different loci in the development of a (complex) disease (e.g., in diabetes, multiple loci are simultaneously involved in the development of diabetes).

Genetic polymorphism is a specific term that describes frequent variation at a specific locus in a genome, resulting in diversity within a population.

The term "Polymorphism" is a Greek word, where "Poly" means many, and "morph" means form. Genetic polymorphism was originally defined by Ford (1940). It specifies different forms of a DNA sequence that creates genetic diversity within a population's gene pool. On average, there is a DNA sequence variation in exactly the same region on a chromosome after about 1000 base pairs in a segment of human DNA carried by many different individuals from around the world. A single base pair that varies between the two homologous chromosomes inherited from the parents assuming that the parents are unrelated when present in more than 1% of the general population is called a *genetic polymorphism*. Polymorphism could be due to deletion, substitution, duplication, or triplication of few bases in the DNA.

After the completion of the Human Genome Project in 2003, the sequence of the entire human genome was identified with their location in the chromosomes. So once the scientific community had the complete human genome data, the next venture was "The International Hapmap project" which was started in October 2002, with the aim of identifying the common patterns of human genetic variation in a different population that affect health and disease. The *HapMap* (short for "haplotype map") is a catalog of common genetic variants called single nucleotide polymorphisms (SNPs). The HapMap project focused only on common SNPs, those where each allele occurs in at least 1% of the population. In 2007, a report on a human haplotype map of over 3.1 million SNPs was published in the journal *Nature* (1).

The majority of these variations in the DNA sequences are selectively neutral changes of little or no functional implication but these can affect how humans develop diseases and respond to pathogens, chemicals, medication, vaccines, and other agents. SNP-based studies have helped tremendously in biomedical researches while comparing the regions of the human genomes between cohorts with and without a disease.

So in addition to structural variability, SNPs are also recognized as a substantial source of genetic variation and influencing phenotypic changes.

Single nucleotide polymorphism

SNP is the most common of all polymorphisms. Almost all common SNPs have only two alleles corresponding to the two different bases in the genome. Genomes of two individuals are 99.9% identical except in identical twins. So out of this 0.1% difference, 80% is represented by SNPs.

For example, on sequencing DNA fragments from three individuals, the result showed the following sequences: GCA**A**CGTTAGA, GCA**G**CGTTAGA, and GCA**T**CGTTAGA, containing a difference in a single nucleotide (shown as highlighted). In this case, we say that there are three alleles: A, G, and T. SNPs are usually assigned a minor allele frequency within a population, which is the lowest allele frequency at a particular locus observed in a population. These differences in single-nucleotide can either influence a variety of traits such as

appearance, disease susceptibility, or response to drugs or most SNPs lead to no observable differences.

The Hapmap project has made it feasible to identify almost 500,000 SNPs in a single DNA sample.

Submicroscopic unbalanced genomic changes called *copy number variations* can detect the cause of congenital anomalies and/or learning disabilities. The methodology of CMA has been discussed in the Cytogenetic: an overview section. High-resolution microarrays are available that include copy number probes as well as SNPs which can reveal that allele is present at that locus as well as the number of copies of that DNA segment. Thus in the field of clinical genetics, array-based methods have been widely accepted as one of the most efficient techniques in terms of their resolution, high coverage, and high-throughput, and they are also referred to as *virtual karyotype*.

Thus, a CMA along with an SNP array has the potential to detect chromosomal abnormalities and obtaining genotype information to identify the origin and mechanism of the chromosomal aberration.

Genetic disorders are mainly classified into chromosomal disorders, single-gene disorders, multifactorial disorders, and acquired genetic diseases. Chromosomal disorders have already been discussed in the cytogenetic section; the following sections discuss the other three main categories of genetic disorders; single-gene disorders, multifactorial disorders, and mitochondrial disorders. In this edition, we have introduced another category of genetic disorders that are known as epigenetic disorders.

Single-gene disorders

Over 10,000 traits or disorders in humans exhibit single-gene, unifactorial, or Mendelian inheritance. However, characteristics such as height, weight, and many common familial disorders, such as diabetes, hypertension, etc., do not usually follow a simple pattern of inheritance. A trait or disorder that is determined by a gene on an autosome is said to show autosomal inheritance, whereas a trait or disorder determined by a gene on one of the sex chromosomes is said to show sex-linked inheritance.

In single-gene disorders, the gene responsible for the disease can be traced through families, and their occurrence in the next generations can be predicted. An *autosomal dominant* trait is one that manifests even in the heterozygous state (i.e., one mutant allele and one normal allele). Or in other words, the mutation is present only on one chromosome which is enough to cause the disease. Autosomal dominant disorders are generally milder than recessive disorders; a few examples are neurofibromatosis, achondroplasia, and Marfan syndrome. *Autosomal recessive* disorders only manifest when the mutant allele is present on both chromosomes, i.e., homozygous. Individuals who are heterozygous for a recessive mutant allele have a normal phenotype but genotypically are carriers. The disorder affects both males and females in equal proportions. Examples of autosomal recessive disorders are beta-thalassemia, spinal muscular atrophy, galactosemia, etc.

Sex-linked disorders can be either X-linked or Y-linked. In *X-linked recessive* mode of inheritance, a mutation in the X chromosome causes the phenotype to be expressed in males who are hemizygous for the gene mutation (i.e., they have only one X chromosome) and in females who are homozygous for the gene mutation (i.e., they have a copy of the gene mutation on each of their two X chromosomes). Carrier females have only one copy of the mutation, so do not usually express the phenotype, although differences in X-chromosome inactivation can lead to varying degrees of clinical expression in carrier females. Examples of X-linked recessive disorders are hemophilia, Duchene muscular dystrophy, etc. *X-linked dominant* inheritance although uncommon, includes disorders that manifest in heterozygous females as well as in males; examples include hypophosphatemic vitamin D-resistant rickets, orofaciodigital syndrome, etc.

Multigenic and multifactorial disorders

In contrast to the monogenic or single-gene disorders, multigenic disorders are caused by mutations in more than one gene. Mutation in any one of these genes alone may not cause the disease, but all these mutations present together can result in a diseased condition.

A trait is called multifactorial if multiple genes are assumed to interact with environmental factors. Most diseases develop as the result of a combination of small variations in genes that can predispose an individual to a disease. Such inheritance is observed in common disorders such as hypertension, diabetes mellitus, neural tube defect, ischemic heart disease, etc. It is very important to identify susceptibility genes for common disorders so that individuals at high risk for these diseases can be identified early and preventive options can be made available to them.

Mitochondrial disorder

In addition to a nuclear genome, our cells also contain a mitochondrial genome; each mitochondrion maintains dozens of copies of its own circular genome, and most human cells contain numerous mitochondria. Mitochondria are organelles, which are found in every cell of the human body except red blood cells. When a cell divides, its mitochondria are distributed to the two

daughter cells. However, mitochondrial segregation occurs randomly and is not as organized as the highly regulated process of mitotic chromosome segregation. Therefore, cells will receive similar, but not identical, mitochondrial DNA populations. Mitochondria have their own set of genes, as well as on nuclear-encoded genes, in order to carry out their function as the ATP-generating (adenosine triphosphate) powerhouses of the cell. Therefore, mitochondrial mutations can lead to profound effects on cellular metabolism and function, especially in tissues that have high-energy demands, such as that of the brain, skeletal muscle, cardiac muscle, and retina. Mitochondrial inheritance is often called "maternal inheritance" because a child inherits the great majority of their mitochondria from their mother through the egg. Mitochondrial DNA contains 37 genes that are all essential for the normal function of the mitochondria. Some of the common mitochondrial disorders are mitochondrial encephalopathy with lactic acidosis and stroke-like episodes (MELAS), myoclonic epilepsy with ragged red fibers (MERRF), etc.

Epigenetic disorders

So far we have been studying the heritable changes in the DNA sequence having certain phenotypic effects. But there are certain changes that affect the gene activity and its function without any change in the DNA sequence, known as an epigenetic phenomenon.

Epigenetics is defined as genetic control by factors other than an individual's DNA sequence. Epigenetic factors control genes and determine which proteins are to be transcribed. On one hand, epigenetic changes are important for normal development, and on the other, they can also lead to certain genetic diseases; the commonest of them are Fragile X syndrome, Prader–Willi syndrome (PWS), Angelman's syndrome (AS), Rett syndrome, alpha-thalassemia, and some form of cancers. It has been observed that the nervous system is more sensitive to epigenetic disturbances because of its complexity, and thus many syndromes involving mental disability are caused by variations in the epigenetic centers in the genome.

For the development of the embryo, both maternal and paternal genomes are important since both are not equivalent. There are specific genes known as imprinted genes that are expressed from single parental allele and have gamete-specific differential expression. Different experimental studies have established that absence or overexpression of these imprinted genes inherited from either the maternal or paternal genome results in developmental abnormalities.

In eukaryotes, almost all cells contain the same genetic code, but not all genes are active at the same time simultaneously. DNA methylation is one of the common epigenetic mechanism by which the cells regulate expression. In this process, a methyl (CH_3) group is added to DNA, onto the C5 position of the cytosine to form 5-methylcytosine, thereby often modifying the function of the genes and thus affecting gene expression. The expression of imprinted genes depends on the parental contribution either the maternal or paternal allele is methylated and only one copy produces protein. The majority of the imprinted genes plays a crucial role in controlling embryo growth and early brain development and continue even later in life (Wilkinson et al., 2007).

PWS and AS represent one of the best examples of genomic imprinting in humans. The loss of expression of the paternally expressed genes causes PWS and loss of maternally expressed genes causes AS, both on chromosome 15q11.2-q13.

PWS is a complex genetic condition that affects multiple parts of the body. Distinct clinical features are observed in these patients. Some of the predominant features are described as below:

Delayed milestones/intellectual delay
Strabismus ("crossed eyes")
Poor physical coordination
Prominent nasal bridge
High, narrow forehead
Thin upper lip
Downturned mouth
Small hands and feet with tapering of fingers
Excess fat, especially in the central portion of the body
Speech delay
Scoliosis (curvature of the spine, often not detected at birth)
Excessive sleeping
Soft skin, which is easily bruised

PWS is a contiguous gene disorder, meaning a deletion that eliminates several genes that are lying in close proximity to one another on the chromosome. It is localized to a 5–6 Mb genomic region on the proximal long arm of chromosome 15 (15q11.2-q13). This region is demarcated by three common deletion breakpoints called BP1, BP2, and BP3 as shown in Fig. 24.4. The proximal region has four nonimprinted genes that are expressed biparentally and lies between the proximal regions of BP1 and BP2. Next to this is the "PWS paternal-only expressed region" containing five polypeptide coding genes (MKRN3, MAGEL2, NECDIN, SNRPN, and C15orf2). Imprinting center (IC) is that portion of the chromosome 15, which spans 5′ exons of the gene encoding the small nuclear ribonucleoprotein N (SNRPN) and Chromosome 15 open reading frame 2 (C15orf2) which controls imprinting. Next to these are two clusters of small nucleolar RNA (Sno RNA) genes.

The "AS region" contains the preferentially maternally expressed genes UBE3A and ATP10A (Cassidy

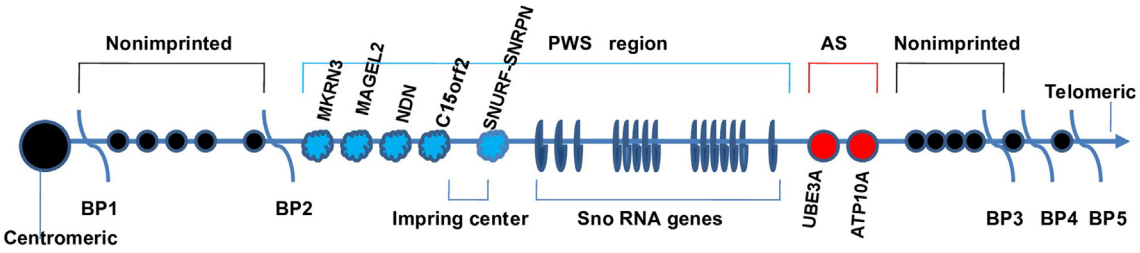


FIGURE 24.4 Schematic diagram showing imprinted genes in chromosome 15.

and Driscoll, 2009). There are different chromosomal and molecular mechanisms that have been identified in PWS (Angulo et al., 2015). Three main mechanisms are as follows:

1. Paternal microdeletion involving the common deletion breakpoints in chromosome 15q11.2-q13 is more frequent and observed in about 70%–75% of cases.
2. Maternal uniparental disomy (UPD) is the next common cause responsible for about 20%–25% of cases. *UPD* is defined when a person receives two copies of a chromosome, or part of a chromosome, from one parent only and no copies from the other parent. In PWS, a person having a deletion in the paternal chromosome 15 will have no active genes in this region and instead will have two maternal copies of chromosome 15 in each cell. This phenomenon is known as *maternal UPD*.
3. Imprinting defect (ID) is responsible for 1%–3% of cases. *Genomic imprinting* refers to a phenomenon where genes are expressed in a parent-of-origin-specific manner. In PWS, the paternal chromosome carries a maternal imprint, while in AS, the maternal chromosome carries a paternal imprint. In this disorder, a microdeletion affecting the 5′ end of the SNURF-SNRPN locus which is the imprinting center (IC) and regulates imprinting in the whole domain causes ID.

Other defects are such as balanced and unbalanced translocations, which, together with ID, are responsible for the majority of familial cases.

Methodology: application of different molecular techniques for diagnosis of genetic disorders

Various techniques for genetic diagnosis are being used to confirm the clinical diagnosis for a number of heritable disorders and infectious diseases. The DNA sample isolated from a patient's cell can be used to screen for innumerable diseases. With the advancement of techniques and increasing knowledge of the pathophysiology of a number of diseases, diagnosis can be made well in advance for better management.

Molecular diagnostic technologies are now being used for the following applications:

Clinical diagnostic testing: Application of genetics in medicine and health has been around for over 50 years. Many common genetic diseases such as thalassemia, Duchenne muscular dystrophy, Hemophilia, Fragile X syndrome, etc., are being diagnosed by molecular tests. These methods not only diagnose but are also utilized for the identification of individuals who are at increased risk of developing certain disorders later in life (e.g., Huntington's disease). In some diseases, such as breast cancer, screening for mutations in *BRCA1* and *BRCA2* genes helps in counseling and surveillance of family members who are at risk of developing breast cancer.

To perform any genetic test, DNA is needed; it can be extracted from virtually any part of the human body, the most common sources being blood and soft tissue samples. DNA can also be extracted from semen, saliva, hair roots, etc. Variations in DNA can be identified by various techniques such as Southern blotting, polymerase chain reaction (PCR), DNA sequencing, or gene expression studies.

Southern blotting

The Southern blotting is a technique named after its inventor, the British biologist Edwin Southern, to detect a specific DNA sequence in DNA samples. It combines a technique of transferring electrophoresis-separated DNA fragments to a filter membrane and probe hybridization to detect the fragments. A Southern blot helps in finding out whether a particular fragment of a gene is present in the genomic DNA or not and whether there is any change in the size of the DNA fragment. Hence, the presence or absence of the gene, large deletions and duplications (or even large gene arrangements) can be detected by Southern blotting.

The Southern blotting technique is technically demanding, low–throughput, and time-consuming. Therefore, newer techniques are being used for identifying large deletions and duplications such as multiplex ligation probe amplification (MLPA) and real-time PCR which are newer modified PCR methods, which allow more rapid detection and quantification of the PCR product. MLPA is an easy to use and high-throughput technique for DNA copy number quantification and is used for many genetic disorders and tumors (Schouten et al., 2002). This technique is also used to investigate the abnormal methylation status of DNA sequences. Presently, 60 probes can be amplified simultaneously in a single reaction, each of which can detect a specific DNA sequence of approximately 60 bases (or nucleotides) in length. Each MLPA probe consists of two oligonucleotides that hybridize to immediately adjacent target sequences followed by ligation of these two oligonucleotides into a single probe. All ligated probes are amplified simultaneously using the same PCR primer pair, one of which is fluorescently labeled. The amplification products are visualized during fragment separation in a capillary electrophoresis instrument or sequencer that yields a specific electropherogram. Methylation-sensitive MLPA (MS-MLPA) probes for methylation detection resemble other MLPA probes, except that their target sequence contains the restriction site of the methylation-sensitive endonuclease HhaI.

Real-time polymerase chain reaction

A real-time PCR that is also known as quantitative PCR, where one can check the amplification of a targeted DNA in real-time, whereas in conventional PCR at the end of the PCR cycles, a gel electrophoresis is carried out to visualize the products. The principle of real-time is based on the detection of the fluorescence produced by a reporter molecule which increases, with the accumulation of the PCR product with each cycle of amplification. These fluorescent reporter molecules include either nonspecific fluorescent dyes that intercalate with any double-stranded DNA or sequence-specific DNA probes consisting of oligonucleotides that are labeled with a fluorescent reporter that permits detection only after hybridization with its complementary sequence. Fig. 24.5A and B shows the application of real-time PCR in the identification of deletion and duplication.

Quantitative fluorescent polymerase chain reaction

In this type of PCR, chromosome-specific DNA sequences are amplified using fluorescent primers. This method uses highly polymorphic microsatellite markers regions that have short-repeated sequences of DNA located on the chromosomes of interest to determine the number of copies of those chromosomes present per cell. In quantitative fluorescent (QF)-PCR, three

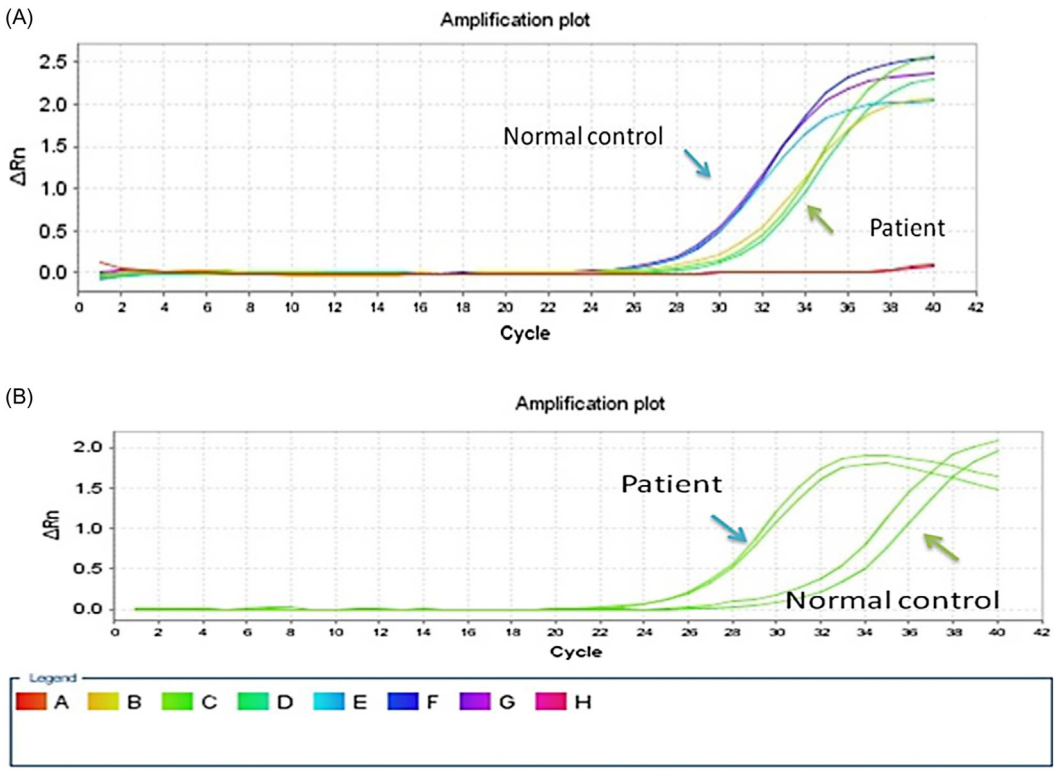


FIGURE 24.5 A: Real time analysis showing heterozygous deletion of 5p region in patient sample relative to normal control B: Real time analysis showing 12p duplication in patient sample relative to normal control.

to five STR markers across chromosomes 13, 18, 21 and each X and Y chromosome are used to detect common aneuploidies like Down syndrome, Turner syndrome, Edward syndrome, and other sex chromosome-related aneuploidies as shown in Fig. 24.6. Normally, the markers are diallelic and are in the ratio of 1:1, while in case of trisomy, the ratio is either 1:1:1 or 2:1, which indicates that one allele is in double dosage as shown in the figure. This is one of the recommended methods of choice for rapid screening of common aneuploidies in prenatal samples as well as maternal cell contamination in the fetal DNA sample (Adinolfi et al., 1997).

DNA sequencing

This process is used to determine the nucleotide sequence of a given DNA fragment. With this basic information, one can locate regulatory and gene sequences, make comparisons between homologous genes across species, and identify mutations. In 1974, an American team and an English team independently developed two methods. The American team, lead by Maxam and Gilbert, developed a "chemical cleavage protocol," while Frederick Sanger designed a procedure analogous to the natural process of DNA replication. Even though both teams shared the 1980 Nobel Prize, Sanger's method became the method of choice because of its expediency and simplicity.

This method uses the chain termination method, and the extension is initiated at a specific site on the template DNA by using a short oligonucleotide "primer" complementary to the template at that region. The oligonucleotide primer is extended using an enzyme, DNA polymerase, that replicates DNA. In addition to the normal dinucleotides (dNTPs) found in DNA, dideoxynucleotides (ddNTPs) are added to prevent the addition of further nucleotides. Dideoxynucleotides are essentially the same as nucleotides but they contain a hydrogen group on the 3′ carbon instead of a hydroxyl group (OH). The DNA chain is terminated because a phosphodiester bond cannot be formed between the dideoxynucleotide and the next incoming nucleotide, resulting in a series of related DNA fragments that are terminated only at positions where that particular nucleotide is used. The fragments are then size-separated by polyacrylamide gel electrophoresis, and the gel is then exposed to either UV light or X-ray, depending on the method used for labeling the DNA (Sanger et al., 1992).

With the advancements in technology, automated sequencing has been developed, so that more DNA can be sequenced in a shorter period of time. This automated procedure, developed by Russell in 2002, is based on the principles of Sanger's method in which reactions are performed in a single tube containing all four ddNTP's, each labeled with a different color dye

Due to limitations like low-throughput, high cost, and the long time taken, Sanger sequencing efforts were made to develop new methods to process millions of reactions in parallel, resulting in very high-speed sequencing referred to as next-generation sequencing (NGS). In 2000, a USA company called Massively Parallel Signature Sequencing (MPSS) Lynx Therapeutics first launched the NGS technologies. The newer methods changed the scientific approaches in both basic and applied research in many scientific disciplines, especially in many branches of the biological field, including plant pathology and plant virology. Millions of small fragments of DNA are sequenced in parallel, and bioinformatics analyses are used to put these fragments together by mapping the individual reads to the human reference genome. NGS can be used to sequence entire genomes or all 22,000 coding genes (a whole-exome), small number of individual genes or transcriptome sequencing, which includes sequencing and analysis of full-length mRNA. Recently, more robust, simplified, and cost-effective platforms of third-generation sequencing have been developed. Helicos Genetic Analysis Platform, single-molecule

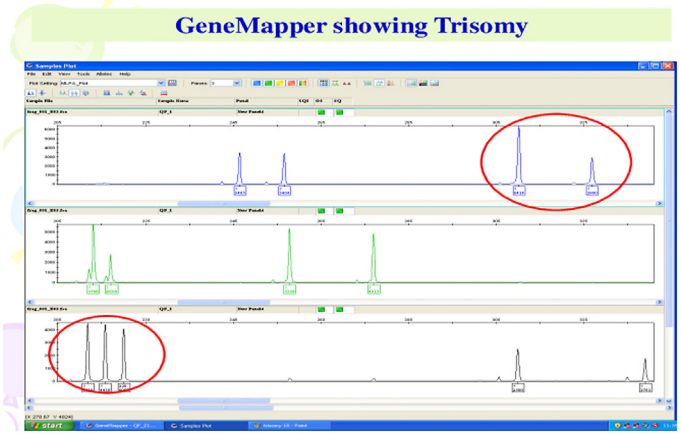


FIGURE 24.6 QF PCR result showing trisomy 21.

real-time (SMRT) sequencing, and MinION are some of the examples of third-generation sequencing.

With newly immerging rapid and novel technologies, the future of genomics seems quite exciting and sequencing the whole genome in still less time and cost will no longer be a dream. One could expect these technologies to help unravel further mysteries hidden in the genome.

Principle

The principle of all molecular genetics techniques is to elucidate the structure and function of genes. Molecular genetics techniques along with certain biochemical methods are involved with the direct study of DNA and RNA. This field started with the invention of recombinant DNA technology and the amplification of a specific region in a gene of interest, and today, the entire genome can be scanned to identify the variants' functions and their possible involvement in a given human disease or disorder.

The following case study should help readers to understand how the theoretical concepts of genetics are applied using the various techniques discussed above for the diagnosis of a genetic disorder.

Case study 1

Prader Willi Syndrome (PWS) as explained earlier is an epigenetic disorder and is diagnosed using a series of specialized genetic tests that are carried out specifically to diagnose this condition. The physical examination and history are very important in making the diagnosis and should be done before genetic testing.

Routine chromosome tests, like karyotype, do not reliably detect the genetic changes of PWS. A chromosomal analysis is required for genetic counseling purposes to identify an interstitial de novo deletion from a balanced or unbalanced chromosomal rearrangement involving the 15q11.2 region. FISH with the SNRPN probe can easily detect deletions of 15q11.2-q13. With the increasing use of CMA in clinical genetics, it has gradually replaced FISH technique for the identification of deletions in PWS and AS. CMA technique can also provide additional information like other chromosomal abnormalities elsewhere in the genome. However, CMA will not identify the rare chromosomal rearrangements (balanced translocations and inversions) involving proximal 15 which are detectable by karyotype or FISH analysis and are important in recurrence risk determination.

DNA methylation analysis can be done by various methods. One is sodium bisulfite treatment of DNA followed by PCR using primers specific for differentially methylated sites within the SNRPN exon 1/promoter regions or restriction digestion of DNA with a methylation-sensitive enzyme followed by PCR.

Methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) where methylation-specific enzymes are used (explained earlier under MLPA technique) is one method by which one can analyze the copy number change as well as methylation of 15q11 region simultaneously. MS-MLPA has become the method of choice as in a single test, it investigates copy number changes and methylation status at several loci, thereby reducing the risk of a false positive or false negative result due to SNPs. Thus, for complex disorders like PWS, it is important to keep in mind that more than one test is also required in certain cases to confirm the clinical diagnosis.

Prenatal diagnosis

Prenatal diagnosis means diagnosis or detecting abnormality in the unborn child during pregnancy as early as possible. This is one of the most important subfield in clinical genetics that involves a multidisciplinary group of medical and scientific professionals. Geneticists and genetic counselers help couples or families to understand the risk of having a child with a genetic disorder and discuss options for prenatal screening and testing. Obstetricians and gynecologists do the ultrasonography scan and aspirate the fetal sample under aseptic condition and then another group of experts in the laboratory carry out the appropriate tests to identify the genetic condition in the fetus.

Prenatal diagnosis is generally divided into two categories, those that are noninvasive and those that involve invasive methods. The noninvasive tests include ultrasound imaging and maternal serum tests. A screening test can only provide risk or a probability, indicating a particular condition. The noninvasive tests are generally offered to all pregnant women, which is a part of routine investigations while the more-invasive tests are generally recommended when screening tests are positive or if some risk factors exist in the family.

Prenatal screening tests (noninvasive): The following screening tests at different prenatal stages helps in finding out the probability of genetic conditions in the fetus.

1. Double marker test: Usually done at 11−13 weeks of pregnancy. In the maternal serum, pregnancy-associated plasma protein-A (PAPP-A) and free beta-human chorionic gonadotrophin (βhCG) is tested, in combination with the mother's age.
2. Ultrasound: A special ultrasound called a nuchal translucency ultrasound is performed between 11th and 14th week of pregnancy. This procedure helps to check the accumulation of fluid at the back of baby's neck. The presence of more fluid than normal, indicates a higher risk of Down syndrome.

The combination of the above two tests serves as screens for structural fetal malformations, Down syndrome, and neural tube closure defects.

Prenatal diagnostic tests: These include invasive methods by which the fetal samples are collected before carrying out diagnostic tests that are capable of giving confirmatory results. Before any invasive prenatal testing, a detailed genetic counseling is done to inform the couple about the risks, benefits, and limitations of the procedure. The following are some of the commonest procedures that are performed depending on the type of abnormality suspected in the fetus.

1. Amniocentesis is one of the commonest procedures which is conducted anytime between 16 and 20 weeks of pregnancy. Amniotic fluid is a clear, slightly yellowish fluid in the amniotic sac which surrounds the fetus in the uterus. This fluid contains fetal cells that are analyzed mainly for chromosomal disorders. The procedural related risk is minimal, which is about 0.5%.
2. Chorionic villus sampling (CVS) is conducted after 11 weeks of pregnancy. This is the placental tissue in the uterus that provides blood and nutrients from the mother to the fetus. CVS testing is most appropriate for single-gene disorders. The procedural related risk is about 0.5%–1%.
3. Cordocentesis (percutaneous umbilical cord blood sampling) is done at a later stage, after 18 weeks of pregnancy. A small amount of blood is drawn from the vein in the umbilical cord. This procedure poses a little higher risk to the fetus about 1%–2% as compared to the other two procedures.

Ethical issues

Genetics is an area of medicine with enormous medical, social, ethical, and legal implications. In India, institutions like the Indian Council of Medical Research (ICMR) have issued statements that provide guidelines to physicians and address some of these concerns regarding genetic counseling and testing during pregnancy. In recent years, with the advent of in vitro fertilization (IVF), genetic testing has been extended to pre-implantation genetic diagnosis of embryos. This can be a useful tool in cases where one or both genetic parents have a known genetic abnormality and testing is performed on an embryo to determine if it also carries a genetic abnormality. It is an alternative option of preventing heritable genetic disease, thereby eliminating the dilemma of pregnancy termination following unfavorable prenatal diagnosis. With the development in genetic technologies, it has opened doors for new approaches to health promotion, prevention, diagnosis and treatment of both rare and common diseases. Now the focus has shifted from single-gene diseases to a new field of research called genomics, in which all the genes present in the human genome are studied, as well as their interaction with each other and the influence of the environment on their functioning. This evolution has led to a more personalized approach to treat common diseases such as cancer, diabetes, and heart diseases and is referred to as "personalized medicine." Although this new approach holds great promise, it also raises issues about confidentiality and consent. The readers must be aware about the ethical aspects of genetic research, genetic testing, privacy, disclosure of genetic information, and freedom of reproductive choices. The World Health Organization in 1998 made a proposal—"Proposed International Guidelines on Ethical Issues in Medical Genetics and Genetic Services"—for the scientific and medical community. The entire content of the proposal has not yet reached a consensus among all nations, since the laws of each nation differ with respect to some particular issues such as abortion after prenatal diagnosis, choices about alternatives in assisted reproduction, and the status of the use of human embryos in genetic research. The following lines from IARC Sci Publ 154:131, 2001 will highlight the sensitive issues and ethical dilemma of genetic screening:

"Many lives could be saved by screening individuals at risk and targeting preventive behavior to them but there will always be risk of making healthy people 'sick' through detecting presence of predisposing genes and potential for stigmatization and discrimination by society, insurance companies and employers."

In India, ethical issues in genetics and genomics are governed by the following guidelines laid down in:

- Statement of Specific Principles for Human Genetics Research ICMR Ethical Guidelines for Biomedical Research on Human Subjects 2000, pages 49–66 and recently a detailed National *Ethical Guidelines* for Biomedical and Health Research Involving Human Participants with a separate section involving children as subjects were published in 2017.
- Ethical Policies on Human Genome, Genetic Research and Services issued by the Department of Biotechnology, GOI, January 2002.

Translational significance

The term "translational research" is now being used often in scientific gatherings at academic centers and

government health departments, in biomedical industries, and in private health research organizations. Translational research emphasizes the practical application of knowledge derived in basic science to the development of a new understanding of disease mechanisms, diagnoses, and therapeutics in humans to improve health. In other words, it endeavors to move "from bench to bedside" or from laboratory experiments through clinical trials to develop therapeutics for direct patient applications.

Promising future

Gene-based therapies are slowly achieving clinical success. Several approaches to genome editing have been developed, which allow genetic material to be added, removed, or altered at particular locations in the genome. Before 2015, genome editing with engineered nucleases such as zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and engineered meganucleases were in use. In 2015, the CRISPR-Cas9 system generated a lot of excitement in the scientific community and was selected by Science as 2015 Breakthrough of the Year. CRISPR-Cas9, is the short form of clustered regularly interspaced short palindromic repeats and CRISPR-associated protein 9, the principle which was adapted from a naturally occurring genome editing system that is existing in bacteria.

This method is being explored in research on a wide variety of single-gene disorders such as cystic fibrosis, hemophilia, and sickle cell disease. It definitely holds promise for the treatment and prevention of such disorders and also for more complex diseases, such as cancer and heart diseases.

World Wide Web resources

Online tools provide the benefits of getting information at the click of a button. Remember that this technology is just a means of delivering information, so it is important to also read books and journals. Although the Internet is one of the greatest recent inventions, it can still be an unreliable resource, so it is always advisable to consult other sources to acquire in-depth knowledge about a subject.

The following are some useful websites:

1. GeneEd
2. DNA from the Beginning
3. Genetic Science Learning Center
4. Understanding Genetics
5. On-Line Abstract and Journal Articles
6. Genetic Disorders and Their Frequency in India.
7. Centre for the Development and Evaluation of Complex Interventions for Public Health Improvement (DECIPHER) project: http://decipher.sanger.ac.uk.
8. The Chromosome Anomaly Collection: http://www.ngrl.org.uk/wessex/collection/.
9. Chromosomal Variation in Man Online Database: http://www.wiley.com/legacy/products/subject/life/borgaonkar/access.html.
10. Cytogenetic Data Analysis System (CyDAS): http://www.cydas.org/.
11. Database of genomic structural variation (bdVar): http://www.ncbi.nlm.nih.gov/dbvar/.
12. Ensembl: www.ensembl.org/.
13. European Cytogeneticists Association Register of Unbalanced Chromosome Aberrations (ECARUCA): www.ecaruc.net.
14. The International Standards for Cytogenomic Arrays (ISCA) Consortium: https://www.iscaconsortium.org/index.php.
15. Small supernumerary marker chromosomes: http://ssmc-tl.com/sSMC.html.

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Further Reading

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Glossary

Locus The position of a gene on a chromosome is called its locus and the corresponding loci on a homologous pair of chromosomes carry genes for the same character.

Allele One of the several alternative forms of a gene at a given locus is called allele.

Genotype and phenotype The genetic make-up of a person is called the genotype, and the clinically manifest characters are known as the phenotype.

Codons Each protein is a long-polypeptide chain molecule formed by joining amino acid molecules. The genetic code consists of 64 triplets of nucleotides. These triplets are called codons, and each codon encodes for one of the 20 amino acids used in the synthesis of proteins.

Transcription The information in DNA is transferred to a messenger RNA (mRNA) molecule by a process called transcription.

Sense strand RNA synthesis occurs in the 5′–3′ direction and its sequence corresponds to that of the DNA strand.

Genetic polymorphism Occurrence of two or more alleles at one locus in the same population having a minimum frequency of at least 1%

Single nucleotide polymorphism It is a variation of a single nucleotide at a specific position in a DNA sequence not resulting in a change in amino acid in the protein within a population.

Haplotype A group of alleles of different genes on a single chromosome.

Copy number variation It is a type of variation involving alteration in the number of copies of a specific region of DNA either due to deletion or duplication.

Epigenetics It is a study of heritable changes in gene expression without a change in the DNA sequence.

Chromosomal microarray It is a microchip-based diagnostic tool to detect deletion, duplication, or unbalanced chromosomal rearrangements.

Methylation It is a process by which methyl groups are added to a substrate.

Uniparental disomy It refers to when an individual receives both the copies of a chromosome or a part of a chromosome from one parent and no copy from the other parent.

Genomic imprinting It is an epigenetic phenomenon where the expression of a gene depend on whether the allele is inherited from mother or from father.

Long answer questions

1. What are the different kinds of mutations?
2. Describe the different kinds of inheritance patterns.
3. What are the structural abnormalities that are commonly observed in chromosomal disorders?
4. What is the significance of SNPs in clinical diagnostics and pharmacogenomics?
5. Explain the phenomenon epigenetics.

Short answer questions

1. How are human chromosomes classified?
2. How would you write the chromosome complement in a female showing translocation of the long arm of chromosome 8 on the short arm of chromosome 11?
3. What would be the sequence of mRNA transcribed from the following DNA strand ATGCGCCATTGTGTC?

4. When does a variation in the human genome can be termed as a "Polymorphism"?
5. How are Prader–Willi and Angelman syndrome different?

Answers to short answer questions

1. Each chromosome has one short arm and one long arm, separated by a centromere. Each chromosome differs in length and also in the position of the centromere. Based on the length and the position of the centromere, chromosomes are classified into A-G groups.
2. 46,XX,t(8q;11p)
3. GACACAAUGGCGCAU
4. A variation is said to be polymorphic when more than one allele is known to occur at that particular gene's locus within a population. Moreover, each allele must also occur in the population at a rate of at least 1% to be considered as polymorphic. Gene polymorphisms can occur in any region of the genome. The majority of these polymorphisms have no phenotypic consequence, meaning that they do not alter the function or expression of a gene. Polymorphisms promote genetic diversity within a population's gene pool.
5. These two disorders, Prader–Willi (PWS) and Angelman syndrome (AS) share the same genetic basis that is genetic imprinting. The genes involved in these two disorders are located in the same region in chromosome 15 long arm. PWS is caused due to lack of paternally expressed genes, and AS is due to a lack of maternally expressed genes. PWS paternal-only expressed region contains five polypeptide-coding genes (MKRN3, MAGEL2, NECDIN, SNRPN, and C15orf2), and AS region contains the preferentially maternally expressed genes UBE3A and ATP10A.

Yes/no type questions

1. Is G-banding the most widely used technique for chromosomal analysis?
2. In mitosis, chromosomes are in most condensed form at anaphase?
3. The distal end of each chromosome called telomeres get shorter with each cell division, and eventually, the cell can no longer divide and dies.
4. Do acrocentric chromosomes have centromere at or near the middle??
5. Robertsonian translocation is the term used when any two acrocentric chromosomes break at their centromeres and fuse to form a single chromosome with a single centromere?
6. AUG is the start codon of a messenger RNA (mRNA) transcript translated by a ribosome in eukaryotes?
7. Synonymous mutation is the term used when a mutation does not alter the amino acid in the polypeptide chain?
8. Epigenetics is defined as genetic control by factors other than an individual's DNA sequence.
9. Prenatal diagnosis means diagnosis or detecting abnormality in the newborn child.
10. Is Maxam and Gilbert sequencing the most common sequencing method used worldwide?

Answers to yes/no type questions

1. Yes—G-banding is the most widely used technique for chromosomal analysis
2. No—Metaphase.
3. Yes—After each division, the ends of telomeres get shorter and shorter.
4. No—Metacentric chromosomes have centromeres located at their middle point.
5. Yes.
6. Yes—AUG is the initiation codon.
7. Yes.
8. Yes—Epigenetics broadly encompasses the effect of the environment on an organism.
9. No—Prenatal diagnosis implies diagnosis before birth.
10. No—Sanger sequencing is the most commonly used method worldwide.

CHAPTER

25

Antibodies and their applications

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Summary

With the creation of hybridoma technology by Georges Kohler, Cesar Milstein, and Niel Kay Jerne in 1975, advances in the diagnosis and therapeutics of clinically important diseases have started to appear. Monoclonal antibodies have started to displace antiquated polyclonal antibodies, and new recombinant products such as chimeric and humanized versions of antibody constructs (scFv, dsFv, diabodies, and bispecific antibodies) are likely to become future immunological reagents that are going to revolutionize diagnostics and therapeutics for life-threatening diseases. In 2015 alone FDA approved 10 therapeutic monoclonal antibodies. This historically high-approval rate is a first approach since therapeutic monoclonals were introduced in 1992.

What you can expect to know

Antibodies against any protein can be made in the laboratory by injecting a test animal (e.g., mouse or rabbit) with a pure sample of the protein. With the development of modern biotechnological methodology, new and novel methods have been developed to produce tailor-made antibodies against desired antigens. These include hybridoma technology that makes monoclonal antibodies, their chimeric and human versions that are less immunogenic, development of phage display for antibody screening, small recombinant antibody constructs such as single-chain variable fragment (scFv), disulfide-linked variable fragment (dsFv), diabody (bivalent antibody construct), and bispecific antibodies that are preferred for imaging tissues, a variety of clinical applications. Advances in biotechnology and molecular biology have provided methods for reengineering mouse antibodies to completely replace the rodent antibody sequence with functionally equivalent human amino acid sequences to form human antibodies from transgenic mice. There is no doubt that in years to come antibodies will become indispensable as chemical and research reagents.

History and methods

Immunodiagnostics: role of antibodies

Introduction

We are constantly exposed to a myriad of bacteria, viruses, fungi, and parasites every single day of our lives. Most of the time we never know this because we are equipped with a defense arsenal, the immune system. This remarkable defense of the human body is constituted by a vast army of cells and associated organs. The immune system is capable of defense against pathogens that may not have been encountered before.

The immune system forms two layers of defense in the human body: nonspecific or innate immunity, and specific or adaptive immunity. Innate immunity is constituted by mechanical barrier (e.g., skin, mucous membrane), chemical barrier (e.g., stomach acid), nonspecific defense processes (e.g., fever, inflammation), and a vast array of pattern recognition receptors (PRRs). PRRs include receptor-like Toll-like receptors, NOD molecules, and many others. PRRs recognize a vast array of conserved microbial motifs (called pathogen-associated molecular patterns or PAMPs) present in microbes. PAMPs are absent from host cells. PRRs bind PAMPs expressed on microbial surface and clear the microbes from the host body. These PRRs can be present in both soluble form in the human body (e.g., pentraxins) or localized on surface of the cell (Toll-like receptor).

Animal Biotechnology.
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In contrast to innate immunity, adaptive immunity is more concerned with specific aspects of immune response. Adaptive immunity is constituted primarily by antibodies, B cells, and T cells. It has two important qualities: (1) high specificity (i.e., it is specifically directed toward specific antigens/pathogens), (2) memory (i.e., it has a unique ability to remember pathogens). This implies that if the pathogen ever tries to infect the host again, the immune system will respond to infection immediately and protect the host. The adaptive immune system is able to protect us because it has the ability to produce billions of distinct antibodies along with billions of T-cell receptors, each of which recognizes a distinct antigen present on the microbial surface, thus initiating the destruction of the invading pathogen.

History

In 1890 von Behring and Kitasato reported the existence of "antitoxin" serum factor that could protect against lethal doses of toxins in humans. This was the beginning of an era where the significance of antibodies was understood and their use in diagnostic and analytical reagents started getting exploited. With the advent of hybridoma technology by Milstein and Köhler in 1975, the way to produce custom-built antibodies in vitro was paved. The hallmark of monoclonal antibodies is their specific binding to a particular antigen, which enables them to find a target precisely in vivo. In order to make mouse monoclonal antibodies less immunogenic, Boulianne, Morrison, and coworkers (1984) engineered recombinant antibodies (chimeric antibody created by coupling the animal antigen-binding variable domains to human constant domains). Going one step further on the path from fully mouse to fully human antibodies was Greg Winter and coworkers, who grafted mouse CDRs on human variable and constant regions to form humanized antibodies. The late 1980s onward saw the development of bispecific antibody constructs with different specificities in their arms, and small genetically engineered antibody fragments such as scFv, dsFv, Fab, and diabodies that found their applications in the medical, pharmaceutical, food, and environmental industries.

Antigens and antibodies

Antigens (or immunogens) can be defined as the foreign molecules that can elicit an immune response. Broadly speaking proteins and carbohydrates are usually antigenic, while lipids and DNA are usually weakly immunogenic/antigenic or nonimmunogenic. Antigens as a whole are not immunogenic; they have small portions on their surface that provoke an immune response. These small restricted portions are called *antigenic determinants or epitopes*.

Antigens (such as proteins and carbohydrates) are large molecules that usually have several antigenic determinants on their surface. Since these biomolecules are present on almost every invading bacteria, virus, and other pathogen, these microbes tend to be strongly antigenic and generate a strong immune response.

Once microbes enter the host body, the immune system gets activated and protects the host through both innate and specific or adaptive immunity. Protection initiated by innate immunity is beyond the scope of this book, and hence will not be discussed here. Adaptive immunity can be antibody-mediated or T-cell mediated. B cells, upon stimulation, produce millions of antibodies that are released in the blood. These antibodies bind to the invading pathogens and clear them from the circulation. This form of immunity is mediated by antibodies (and B cells) and is called humoral immunity. The other form of adaptive immunity is called cell-mediated immunity and is mediated by T cells. T cells work in a similar manner as antibodies. T cells are equipped with specific receptors (similar to antibodies) that recognize antigens. However T cells do not recognize antigens "alone" like antibodies. They recognize antigens only when they are associated with cell surface antigen presenting molecule: Class I and II major histocompatibility complexes (MHCs). In other words T-cell receptors recognize antigen + MHC associated complexes while antibodies/B cells recognize antigens "alone." T cells can be subdivided into two types of antigen-specific cells: cytoxic T cells (Tc cells) and helper T cells (T_H cells). Class I MHC + antigen can bind and activate only T_C cells while class II MHC + antigen can stimulate T_H cells. Upon activation T_C cells secrete small molecules called *perforins* that perforate and kill infected target cells. Stimulation of T_H cells causes release of small messenger molecule *cytokines* that summon other immune cells to the infected site. This entire operation, whether it involves antibodies or T cells, mops up the invading pathogen and clears the host system of infection.

Antibodies are unique in their affinity and exquisite specificity for binding antigens, a quality that has made them one of the most useful molecules for biotechnology applications. An important significance of antibody-based applications is that over a third of the proteins currently undergoing clinical testing in the United States are antibodies. Before we move on to select applications of antibodies with possible relevance to biotechnology, let us briefly review the structure and functions of antibodies.

A typical antibody molecule consists of four polypeptides. Two identical polypeptide chains of $\sim$450 amino acids (called heavy chains) and two identical chains of $\sim$250 amino acids (called light chains).

These four protein subunits are arranged in a Y formation. Disulfide bonds between light chain–heavy chain and between two heavy chains hold the antibody molecule. Each light and heavy chain consists of a constant region and a variable region. Starting from the N-terminal, first 110 or so amino acids constitute the variable region. This variable region is variable in sequence and composition among antibodies of different specificities. The rest of the polypeptides in both the light and heavy chains constitute the constant region. This region shows a similar sequence and almost the same composition among different antibodies. The variable region is located at the tips of the arms of the Y-shaped molecule, and the constant region is located at the stem of the antibody. The variable region of light and heavy chains constitutes the antigen-binding site. There are three regions within variable regions (of both light and heavy chains) that show a greater degree of variability than the rest of the region. These regions (three in each chain) constitute six hypervariable regions or complementarity determining regions (CDRs). CDRs are involved in binding the antigens/antigenic determinants.

Based on the minor differences in the constant region of the heavy chain, antibodies are categorized into five different classes: IgG, IgM, IgA, IgD, and IgE. The structures and few important functions of antibodies are listed in Table 25.1. From a biotechnology perspective, the most important class of antibodies is IgG, followed by IgM and IgA. The IgG antibody is the most abundant antibody in the blood. About 80% of the total serum immunoglobulin are IgG. IgG is the only antibody that can cross the placenta (i.e., move from mother to fetus). It is also the main antibody that is involved in opsonization (coating of microbes so that they are easily phagocytosed) and complement activation. IgM provides the first adaptive defense against invading pathogens as it is produced first in the primary immune response. Being pentameric and equipped with ten binding sites, the IgM antibody is a very effective agglutinator. IgA antibodies are the second most common antibody found in blood after IgG. They protect the exposed surface of the body against a plethora of pathogenic organisms. IgE also protects external mucosal surfaces from the assault of pathogens, but is more known for its role in allergic reaction. IgD antibodies are primarily expressed on B-lymphocytes and are probably involved in lymphocyte activation and suppression. IgD cannot bind mast cells or activate the complement system. Its concentration in serum is the lowest among all the classes of antibodies.

Polyclonal and monoclonal antibodies

In humans, the immune system is capable of creating millions of antibodies from which suitable antigen-binding antibodies are selected. Envious of this unsurpassed powerful system for making such exquisite binding sites, scientists have been investigating for decades various strategies to build tailor-made binding sites. The first breakthrough came in 1975 with the development of innovative hybridoma technology. Monoclonal antibody technology—hybridoma technology remains one of the core technologies of biotechnology, and hundreds of diagnostically relevant hybridoma have been developed to date.

Principle

Before going into the details of this technology, let us briefly discuss how antibodies are formed when a pathogen enters a host body. A pathogen (or an antigen), due to its large size, have several antigenic determinants. Each antigenic determinant stimulates a B cell of single specificity. Since there are many antigenic determinants on an antigen, several different B cells (having different specificity) are stimulated. Upon stimulation these B cells start secreting antibodies. Although antibodies are formed against a single antigen, they are directed toward different antigenic determinants of this antigen. Hence they have different microspecificities and affinity. These antibodies that are directed toward different antigenic determinants of one antigen and are the product of different clones of cells are called *polyclonal antibodies*. Polyclonal antibodies have different microspecificities and affinities toward an antigen, or more specifically, antigenic determinant. Polyclonal antibody preparations have been used for several decades to induce passive immunization against a

TABLE 25.1 Class and function of antibodies.

Class	Subclass	Light chain	Heavy chain	Structure	Important function
IgG	IgG1, IgG2, IgG3, IgG4	λ or κ	Γ		Complement activation, toxin neutralization, opsonization
IgA	IgA1, IgA2	λ or κ	A		Prevents viral/bacterial attachment on external surface
IgM	None	λ or κ	μ		Agglutination, killing microbes by complement activation
IgD	None	λ or κ	Δ		Lymphocyte activation
IgE	None	λ or κ	E		Counters parasitic infections, involved in allergic reactions

variety of pathogens and their toxins, including botulinum antitoxin, tetanus antitoxin, diphtheria antitoxin, and viper venom antisera (all of them raised in horse). These antibody preparations are used prophylactically or therapeutically for preventing or treating a number of diseases.

Polyclonal antibodies constitute a minor component in the complex mixture of serum proteins in which they are present. Therefore, polyclonal antibodies lack the degree of purity and specificity required for many current immunological techniques where increased assay sensitivity is required. Theoretically if a single clone of B cells can be isolated from an animal and grown in culture, it should provide a continuous source of antibody that has single specificity. Unfortunately B cells live for only a few days as they survive poorly once they are taken out of the body. The solution to this problem was invented by Köhler and Milstein in 1975. They fused a rodent's mortal antibody-producing cell with an immortal tumor cell (which does not have the ability to produce antibody) to form an immortal antibody-producing cell. These cells were then isolated and cultured to become a source of antibody of single specificity. These antibodies are directed toward a single antigenic determinant and secreted by a single clone of cells called monoclonal antibodies. Once a pure culture of immortal hybrid cells that secretes monoclonal antibodies has been isolated, it can be grown in a culture dish or grown as ascite tumors in a mouse; it could also be frozen and reused later (Fig. 25.1). These hybridomas will grow and divide and will mass produce antibodies of a single type (i.e., monoclonals).

Hybridoma technology and methodology

Let us discuss this technique in detail. A laboratory animal (such as the mouse) is immunized with an antigen. The spleen of this animal is removed and the antigen-stimulated B cells are then isolated. These antigen-stimulated B cells, which are mortal antibody-producing cells, are then fused with immortal nonantibody-producing cells. These nonantibody-producing immortal cells are special cells that have been developed by Milstein and Köhler. They are myeloma cells (cancerous B cells) that do not have the ability to produce antibodies, but can divide indefinitely. Isolated B cells are then fused with immortal myeloma cells (with the help of polyethylene glycol (PEG) or inactivated sendii virus) that fuse juxtaposed lipid bilayers of both the cells to form hybrid cells or hybridoma cells. These hybridoma cells are then grown in medium containing aminopterin and hypoxanthine. Myeloma cell lines lack the enzyme *hypoxanthine guanine phosphoribosyl transferase* (HGPRT), a key enzyme of purine synthesis by the salvage pathway. The other de novo pathway is functional for these cells. The de novo synthesis of nucleotides requires tetrahydrofolate, which is derived from dihydrofolate. This conversion of dihydrofolate to tetrahydrofolate (and hence nucleotide) is blocked by aminopterin. In a medium containing aminopterin, only those cells that have obtained the HGPRT gene from B cells (and hence have a functional salvage pathway) and an immortality trait from myeloma cells multiply, all others die. Hence only the cells that carry the immortality of myeloma cells and the antibody-producing capacity of B cells survive and all the other cells (including unfused cells) are eliminated. The multiplying and expanding cells are cloned by growing single cells as colonies. The antibodies produced by them are screened for antigen-binding activity. Monoclonal antibodies are produced in low yield in culture bottles ($\sim$20 μg/mL) but its yield can be increased by growing hybridoma cells in ascitis in the peritoneal cavity of mice ($\sim$4–5 mg/mL).

Important facts about polyclonal antibodies:

- Polyclonal antibodies recognize multiple epitopes on any one antigen, hence serum obtained after immunization with antigen will contain a heterogeneous mixture of antibodies of different affinities and microspecificities.
- They are constituted mainly of the IgG class of antibodies.
- They are inexpensive to produce as the technology/skills required for their production is much cheaper.
- The time required for producing polyclonals is usually short.

Advantages of using polyclonals:

- Polyclonals recognize multiple epitopes on any antigen.
- These antibodies bind target proteins on multiple epitopes and hence make the protein more visible. Multiple epitopes generally provide better detection.
- These are better suited for easy detection of denatured proteins as they are more tolerant of minor changes in the antigen (e.g., heterogeneity of glycosylation) or change in the exposure of antigenic determinants upon slight denaturation.
- Polyclonals are better suited for identifying proteins of high homology to the immunogen as there are chances that in a polyclonal mixture some antibodies will react with highly homologous, common antigenic determinants.
- Inexpensive to produce.
- Relatively quick to produce.

Disadvantages of using polyclonal antibodies:

- They are prone to batch variability (i.e., the antibody population produced against the same antigen in the same animal may not be exactly the same).

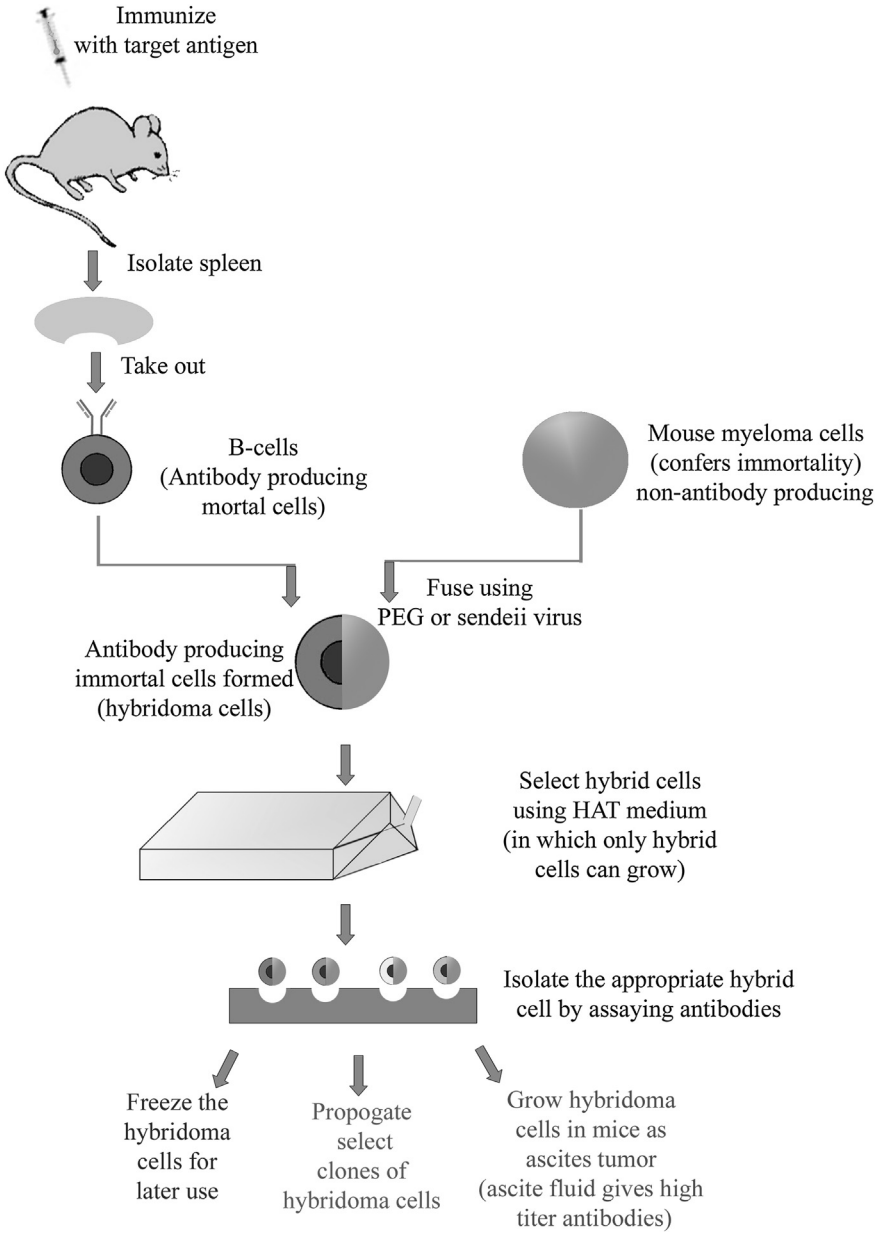


FIGURE 25.1 Production of monoclonal antibodies (a simplified overview).

- Highly purified antigens should be used otherwise it might lead to production of cross-reactive antibodies.
- Polyclonal antibodies are not useful for probing specific domains of antigens because the antiserum will contain a "mixture" of antibodies that will usually recognize many domains. Cross-reactivity is one of the major problems of polyclonal antibodies as they contain antibody subpopulations that recognize different antigenic determinants on the same antigen.

Important facts about monoclonal antibodies:

- They are directed against only one epitope (antigenic determinant) on an antigen.
- Monoclonals have only one antibody subtype. They all belong to one class and subclass and have the same affinity and specificity.
- High technology and training is required for producing and maintaining monoclonals.
- The time scale required for producing monoclonals is quite long.

Advantages of using monoclonal antibodies:

- Once hybridomas producing monoclonals are made, they are a constant and renewable source of antibodies and all batches of monoclonals are identical. Monoclonals are produced by a colony of cells derived from a single hybridoma. Each cell

contains identical genetic material and produces homogenous antibodies that recognize a single antigenic determinant.
- Monoclonals detect one epitope/antigenic determinant on any antigen. This offers the following advantages:
- Monoclonals specifically detect one target epitope, hence they are less likely to cross-react with other proteins.
- Because of their exquisite specificity, monoclonal antibodies are excellent as the primary antibody in an assay or for detecting antigens in tissue. They give significantly less background staining than polyclonal antibodies.
- Monoclonals give highly reproducible results.

Disadvantages of monoclonals:

- If an antigenic determinant is lost through chemical treatment of the antigen, monoclonals will not be able to detect it. Polyclonal antibodies can probably detect such an antigen as it contains antibodies that can bind more than one antigenic determinant.
- High cost and training are required for producing monoclonals.
- Trained personnels are required for the technology.

Application of monoclonal antibodies

Monoclonal antibody production technology has revolutionized the world of biotechnology. Continuous advances in recombinant DNA technology over the years have provided numerous ways to design monoclonals that are more robust and efficient as compared with their original murine versions. Monoclonal antibodies have been used as diagnostics, therapeutics, research reagents, and drug targeting agents for various diseases. Hybridoma technology, along with recombinant DNA technology, have successfully led to the development of chimeric antibodies, humanized antibodies, antibody constructs, and bispecific antibodies; all these have enormous potential for diagnostic and therapeutic uses.

With the development of monoclonal antibody production technology and the availability of tailor-made antibodies that have desirable qualities (such as exquisite specificity and high affinity), a perfect antibody reagent became available to scientists and clinicians. Monoclonals slowly started replacing polyclonals as the preferred reagent for both diagnostics and therapeutics.

Immunosorbent chromatography

The classic use of antibodies involved their use as solid-phase binding reagents. Use of monoclonal antibodies enabled binding and purification of desired proteins that displayed a single antigenic determinant. For example, there are four hormones—chorionic gonadotropin, lutropin, thyrotropin, and follitropin—that have two chains, α and β. The α chain is common to all and the β chain is unique to each one. When polyclonal antibodies are formed against any one of them, it cross-reacts with all of them, as they contain antibodies to the α chain (which is common to all). However when monoclonals are formed against the β chain of any hormone, it detects only that particular hormone and not the other three. Monoclonal antibodies can be coupled onto solid support, such as agarose gel or sephadex matrix, and can be used as an immunosorbent column that can be used to isolate a single protein when a mixture of proteins (such as serum) is passed through it. Since monoclonal antibodies are specific for a single epitope, usually a single protein gets bound to the solid-phase monoclonal antibodies and the rest of the unbound proteins get eluted and discarded. This target protein, which is now highly purified, is eluted and used. Use of monoclonals has allowed single-step purification of novel proteins such as human interferon and human clotting factor. Use of polyclonals for similar purification would have resulted in purification of several proteins (instead of one), as polyclonal antibodies would have cross-reacted with other proteins present in the mixture. Because of their avidity and specificity, monoclonal antibodies have become indispensable for protein characterization and purification.

Blood typing reagent

Monoclonals have been used as preferred blood typing agents for several decades. The large volume requirements for high-quality ABO and Rh typing reagents is now made possible with the availability of monoclonal antibodies. Superior detection reagents can now be prepared from blends of at least two antibodies (i.e., anti-A and anti-B monoclonals) to optimize the intensity of agglutination for slide tests, thus making them easily visible as well as to detect weaker subblood groups such as Ax and Bw. This was practically unthinkable with polyclonal antibodies.

More sensitive in RIA

Monoclonals are several-fold more sensitive than polyclonals in detecting an antigen in serum or other body fluids. Concentration of Transcortin (cortisol-binding protein) was determined by radioimmunoassay using both polyclonal and monoclonal antibodies. It was found that monoclonal antibodies detected cortisol at several hundred-fold lower concentrations as compared to polyclonal antibodies. Monoclonal antibodies

are used in radioimmunoassay to monitor the course of epithelial ovarian cancer.

Positive and negative selection of cells

Positive and negative selection of cells is one of the very important classical applications of monoclonal antibodies. In positive selection, monoclonals bind to the target cell (that we want to isolate) and separate it from rest of the contaminating cells. In negative selection all the unwanted/contaminating cells are bound to monoclonal antibodies and these antibody-coated cells are then removed from the system and leave only the cells of interest. A brief explanation follows for these cell selection processes.

Negative selection

CD4 + T cells can be isolated from blood by depletion of all non-T cells (B cells, macrophages, and natural killer cells); cells that remain in solution afterward are CD4 + T cells. Blood cells are incubated with a mixture of monoclonal antibodies to coat unwanted cells. The cells are targeted for removal by monoclonal antibodies that bind cell surface antigens present on contaminating cells and not on CD4 + cells such as CD14, CD16, CD36, and CD123. Moreover these antibodies are bound on dextran-coated magnetic beads. Subsequent exposure to a strong magnetic field removes the unwanted bead-coated cells, leaving behind the desired cell population.

Positive selection

Positive selection can be best illustrated with the following example. Metastatic carcinoma (cancer of epithelial origin) usually expresses cytokeratin 7/8 protein, which can be recognized by anticytokeratin antibody-1. Such malignant cells are detected by positive selection of such cells in peripheral blood, bone marrow, and lymphoid tissue of patients with metastatic carcinomas. Anticytokeratin antibody is coated on magnetic microbeads and incubated with disseminated cells of metastatic carcinomas. These antibodies bind and isolate/enrich tumor cells that display cytokeratin 7/8. It is reported that an enrichment of up to 10,000 times of desired cells can be achieved by this method. The desired cells are separated from the rest of the cells using a magnetic cell separator.

Diagnostic and therapeutic application

Ever since the first report of the successful use of monoclonal antibody for the treatment of human B-cell lymphoma in 1986 (muromonab-CD3/orthoclone OKT3), hundreds of monoclonal antibodies have been incorporated into standard therapeutic and diagnostic applications for various diseases. These monoclonal antibodies have proved to be an immensely useful scientific research and diagnostic tool.

Owing to their unrivalled specificity, monoclonal antibodies have also been in the spotlight as potential therapeutics for a variety of diseases, including cancer, infectious disease, cardiovascular disease, and transplantation disease. Major applications of monoclonal antibodies can be broadly divided into two main categories: (1) diagnostic and (2) therapeutic applications.

During the past several decades, monoclonals have found tremendous applications in diagnostics, therapeutics, and drug targeting. The diagnosis of any infectious disease often requires the demonstration of the pathogen or a specific antibody against the pathogen or its toxin. In some infectious diseases, such as reproductive or respiratory infections, the pathogen can be demonstrated throughout the course of infection, while for others, it is visible for a short period of time. Specific antibody-based tests identify the pathogens associated with the disease or toxins they secrete or any other protein they might inadvertently release. Monoclonal antibodies recognizing unique antigenic determinants on pathogens/toxins can be developed. MAb can recognize a single antigenic determinant characteristic of a pathogen. This restricted reactivity allows for precise identification of the organism of interest, which is the major advantage of monoclonals over polyclonal antisera. In the case of a pathogen having a subtype defined by unique antigenic differences, specific monoclonal antibodies can be used, whereas conventional antisera need laborious absorption to remove cross-reactive antibodies. Specific monoclonal antibodies of diagnostic value have been tailor-made and successfully used to detect pathogens such as Leishmania, Trichomonas, Trypanosoma, and several others. With enhanced sensitivity and exquisite specificity of monoclonal antibodies, diagnostic test systems that detect a number of animal viruses have also been developed. Monoclonal antibodies have revolutionized the laboratory diagnosis of various diseases and are employed as diagnostic reagent for biochemical analysis and as a tool for diagnostic imaging of diseases. Animal viruses such as HIV, herpes virus, rotavirus, rabies virus, human papilloma virus, and chikengunya virus are now being detected by immunodiagnostics. MAbs are successfully used in diagnostic imaging of cardiovascular diseases, cancers and sites of bacterial infection. MAbs tagged with radiolabel can be used to localize the atherosclerotic lesions by imaging technique.

Both the diagnostic and therapeutic properties of monoclonal antibodies exploit the unrivalled specificity of monoclonals. Once the correct target antigen has been identified, monoclonal antibodies can be raised against an antigen/antigenic determinant. This

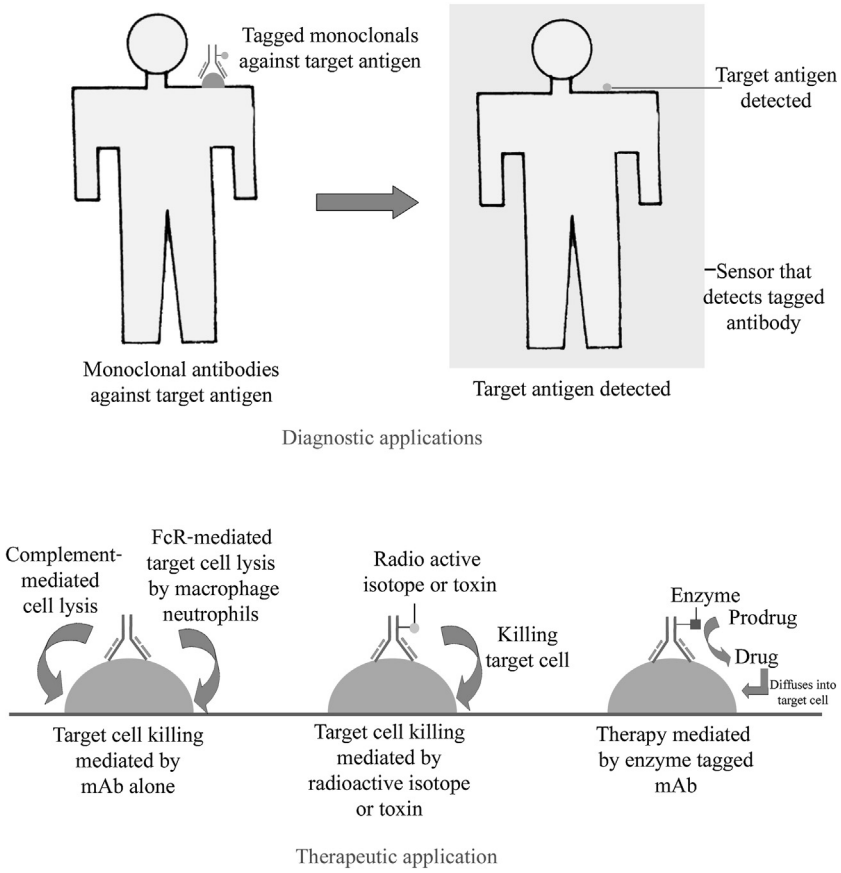


FIGURE 25.2 Major applications of monoclonal antibodies.

monoclonal antibody can be used for diagnostic or therapeutic purposes. Once injected this antibody will build to a specific target cell and not to the multitude of other cells available. Monoclonal antibodies can be used alone (naked monoclonal antibodies) to trigger the desired response or can be linked with toxic payloads such as radioisotopes or toxins or drugs/prodrugs (conjugated antibodies) to have the desired effect (Fig. 25.2).

The proper usage of monoclonal antibody preparations is dependent on the fact that the target cell expresses antigen/antigenic determinants that are unique to that particular cell and not to neighboring cells. This is common among cancerous cells. Transformation of a normal cell to a cancerous state is normally associated with expression of unique surface antigens on tumor cells that appear foreign to the host immune system. Such antigens (called tumor antigens) are usually not expressed on normal cells, or expressed at very low concentration. Initially lot of efforts was focused on generating antisera with some degree of specificity for cancerous cells. However despite repeated claims of success and ensuring controversies only two marker antigens were discovered: CEA (carcinoembryonic antigen for colon cancer) and α-fetoproteins (for hepatocellular cancer). Identification of such antigens set the stage for selective killing of cancerous cells thus revolutioning biological sciences. Targeting of such antigens by naked monoclonal antibodies can have the desired effect. In other words they can be used to kill the target cell by antibody-dependent cell-mediated cytotoxicity (ADCC). For example in B cell cancer of mouse, monoclonal antibodies targeted toward CD20 and CD10 antigens induced ADCC or complement-mediated cell lysis. Some antitumor effects of rituximab (a monoclonal antibody used in the treatment of non-Hodgkin's lymphoma) are used because of complement-mediated cell lysis. Another example of the use of naked monoclonal antibody is alemtuzumab (Campath), which is used to treat patients with chronic lymphocytic leukemia (CLL), cutaneous T-cell lymphoma (CTCL), and T-cell lymphoma. It is also administered in some other conditions for immunosuppression for bone marrow transplantation, kidney transplantation, and islet cell transplantation. Alemtuzumab binds to CD52 antigen, a protein present on the surface of mature B and T cells. After treatment with this antibody, these CD52-bearing cells are targeted for destruction.

Diagnostic applications in cancer: For diagnostic purposes, most monoclonal antibodies are linked with a gamma emitter so that radiation can pass through several layers of cells for detection purposes. The most

commonly used radioisotope is Technetium (T_c^{99m}). Some monoclonal antibodies commonly used for diagnostic purposes include CEA-Scan (mouse monoclonal antibodies that detect CEA), Indimacis 125 (mouse monoclonal antibodies that detect tumor antigen CA-125 expressed in ovarian adenocarcinoma), and several others that are currently used in diagnostic imaging. Addition of antibody construct such as minibody (engineered antibody fragment having V_L-V_H portion) that detects and images CEA, affibody (an antibody mimetic) that targets ERBB2, single-chain variable fragment (discussed later) that targets CC49 antigen, added to spectra of diagnostic antibodies. Immunoscintigraphy is a better diagnostic tool than the imaging techniques as it can differentiate between cancerous and noncancerous growth.

Therapeutic applications in cancer: As on May 1, 2016, FDA has approved 62 therapeutic antibodies till its first introduction in 1992. In 2015 a record high of 10 therapeutic antibodies were approved while in 2016 already saw an approval of four therapeutic antibodies. Monoclonal antibodies can also be used to deliver cytotoxic payloads directly to tumors by conjugating them with radioactive isotopes or toxic chemicals, drugs, or prodrugs (Table 25.2). Monoclonal antibodies attached to a radioactive substance, drug, or toxin are called conjugated monoclonal antibodies. The monoclonals are used as a targeting device to take these payloads directly to the cancer cells. Conjugated monoclonals circulate in the body until they can find and bind the target antigen. They then deliver the toxic payload where it is needed most. This lessens the damage to normal bystander cells in other (or neighboring) parts of the body.

Conjugated monoclonal antibodies can be divided into several different types depending on what they are linked:

- Monoclonals linked with chemotherapeutic drugs are referred to as chemo-labeled. For example, one of the chemo-labeled antibodies approved by the FDA is brentuximab vedotin (Adcetris), which targets the CD30 antigen found on B cells and T cells. The antibody is attached to a chemotherapy drug called monomethyl auristatin E (MMAE). It is used to treat Hodgkin's lymphoma and anaplastic large cell lymphoma.
- Monoclonals linked with radioisotopes are referred to as radiolabeled. Ibritumomab tiuxetan (Zevalin) and tositumomab (Bexxar) are examples of radiolabeled monoclonals. Both these antibodies are directed against the CD20 antigen, but they each have a different radioactive particle attached. They deliver their toxic payloads directly to cancerous B cells and are used in treating some types of non-Hodgkin's lymphoma.
- Monoclonals attached to toxins are called immunotoxins. Immunotoxins have cytotoxic poisons (toxins) attached to them, which makes them similar in many ways to chemo-labeled and radiolabelled monoclonal antibodies. Although antibodies attached to *Pseudomonas*, exotoxin-A, and ricin toxin are under clinical trials, till date no immunotoxins are approved to treat cancer, although several of them are being explored.

Radioisotopes commonly linked to monoclonal antibodies for cytotoxic effect include strong emitters such as iodine-131, 124, 125, and yttrium-90. I^{131} can be used for both radiotherapy and imaging. For selective delivery of drugs to target cells, several types of drugs and prodrugs have also been linked to monoclonal antibodies and targeted selectively to tumor sites. Chemotherapeutic drugs, such as adriamycin, auristatins, methotrexate, doxorubicin, and prodrugs such as etoposide, have been linked to mAb and selectively delivered to tumor sites with moderate success.

Choice of linkers

The large size of intact antibody–drug-linked conjugates prevents them from crossing plasma membranes (such as lysosomal membranes) once they are endocytosed. We know that drugs conjugated to antibodies can exert their specific action only after dissociation from the antibody and subsequent diffusion to their targets in the cytoplasm. Studies have revealed that, upon internalization, the conjugates were delivered to lysosomes, where their antibody moiety gets completely digested and releases protein-free derivatives of the cytotoxic drug.

With this in mind, scientists have designed various linkers that are relatively stable outside the cells (i.e., in the blood), but labile once the antibody–drug-linked conjugate is inside the cell. For example, anthracyclin drugs (such as doxorubicin and calicheamicin) were conjugated to antibodies via hydrazone-based linkers that are relatively stable at neutral pH of the blood, but are hydrolyzed in the acidic environment of phagolysosomes. Similarly another cytotoxic class of drug, toxoids, were coupled to antibodies via pH–labile ester bonds that break at low pH. Similar drugs, auristatins, were attached via disulfide linkage. These can get cleaved by disulfide exchange with thiols such as reduced glutathione, which is about 1000-fold higher concentration inside cells than in blood. Cytotoxic drugs such dolastatins and cyclopropylindole have

TABLE 25.2 Antibodies approved as drugs by the Food and Drug Administration (FDA).[a]

Product	Directed against (antigen)	Therapeutic area	Trade name
Murine monoclonal			
Muromonab-CD3	CD3	Arthritis inflammation	Orthoclone OKT3
Arcitumomab	Carcinoembryonic antigen	Metastatic colorectal cancer (detection)	CEA-Scan
Tositumomab	C2O	Oncological disease	Bexxar
Ibritumomab tiuxetan	C2O	Non-Hodgkin's lymphoma	Zevalin
Nivolumab	PD-1	Nonsmall cell lung cancer	OPDIVO
Imciromab	Cardiac myosin	Cardiovascular disease	Myoscint
Capromab pentetate	Tumor surface antigen PSMA	Prostate adenocarcinoma (detection)	Prostascint
Secukinumab	IL-17A	Crohn disease, infections, tuberculosis	Cosentyx
Dinutuximab	GD2	Neuroblastoma	Unituxin
Chimeric			
Basiliximab	C25 (IL-2-receptor)	Prophylaxis of acute organ rejection	Simulect
Infliximab	TNF-α	Crohn's disease	Remicade
Rituximab	CD20	Non-Hodgkin's lymphoma	Rituxan
Antibody fragments			
Nofetumomab (murine Fab)	Carcinoma associated antigen	Small-cell lung cancer (detection)	Verluma
Igovomab (murine $(Fab)_2$ fragment)	Tumor antigen−CA	Ovarian carcinoma	Indimacis 125
Sulesomab (murine Fab)	Granulocyte antigen NCA-90	Osteomyelitis (detection)	Leukoscan
Abciximab (chimeric Fab)	Platelet surface receptor	Prevention of blood clot	Reopro
Humanized			
Daclizumab	CD25 (IL-2-receptor)	Prevention of acute kidney transplant rejection	Zenapax
Mepolizumab	IL-5	Severe asthma	Nucala
Gemtuzuumab	CD 33	Acute myeloid leukemia	Mylotarg
Trastuzumab	Human epidermal growth factor receptor (Her-2)	Metastatic breast cancer	Herceptin
Ranibizumab	Vascular endothelial growth factor (VEGF) receptor	Eye diseases	Lucentis
Palivizumab	Respiratory syncytial virus F Protein	Respiratory tract disease	Synagis
Human phage display/synthetic antibody			
Adalimunab	TNF-α	Immune disorders, Crohn's disease	Humira
Xenomouse			
Panitumumab	Epidermal growth factor receptor (EGFR)	Metastatic colorectal cancer	Vectibix

[a]*Only a few representative examples are given.*

been conjugated via peptide linkers containing a valine−lysine sequence. These are stable in blood but get degraded in lysosomes, presumably by enzymes such as cathepsin B or β-glucuronidase.

Chimeric and humanized antibodies

Ideally infusion of murine monoclonal antibodies for therapeutic purposes should generate an immediate immune response. These monoclonals have been

designed for specific antigens on target cells. Surprisingly initial clinical trials involving administration of monoclonal antibodies into human subjects proved quite disappointing. There were three main reasons for this:

- Monoclonal antibodies were initially produced in mouse, and hence these antibodies were treated as foreign bodies by the human system. Introduction of these murine monoclonals elicited a strong immune response that entailed cleaning of monoclonals before they reached the target site. Such an immune response that occurs in the human body after the administration of mouse antibodies is also known as the human antimouse antibody (HAMA) response. A single injection of murine monoclonal elicited an immune response in about 55%–80% of all patients; and human antimouse antibodies are usually detected within 2 weeks of antibody administration. Such an immune response in human subjects usually destroys subsequent administration of therapeutic monoclonals, severely limiting its efficacy.
- The human effector functions (complement activation and binding to Fc receptors) were poorly recruited by the Fc regions of murine monoclonal antibodies.
- Murine monoclonals displayed a shorter half-life in serum when administered to humans.

Methodology

Genetic engineering partly solved these problems by providing an alternative route. Since almost all the above problems were associated with Fc regions of murine monoclonals, the mouse Fc region was replaced by their human counterparts and its Fab region (which recognizes antigen) was left intact. The strategy entailed production of a new form of monoclonal antibody consisting of mouse variable (Fab) regions and human constant (Fc) regions. Such monoclonals are referred to as *chimeric antibodies*. Briefly production of chimeric antibodies is similar to production of monoclonals, as both involve fusion of mouse antibody-producing cells with immortal myeloma cells that cannot produce antibody. For synthesizing chimeric antibodies, mouse B-cell DNA is isolated and its constant region is replaced by its human counterpart. The mouse variable region is left intact. This chimeric mouse–human gene of the antibody is then introduced into mouse myeloma cell lines for the production of chimeric monoclonal antibodies. Such chimeric antibodies have been shown to be less immunogenic (chimeric antibodies were immunogenic in about 1% of human patients), have an extended serum half-life (about 250 hours as compared to 35 hours for murine monoclonals), and exhibit increased Fc-mediated effector functions as chimeric antibodies carry a human Fc region. It has been estimated that the constant region contributes 90% of the immunogenicity of chimeric antibodies, with the variable region contributing the remaining 10%. Rituximab is an example of chimeric monoclonal antibody used in the treatment of patients with B-cell varieties of non-Hodgkins lymphoma and in combination with methotrexate, used to treat symptoms of adult rheumatoid arthritis.

The entire Fab region of an antibody is not essential for binding of the antigen. Regions within the variable region of light and heavy chains that bind the antigenic determinant are referred to as *complementarily determining regions (CDRs)* or *hypervariable* regions. Overall, each antigen-binding site (one in each arm) has six CDRs, three from the light chain and three from the heavy chains. Further humanization of the chimeric antibody was undertaken to reduce the immunogenicity of monoclonals to the minimum. This involves cutting out the nucleotide sequences of these six CDRs from the mouse antibody and splicing them into human antibody genes (i.e., in both light and heavy chains). Such hybrid antibodies that carry only murine CDRs in an otherwise human antibody are referred to as *humanized antibodies*. This strategy, termed CDR grafting, decreases the amount of murine sequence in a monoclonal from 30% (in chimeric antibody) to 3% (in humanized antibody), and greatly reduces the risk of HAMA. Humanized antibody is a mouse–human chimera encoded by a recombinant gene whose antigen specificity is determined by variable region derived from mouse DNA and its isotype is derived from human DNA. Humanization has overcome major hurdles that limited the therapeutic effectiveness of murine monoclonals. Humanized monoclonal anti-IgE can be used in immunotherapy to control type-I hypersensitivity. Bevacizumab is a humanized monoclonal antibody approved for the treatment of metastatic colorectal and nonsmall cell lung carcinoma.

Phage display technology: screening recombinant antibody libraries

A phage display antibody library is a collection of bacteriophage particles that display antibody (fragments) protruding from their surface. Antibody fragments are cloned into bacteriophage such that antigen-binding variable regions are displayed on the phage surface, providing a phenotype that can be used to select the phage as well as identify the genotype of recombinant antibody clone. The phage particles that are normally chosen are M13 or fd phage vector. Both vectors are bacteriophages and contain outer coats made up of protein. The antibody (fragments) genes are so ligated into the phage DNA that they are expressed on the coat proteins. The antibody fragment is ligated/fused with gene III protein

of M13 (which is expressed as five copies on the surface of phage) or gene III protein of fd phage virion (which is expressed about four copies per virion). The phage particles are then incubated with their host *Escherichia coli*, which facilitates phage replication. Bacteriophage DNA then directs the synthesis of fusion gene products containing the protein of interest in the coat. The entire phage library can then be screened in order to identify the antibody fragment of interest as described below.

Methodology

In 1985 Smith described for the first time that a foreign DNA sequence could be cloned into filamentous bacteriophage and that such cloned sequence can be expressed on the surface of the phage particle. To clone antibody fragments, V_H and V_L mRNA are isolated from hybridoma, spleen, or lymph cells. mRNA is reverse transcribed into cDNA and amplified by polymerase chain reaction. V_H and V_L genes are then fused with a short linker gene that codes for $(Gly_4Ser)_3$ sequence. This V_H−linker−V_L gene construct codes for an antigen-binding fragment that is referred to as a single-chain variable fragment (scFv). This gene construct is then fused with gene III protein of M13 or fd phage virion. The phages are then incubated (transformed) with *E. coli*. Phage particles transform *E. coli* and replicate inside the bacterial cell. The fusion gene product is expressed on surface coat proteins of newly formed phage particles.

The phage particles are then screened to identify those phages that code for the protein of interest. This selection process is called *biopanning* or *affinity screening*. In biopanning, the library of phages displaying the antibody fragments (or its corresponding ligand) is passed over immobilized antigen (or any other target molecule) bound to a column/bead/membrane. All the phages that display desired antibody fragments are retained on the solid support. The bound phage is then subsequently eluted by changing the pH of the elution buffer or by adding competing ligands in the eluting buffer (Fig. 25.3). The eluted phage particle can then be incubated again with *E. coli* and this process may be repeated to isolate those antibody fragments that bind with highest specificity or affinity. In 2002, adalimumab, a phage display-derived antibody, became the first marketed fully human monoclonal antibody product. This fully human antibody was not rapidly cleared by human patients' immune systems and displayed a half-life of 2−3 weeks.

Recombinant antibody library can be of two types: (1) immune antibody libraries, and (2) nonimmune antibody libraries. Immune libraries are those that

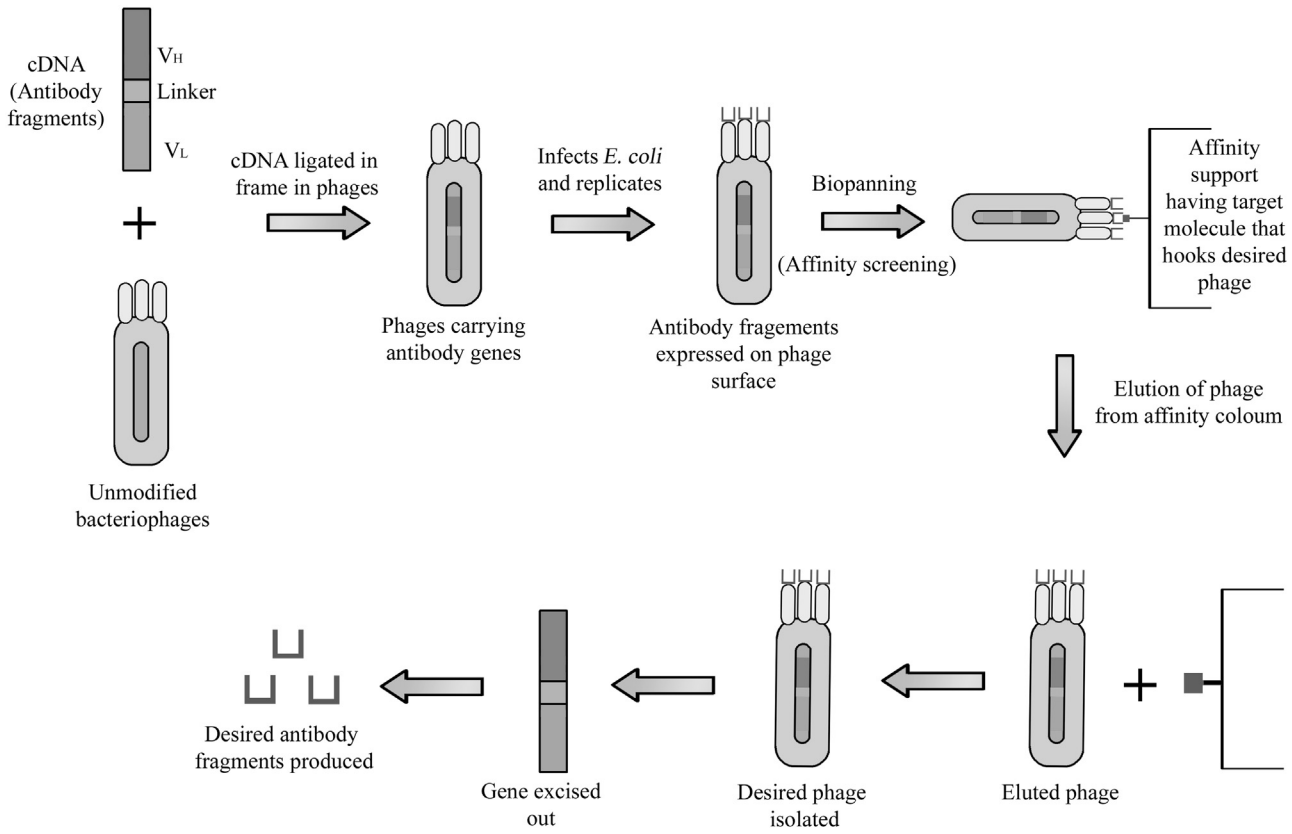


FIGURE 25.3 Phage display technology for antibody screening.

contain genes/cDNA coding for antibody/antigen-binding fragment derived from B-lymphocytes that have been previously immunized with target antigen. Such libraries have a V_H/V_L gene pool cloned from B cell obtained from immunized animals or naturally infected or immunized humans. Nonimmune antibody libraries are produced just as immune antibody libraries but using B cells from nonimmunized donors. These include healthy unimmunized humans or animals not immunized with target antigen. Needless to mention, nonimmune libraries will generate a small number of positive clones for any antigen screened.

Antibody constructs

In addition to single-chain variable fragment (scFv), other commonly used antibody constructs include disulfide-linked variable fragment (dsFv), antigen-binding fragment (Fab), diabody, and bispecific antibodies. In dsFv, V_H, and V_L chains are expressed as separate polypeptides in bacteria. These two chains are covalently assembled by engineering an interchain disulfide bond to the dsFv antibody. Antigen-binding fragments that do not have this specially constructed interchain disulfide bond have also been produced (called Fv fragments), but were found to be prone to aggregation and dissociation.

Fab antibody fragment constructs are similar to dsFv fragments but have an extra constant domain in both the light and heavy chains. Fabs consist of two polypeptide chains expressed in bacteria. One contains the polypeptide-coding region of the light chain variable domain plus the constant domain, and the other contains the heavy chain variable domain plus one constant domain. Just as in dsFv, light and heavy chains are linked by a disulfide bond. It should be noted that scFv contains two disulfide bonds whereas Fabs contain five disulfide bonds. Since Fabs are relatively large constructs and they have a large interface of light chain and heavy chain along with the presence of disulfide bonds, they show increased stability. Even though Fabs require the association of two chains, they often occur quite efficiently in bacteria.

Recombinant antibody fragments such as scFv, dsFv, and Fab can be expressed efficiently in microbes, particularly in *E. coli*. Since disulfide bonds form in an oxidizing environment and proteins are synthesized in cytosol (which is highly reducing), these proteins are exported from the cytosol into the periplasmic space. The periplasmic space is the compartment between inner and outer membranes of the bacterial cell. This space is highly oxidizing in nature. Export into this space is easily accomplished by attaching one of the well-characterized leader peptides to the N-terminus of the antibody fragment. Proteins expressed in the periplasm can be recovered from osmotic shock or from total cell lysate. Moreover prolonged high-level expression of antibodies at 37°C renders the outer membrane of *E. coli* permeable, and proteins and protein fragments can be recovered from the culture media.

Diabody

Recombinant DNA technology was able to generate small bivalent antibody constructs (diabody) consisting of two V_H and two V_L domains. In a diabody, each V_L domain is connected with one V_H domain in scFv by a short linker. This construct is similar to scFv fragments with the difference that the peptide linker is shorter (range from 3 amino acids to $\sim$10 amino acids) than that is present in the scFv fragment. The shortening of the linker does not allow bending of the polypeptide chain and formation of the antigen-binding site from the V_L and V_H domain from the same "scFv" fragments. Hence two such scFv chains associate or dimerize to form two antigen-binding sites. Each antigen-binding site of the diabody is constructed by a V_H domain of one "scFv" fragment and a V_L domain of the other "scFv" fragment. The resulting diabody has two antigen-binding sites pointing in opposite directions. If two of the same scFv fragments are allowed to dimerize, a bivalent antibody construct results, which is similar to the antibody but lacks an Fc region. If two different "scFv" fragments are used, the resulting diabody will form two different antigen-binding sites simultaneously. Such diabodies are referred to as bispecific antibodies or bispecific diabodies (e.g., diabody−vcF4 that is anti-CD30 and is conjugated with the potent antitumor drug monomethyl auristatin). Bivalent and bispecific antibodies have immense potential for practical application such as in immunodiagnosis and therapy.

Human antibodies from transgenic mouse

Advances in molecular biology involving the manipulation of gene sequences in vitro and the expressions of these manipulated sequences in bacterial, fungal, and mammalian cell culture provided methods for reengineering mouse antibodies to partially replace the rodent antibody sequence with a functionally equivalent human amino acid sequence, thus reducing the overall immunogenicity without destroying the recognition properties of the original antibody. These methods for reengineering rodent monoclonal antibodies via chimerization and humanization proved to be quite successful. Shortly after that new technologies were developed that allowed the

production of novel monoclonal antibodies from human immunoglobulin gene sequences. These new technologies can be broadly divided into two categories (1) reassembling recombinant human antibodies from specially constructed gene libraries such as phage display antibody libraries and (2) synthesizing human antibodies in transgenic animals comprising a human germline configuration of immunoglobulin genes.

Methodology

In 1985 Alt et al. for the first time suggested that transgenic technology could be useful for generating new human monoclonals starting from an un-rearranged, germline-configuration transgene. In 1994 two groups reported generation of mice that carried a human immunoglobulin germline sequence and disrupted mouse immunoglobulin genes. They disrupted the endogenous mouse heavy and light chain genes and introduced a human transgene encoding heavy and light chains into a mouse embryonic stem cell. The egg was kept in culture during the first few days of embryonic development. The engineered embryos were then implanted into the womb of a female mouse. The implanted embryos developed normal (but transgenic) animals. Once born, those animals that carry the transgene and maintain it stably were called founder animals.

Founder mice (carrying human antibody transgene) appeared to carry out VDJ recombination, class switching, and somatic hypermutation of the human germline antibody genes in a normal fashion, thus producing high-affinity antibodies with completely human sequences. These animals expressed both IgM and IgG. Despite the fact that human immunoglobulin transgenic mice expressed hybrid B-cell receptors (i.e., human immunoglobulin, mouse Ig, mouse Ig, and other signaling molecules), B cells developed and matured as normal B cells. The transgenic mice could be injected with the desired antigen and the resulting antibody isolated. Moreover monoclonal antibodies could also be prepared by standard hybridoma technology. Half-lives of human antibodies recovered from such transgenic mice were similar to antibodies recovered from humans. For example, in monkeys, the half-life of transgenic mouse-derived human antibodies was found to be 9.5−11 days, similar to the half-life of human IgG (9.6 days), and different from murine IgG, which had a half-life of 1.9 days. In 2006, panitumumab (which inhibits epidermal growth factor receptor, EGFR) became the first marketed monoclonal antibody from a transgenic mouse platform. Since then, around 70 different therapeutic drugs derived from phage display or transgenic mouse platforms have entered human clinical testing.

Bispecific antibodies

Bispecific antibodies are those antibodies or antibody constructs that have dual specificity in their binding arms. Naturally occurring antibodies are monospecific and have the same specificity in both their antigen-binding arms. Bispecific antibodies usually do not occur in nature, but are constructed by recombinant DNA or cell-fusion technologies. The classic approach to constructing and producing bispecific antibodies is Quadroma technology. This technology involves fusion of two hybridoma cell lines that produce the desired monospecific antibodies. These two hybridoma cell lines are fused to generate a Quadroma cell that expresses monoclonal antibodies with the desired specificities of the bispecific antibody. Bispecific antibodies carry two different antigen-binding sites. The specificity of one arm is different from the other. Such pairing of two different halves of the antibody has one other effect on antibody structure. The Fc portion of the bispecific antibody does not resemble any of the constituting monospecific antibodies (i.e., it is different from Fc portions of either of the parent monospecific antibodies). The "new" Fc portion may have desired properties or may generate an undesirable effect. To avoid an undesired effect, bispecific Fab fragments (or specifically $F(ab)'_2$) can be prepared by enzymatic digestion, or, better still, by recombinant DNA technology.

The vast majority of bispecific antibodies were designed to redirect cytotoxic effector cells (T_c cells, NK cells, neutrophils) toward targets cells (such as tumor cells). These bispecific antibodies were developed in which one arm of the bispecific antibodies recognized target cells such as tumor cells and the other arm activated effector cells.

This resulted in bringing tumor cells in close proximity to activated effector cells that ultimately caused elimination of tumor cells causing cancer. A number of bispecific antibody constructs are currently being made and tested in animal models or are undergoing clinical trials. The best characterized molecules found on effector cells against which bispecific antibodies have been made include molecules such as CD64 (IgG receptor) and CD89 (IgG receptor), which are present on neutrophils, monocytes, and macrophages; CD16 (IgG receptor) on NK cells; and CD3 (signal transduction unit), which is present on T cells. One arm of the bispecific antibody binds these molecules on effector cells and the other arm binds to the tumor cell triggering lysis of target tumor cells. A recombinant (CD3 × CD19) bispecific antibody was highly effective against CD19-positive lymphoma cells in clinical trials. Another CD16 × CD30 bispecific antibody is also being explored for Hodgkin's lymphomas in animal models. Catumaxomab, a mouse bispecific against CD3X epithelial cell adhesion molecule

(EPCAM) has recently been approved in European unions for EPCAM positive tumors.

Bispecific antibodies can also be used for specific targeting of tumor cells by toxins, chemotherapeutic drugs, or radioisotopes, as shown by in vitro and in vivo studies. Therapeutics based on bispecific antibodies have not yet yielded the anticipated clinical success. However bispecific formats are still being actively pursued in preclinical and early clinical development of bispecific antibodies. The critical selection of the antigen for tumor targeting, as well as the complexity and cost of producing bispecific antibodies, are the main issues that need to be explored before the full potential of bispecific therapeutics can be realized.

Ethical issues

Antibodies have been the paradigm of binding proteins with desired specificities for more than one century, and during the past decade their recombinant or humanized versions have entered clinical applications with remarkable success. Today more than 20 different antibodies have been approved in Europe and the United States, providing considerable market potential for the pharmaceutical and biotech industry. There are several reasons for the remarkable success of antibodies as a class of biological drugs. First they can rather easily be generated against a wide range of target molecules by classical immunization of animals followed by protocols for monoclonal antibody preparation or more recently by in vitro selection from cloned or synthetic gene libraries. However the use of mice to produce monoclonal antibodies has been marred with controversy. The production of monoclonal antibodies using animals (such as mice) entails procedures that have the potential to cause considerable pain and distress to animals. Not only this use of monoclonal antibodies, but their derived products have perils as these products are of animal origin and are therefore recognized and dealt with as foreign entities in human trials. Some important ethical issues related to these are discussed below.

Risk to animals:

- Monoclonal production involves the use of mice. These mice are used to produce both antibodies in abdominal fluid (ascites) and to grow abdominal tumor cells (hybridomas). Both these procedures inflict pain, discomfort, abdominal discomfort, and indigestion in animals. The removal of ascites fluid may cause shock in the animals, which results from rapid fluid loss.
- In order to achieve an enhanced immune response in the mouse, adjuvants are used. Adjuvants release the antigen into the mouse over a long period of time, which often results in painful lesions at the site of injection. The use of adjuvants, such as Freund's Complete Adjuvant (FCA) or Freund's Incomplete Adjuvant (IFA), is quite common. Freund's adjuvant creates a severe inflammatory response in animals that enhances the immune response; however it also causes tissue necrosis, ulceration, self-trauma, hunching, decreased appetite, and weight loss. Freund's Complete Adjuvant has actually been banned in the Netherlands and the United Kingdom due to these reasons.
- Pristane is a chemical that is injected into a mouse's peritoneal cavity; it creates an environment conducive to rapid growth of fluid-producing tumors. It induces granulomatous reactions and prevents peritoneal fluid drainage. Consequently pristane increases monoclonal antibody-rich fluid yield. However its usage has been associated with weight loss, hunched appearance, and inactivity and distress in mice.
- During the production of monoclonals, the mouse's spleen is removed to provide a source of antibody-producing cells. This involves killing the animal that has already undergone quite a lot of discomfort.

Risks to participants in human trials:

Apart from the ethical issues involving animals, there are concerns regarding the safety and efficacy of monoclonals and their derived products that are administered as drugs in "First in" human trials. In March 2006 six healthy volunteers took part in a Phase I clinical study conducted for a CD28 super-agonist antibody (TGN1412) in London. Even though the dose of antibody was 500 times smaller than that found safe in animal studies, within minutes all six volunteers suffered multiple organ failure, probably as a result of CD28 antibody-induced activation and proliferation of regulatory $CD4^+$, $CD25^+$, and T cells (that play an important role in autoimmune diseases) that started attacking the body tissues. All the volunteers survived, but it raised serious issues about the conduct of drug trials.

Armed with current scientific knowledge about monoclonal antibodies and antibody constructs, scientists and ethical committees around the globe must tackle the issues raised above and make informed decisions about their production and use. As a society, we must balance the advantages that a new medicine brings with the dangers that its production and use might induce. Only then can we make balanced decisions at an individual, local, national, and global level about the ethical use of pharmaceutical drugs, including monoclonal antibodies and their constructs.

Clinical corelation of the chapter content

1. The use of antibodies a "magic bullets" to treat the disease was first Antibody–drug conjugates for cancer therapy proposed more than 100 years ago by founders of chemotherapy Paul Ehrlich. Antibody–drug conjugates (ADCs) are monoclonal antibody conjugated to cytotoxic agents. The use of antibodies that are specific to tumor cell surface protein, and, thus have tumor specificity and potency not achievable with traditional drug.

   ADCs are tripartite drugs comprising a tumor-specific monoclonal antibody conjugated to a potent cytotoxin via a stable linker. Monoclonal antibody exert their therapeutic effect by tumor-specific cell surface antigen, once bound they kill tumor cell by one or more following mechanisms:
   - Abrogation of tumor cell signaling, resulting in apoptosis.
   - Complement-dependent cell-mediated cytotoxicity (CDCC).
   - Exertion of inhibitory effect on tumor vasculative and stroma.

List of some ADCs in the market

ADC	Sponsor	Indication	Target antigen	Antibody type	Linker
Anti-PSMA ADC	Progenics	Prostate cancer	PSMA	Human IgG1	Cathepsin cleavable valine-citrulline
Lorvotuzumab mertansine (IMGN-901)	Immunogen	Small-cell lung cancer	CD56	Humanized IgG1	Disulfide SPP
Labetuzumab-SN-38	Immunomedics	Colorectal cancer	CEA (also known as CD66e)	Humanized IgG1	Lysine

2. Use of monoclonal antibody in demyelinating disease.

   Monoclonal antibody therapy is a new treatment strategy for many types of diseases including cancers and autoimmune diseases, realizing its high efficiency and tolerability. Several monoclonal antibodies have been suggested to decrease the incidence of clinical relapse and disease activity in multiple sclerosis (MS) and neuromyelitis optica (NMO).

   In MS anti-a4 integrin (natalizumab), anti-CD52 (alemtuzumab) and anti-CD25 (daclizumab) have been show to effectively reduce relapses as approved by FDA.

   While in NMO, rituximab, anticomplement C5 (eculizumab), and anti-IL-6 receptor (tocilizumab) have been suggested to be effective and some of these are now under clinical trials.

3. Efficacy of monoclonal antibody therapy for high-risk neuroblastoma.

   High-risk neuroblastoma (HRN) is primarily a childhood cancer with 5 years survival rate of 40%–50%. There are number of treatments available to treat HRN including induction chemotherapy, surgery, myeloablative chemotherapy with autologous hematopoietic stem cell transplant, and radiation therapy.

   Dinutuximab monoclonal antibody is the first anti-GD2 approved in combination with GM-CSF, IL-2, and RA for maintenance treatment of pediatric patients with HRN.
4. Monoclonal antibody for non-Hodgkin lymphoma.

Naratuximab emtansine also known as (IMGN529) and debio 1562 is an antibody–drug conjugated to the maytansine-derived microtubule disruptor, DM1 that contains humanized antibody IgG1 antibody against cell surface antigen CD37 found on malignant B-cell. IMGN529 has shown promising preclinical and clinical activity in non-Hodgkin lymphoma including diffuse B-cell lymphoma.

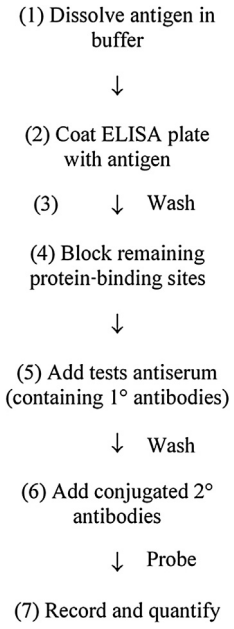
Translational significance

The effective use of accumulated knowledge across various antibody development programs remains a major challenge. Translation of these strategies for monoclonal antibodies that takes science from the bench to the bedside also remains a major challenge. This conversion requires balanced integration of relevant knowledge with respect to target antigen properties, antibody design criteria, their in vivo and in vitro efficacies, and a conscience as to how it can be used for betterment of the human population. Monoclonal and polyclonal antibodies have a broad spectrum of effects and uses in the everyday lives of citizens and scientist across the globe. Table 25.3 lists some important immunodiagnostic kits that are being routinely used today. ELISA is one of the most common immunotechniques employed in these kits and is briefly discussed below (Flow Chart 25.1):

1. Dissolve the antigen in an appropriate buffer. The range is usually 1–10 μg/mL, although it has to be optimized for each antigen.
2. The antigen is then coated or immobilized onto a microtiter plate by incubating the antigen overnight in the wells of the plate.
3. Washing is performed to remove unbound antigen.
4. Once unbound antigen is removed, a concentrated solution of noninteracting proteins, such as bovine serum albumin (BSA) or casein, is added to all wells. This step is known as blocking because these

TABLE 25.3 Some commonly used immunodiagnostic kits.

Diagnostic kit purpose	Immune reaction/immuno techniques employed	Antibody type used	Commercial product available
Blood group typing	Agglutination	Polyclonal/monoclonal	Alba sera, Alba clone
Detection of			
• Analyte	Immunoaffinity	Polyclonal/monoclonal	Randox colum for detecting salbutamol
• Toxins	ELISA	Polyclonal	Alfastar for detecting alfatoxins
• Virus	Immunoaffinity capture	Polyclonal	Abcap antibodies to detect enteric virus in water
• Protozoa	ELISA	Monoclonal	Epituub *G, Lambilia* detection kit
• Bacteria	Agglutination	Monoclonal	Bengals screen kit to detects *Vibrio cholerae*
Cell isolation	Negative selection of cells	Polyclonal	$CD4^+$ isolation kit (easy sep kit)
	Positive selection of cells	Polyclonal	Isolation of any blood cells(easy sep kit)
Autophagy	Flow cytometry	Monoclonal	Flow cellect autophagy
Cytokine	Antibody array	Monoclonal	Detection of cytokine at pg level (Abcam arrays)



FLOW CHART 25.1 Enzyme-linked immunosorbent assay (ELISA).

proteins block nonspecific adsorption of other proteins to the binding sites on the plate.

5. This is followed by application of primary (1°) antibody onto wells of the ELISA plate. Primary antibody is directed toward the antigen that is to be detected. After this step, unbound antibodies are removed by washing.
6. The microtiter plate is then incubated with secondary (2°) antibody, which can be monoclonal or polyclonal in nature and has a probe attached to it. The probe can be an enzyme probe, florescent probe, radioactive probe, etc. The secondary antibody binds the primary antibodies and helps to amplify the signal obtained by the reaction between the antigen and primary antibodies. The plate is then washed to remove excess unbound secondary antibody–probe conjugates from the plate.
7. The next step is dependent on the probe attached to the secondary antibody. If the secondary antibody is attached with an enzyme probe, then the membrane is incubated with the appropriate enzyme substrate to produce the colored reaction product, which can be detected spectrophotometrically. If however the probe is fluorescent or radioactive, then an appropriate imaging and documentation system is required.

World Wide Web resources

In this technological age, there is a flood of information from the Internet. These web resources have made huge amounts of information available at the click of a button. However such information has its own pros and cons. Listed below are a few important ones:

Pros

- The information is available to anyone, anytime.
- Quick access: Just go to Google and click.
- Sites can provide additional information as links on the same topic on the same site.

- The site can be frequently updated (even daily) to provide the latest information.
- The author does not need to be present to provide someone information.
- Information is easy to gather and print.
- It is easy to use the information to create your own resources.

Cons

- A computer with Internet access is required. This is still not common in third world countries.
- Sites may not be authentic. Try using sites such as PubMed (http://www.ncbi.nlm.nih.gov/pubmed), or sites that end with.edu or.gov, or other reputed pages. These should provide reliable information.
- Sometimes scientific information is available on a paid site, which is usually expensive.
- There is no opportunity to ask questions or have things explained in greater detail, though some forums have provisions to post questions.
- Last but not the least, a computer may crash or a site may be removed from the Internet at anytime by its creator.
- Note: Avoid using personal pages to gather scientific information unless they are reputed. Personal pages may provide inaccurate information, as anyone can update any fact on a personal page.

Websites

1. Mike Clark's homepage: www.path.cam.ac.uk. This is a wonderful site for antibody structure, function, and different uses of antibodies.
2. Antibody resource page: http://www.antibodyresource.com. Battery of easy resource sites ranging from structure to therapeutic aspects of antibodies to immunology journals.

Further reading

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Glossary

Antigen (immunogen) Foreign molecule (usually) that can elicit an immune response. Protein and carbohydrate are usually antigenic, while lipids and DNA are weakly immunogenic/antigenic or nonimmunogenic (e.g., bovine serum albumin, capsular polysaccharide of bacteria).

Bispecific Antibodies Antibody or antibody construct that has a dual specificity in the binding arms. Bispecific antibodies usually do not occur in nature, but are constructed by recombinant DNA or cell-fusion technologies (e.g., recombinant (CD3 × CD19) bispecific antibody).

Monoclonal Antibodies Antibody directed against single antigenic determinant and produced by a single clone of cells (e.g., CEA-Scan, a mouse monoclonal antibody that detect human carcinoembryonic antigen).

Diabody An antibody generated by a recombinant DNA technology antibody construct consisting of two variable heavy chain region and two variable light chain region domains (e.g., C6.5, which target human growth receptor 2).

Epitopes (Antigenic Determinants) Small portions of antigens that provoke an immune response.

Humanized Antibodies Hybrid antibodies that carry only murine Complementary Determining Regions (CDRs) in an otherwise human antibody are referred to as humanized antibodies (e.g., Trastuzumab, an antibody that is directed against human epidermal growth factor, Her-2).

Hypervariable region or Complementary Determining Regions (CDRs) The regions within the variable regions of light and heavy chains that bind antigenic determinants.

Phage Display Technology A biotechnological technique that allows expression of exogenous peptides on the surface of filamentous bacteriophage (e.g. adalimumab, a phage display-derived antibody).

Polyclonal Antibodies Antibodies that are directed toward different antigenic determinants of one antigen and are product of different clones of cells. Although antibodies are formed against a single antigen, they are directed toward different antigenic determinants of this antigen.

Abbreviations

ADCC	antibody-dependent cell-mediated cytotoxicity
CDR	complementary determining regions
CEA	carcinoembryonic antigen
Dsfv	Disulfide-linked variable fragment
EGFR	epidermal growth factor receptor
Fab	antigen-binding fragment
HAMA	human antimouse antibody
HGPRT	hypoxanthine guanine phosphoribosyl transferase
MHC	major histocompatibility complex
NK Cell	natural killer cell
PAMPs	pathogen-associated molecular patterns
PRR	pattern recognition receptors
Scfv	single-chain variable fragment

Long answer questions

1. What are monoclonal antibodies? How are they different from polyclonal antibodies? Discuss some of their important diagnostic and therapeutic applications.
2. What are bispecific antibodies? How are they made? Discuss their important potential applications in medical biology.
3. What is phage display technology? Discuss, how can it be used to screen recombinant antibody fragments via a biopanning method.
4. Briefly discuss recombinant antibody fragments such as scFv, dsFv, and Fab. How are they different from diabodies?
5. Differentiate between chimeric and humanized antibodies. What are their advantages over murine monoclonal antibodies?

Short answer questions

1. Human antimouse antibody (HAMA) response that occurs in human after administration of murine antibodies is minimal in which type of monoclonal antibody? Why?
2. What is the role of aminopterin in HAT medium used to screen hybridoma cells?
3. What is the difference between scFv and a diabody?
4. Can a diabody act as a bispecific antibody?

5. List two main differences between monoclonal and polyclonal antibodies.

Answers to short answer questions

1. Since HAMA is primarily directed against the murine part in monoclonal antibodies, it will be lowest where the murine component is the least, which is the case in humanized antibodies. Therefore humanized versions of monoclonal antibodies will have a minimal HAMA response.
2. Aminopterin is a powerful inhibitor of the enzyme dihydrofolate reductase, which is the key enzyme in de novo synthesis of DNA. Mutant tumor cells have only one (*de novo*) pathway working in them for DNA (purine) synthesis, so adding aminopterin is done to kill any unfused tumor cells that are still in culture. Hybridoma cells (fusion products of tumor cells and antibody-producing cells) survive because another pathway (salvage pathway) is working in B cells and provides purines for DNA synthesis.
3. In a single-chain variable fragment (scFv), V_H and V_L domains are linked together by a short peptide linker that is usually 15−20 amino acids long, while in diabodies two scFv are assembled together. Moreover monomers in diabodies have short linkers that are usually five amino acids long.
4. Bispecific antibodies are those antibodies or antibody constructs that have dual specificity in their binding arms. Naturally occurring antibodies have the same specificity in both arms. Yet a diabody can act as a bispecific antibody if it is so constructed that it has two different specificities in its two different "arms."
5. The first difference between these antibodies is that monoclonal antibodies are directed against single antigenic determinants while polyclonal antibodies are directed against several antigenic determinants on the same antigen. Second monoclonal antibodies are derived from cells that are exact replicas or clones of each other and have the same parent cell. Polyclonal antibodies are derived from different B cells.

Yes/No type questions

1. A polyclonal antibody can bind to several antigenic determinants on an antigen
2. Monoclonal antibodies can detect single antigenic determinant only
3. In monoclonal antibody technology, tumor cell that can replicate endlessly are fused with B cells that produces immortal antibody to form a hybrid cell called myeloma
4. Two hypervariable regions of light chain and three hypervariable regions of heavy chain constitute a single antigen-binding site on one arm of antibody
5. Humanized antibodies are formed by gene cutting the CDRs from mouse antibody and ligate them with human antibody genes
6. B-cell from a spleen are isolated from a mouse, injected with target antigen, to produce monoclonal antibody
7. Myeloma cells are cancerous white blood cells
8. The time required for the production of polyclonal antibody is long
9. Polyethylene glycol or sendai virus is used for the fusion of B-cell and immortal myeloma cell in hybridoma technology
10. Myeloma cell lines used in hybridoma technology have functional HGPRT enzyme

Answers to Yes/No type questions

1. Yes
2. Yes
3. No. The resultant cell that is formed after fusion is called hybridoma.
4. No. Three hypervariable light chains and three hypervariable heavy chains constitute a single antigen-binding site.
5. Yes
6. Yes
7. Yes
8. No. The production of polyclonal antibody usually takes shorter period of time.
9. Yes
10. No. Myeloma cell lines have nonfunctional HGPRT, a key enzyme for purine synthesis by salvage pathway. But they synthesize purine by De novo pathway.

CHAPTER

26

Vaccines: present status and applications

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Summary

Vaccine development and its usage over the years have accomplished significant reduction in infections and diseases. Improved knowledge of immune protection and advancements in genetic engineering have led to the introduction of variety of new types of vaccines through manipulation of DNA, RNA, proteins, and sugars. Creation of attenuated mutants, expression of potential antigens in live vectors, and purification and direct synthesis of antigens in new systems have immensely improved the vaccine technology. Both infectious and noninfectious diseases are now within the realm of vaccinology. The profusion of new vaccines has enabled targeting of new populations for vaccination to cure and remove infectious microbes from their natural reservoirs. But, parallel to ancient infection like malaria and new infections like human immunodeficiency virus (HIV), potent vaccines are still elusive and render a bigger challenge to the scientific world.

What you can expect to know

After studying this chapter, readers are expected to have knowledge and conceptual understanding of the historical prospect of vaccine development, an underlying concept of vaccine types, their merits and demerits, modern vaccines, ethical issues related to vaccine, and their future challenges.

Introduction

The immune system is remarkably versatile defense system that has evolved to protect animals from invasion of pathogenic microorganisms. It is able to generate an enormous variety of defense cells and molecules capable of specifically recognizing and eliminating an apparently unlimited variety of invasive pathogens. These cells and molecules act together in a dynamic network to defend the hosts. Immune recognition is remarkable for its specificity. The immune system is able to recognize subtle chemical differences that distinguish one antigen from another. Furthermore, the system is able to discriminate between foreign molecules and body's own cells and proteins. The term immunity is derived from Latin words "immunis" and "immunitas," meaning "to exempt." The first record of deliberate attempt to induce immunity was performed by the Chinese and Turks in the fifteenth century. Various reports suggest that dried crusts derived from smallpox pustules were either inhaled into nostrils or inserted into small cuts in the skin to impart immunity against smallpox, a technique called *variolation*. Buddhist monks used to drink snake venom to confer immunity to snake bites. In 1718, Lady Mary Wortley Montagu observed positive effects of variolation on the native population and attempted the technique on her own children. English physician Edward Jenner is considered the founder of vaccinology. The variolation technique was significantly improved and tested by him, observing the fact that milkmaids who had contracted mild disease cowpox were subsequently immune to fatal smallpox disease. Jenner reasoned that the introduction of fluid from a cowpox pustule into people (i.e., inoculating them) might protect them from smallpox by inoculation of an 8-year-old boy with fluid from a cowpox pustule and later deliberately infected the child with smallpox. As predicted by him, the child did not develop smallpox symptoms. It was experimentally supported by Louis Pasteur who succeeded in growing bacterium thought to cause fowl cholera in culture and then showed that chickens variolated with cultured bacterium developed cholera. When these variolated chickens were

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inoculated with potent live bacterium, they developed cholera, but soon recovered and were completely protected from the disease. Pasteur hypothesized and proved that aging had weakened the virulence of the pathogen and that such an attenuated strain might be administered to protect against the disease. He called this attenuated strain a *vaccine* (Latin word *vacca*, meaning "cow"), in honor of Jenner's work with cowpox inoculation. Although Pasteur proved that vaccination worked, he did not know the reason behind protection.

Following are some key milestones in vaccine development:

1885: Louis Pasteur, known for his animal vaccines, injected a rabies vaccine into two people and caused controversy. At that time, very few people were comfortable with the idea of introducing a deadly, live virus into a human being.

1896: Vaccines for cholera and typhoid were developed using killed versions of bacteria.

1897: A killed vaccine for the plague was developed.

1923: A powerful toxin from diphtheria bacterium was chemically inactivated and used as a "toxoid" to kill bacteria. Before this toxoid vaccine, more than 200,000 cases occurred annually with 15,000 deaths.

1926: A killed vaccine for pertussis (whooping cough) was developed using the whole pertussis organism.

1927: A tetanus toxoid was developed. Before this toxoid vaccine, there were ~600 cases reported annually in the United States with ~180 deaths. By late 1940s, tetanus was combined with diphtheria and pertussis as the children's vaccine known as DTP.

1954: Jonas Salk developed a killed poliovirus that decreased paralysis cases from 20,000 in 1952 to 1600 in 1960.

1961: Alfred Sabin developed an oral polio vaccine using a live virus, which was easy to take and was successful at eliminating the spread of polio.

1963: A safe and effective measles vaccine was developed that reduced the number of cases from four million in 1962 and 3000 deaths, to 309 cases in 1995, with no death.

1964: A killed rabies vaccine was developed and required 30 painful shots in the abdomen. A newer version was developed by 1980 that required only five shots in the arm to protect against deadly rabies.

1967: A vaccine for mumps was licensed, reducing the incidence from ~200,000 cases annually with ~30 deaths to ~600 cases with no death.

1970: Several strains of rubella were attenuated to make vaccine. Between 1964 and 1965, there were ~12 million cases leading to birth defects in 20,000 children. By 1971, measles, mumps, and rubella vaccines were combined into a single shot known as measles, mumps, rubella (MMR).

1970s and 1980s: Meningococcal, pneumococcal, and *Haemophilus influenzae* type b (Hib) vaccines were developed using a part of the bacterial cell wall to develop a safe antigen (conjugate vaccine). These vaccines protected against life-threatening meningitis, blood infections, and various types of pneumonia.

1986: A vaccine for hepatitis B was licensed with an antigen that is cloned rather than grown.

1990: A killed vaccine for hepatitis A was developed.

1995: A varicella (chicken pox) vaccine was licensed for use in children.

1996: The first "DTaP" vaccine was approved using only part of the pertussis organism, combined with diphtheria and tetanus, and reduced annual pertussis deaths significantly after the DTP vaccination.

2000: Premature death related to influenza is estimated at 20,000 annually. Influenza vaccine use reaches 70 million doses.

2010: First therapeutic vaccine based on blood cell infusion was licensed for prostate cancer.

2013: First commercial vaccine by reverse vaccinology representing a novel multicomponent recombinant vaccine against meningococcus type B was developed and commercially named Bexsero.

List of Nobel Prizes awarded in the field

Recipient	Year	Work
Max Theiler	1951	Yellow fever vaccine development
Emil Adolf von Behring		Serum therapy
Bruce A. Beutler, Jules A. Hoffmann, Ralph M. Steinman	2011	Improved vaccines against infections and different cancers
John Enders, Thomas Weller, and Frederick Robbins	1954	Growth of poliovirus in cultures of various tissues
Harald zur Hausen	2008	Connection between the human papillomavirus and cervical cancer

Jonas Edward Salk and polio vaccine

Interestingly, the work by Salk and Sabin did not receive the Nobel Prize. Nobel Archives reveals that when the names of Jonas E. Salk and Albert Sabin were nominated (1955), the clinical trial results of Salk's killed polio vaccine were awaited. In 1956, the search committee concluded that Salk has not developed anything principally new, but only exploited the preexisting discovery made by others and found the development of poliomyetilis vaccine protocol unsuitable for the prize. It is really bizarre that a work that saved the lives of thousands of people suffering from polio was inadmissible for the Nobel Prize.

Infectious diseases attributes for >45% of total deaths in developing countries (https://www.who.int/healthinfo/global_burden_disease/en/). Vaccination is the most effective means to prevent infectious diseases. The World Health Organization (WHO) information showed the mortality of >15 million children across the globe due to infectious diseases, especially, in the developing countries. These lives can be saved by mass immunization programs. The success of any mass immunization program largely depends on the availability of cost-effective and immunoprotective vaccines against the dreadful communicable diseases. There are many issues associated with the production of vaccines (Fig. 26.1).

Vaccines work by "teaching" our immune system to recognize and remember the foreign pathogenic bacteria or viruses. After vaccination, our immune system stores the antigen-specific memory cells. If there is recurrent exposure to the actual disease in future, our immune system then opsonizes the bacteria or viruses much more quickly. Vaccines are made by manipulating germs or parts of germs.

Types of vaccines

Traditional vaccines

The development of vaccines against deadly pathogens represents an important advancement in the history of modern medicine. In the past, traditional vaccination has relied on two specific types of microbiological preparations to produce vaccines for immunization and generation of a protective immune response: first, living infectious microbe prepared in a weaker state that unable to cause disease when used as a vaccine; second, inert, inactivated, or subunit preparations of antigens.

Scientists are the best judge to decide the vaccine type based on the disease-causing agent and the natural behavior/course of the disease. Whole virus vaccines, either live or killed, constitute the majority of vaccines in use at present. However, recent advancements in molecular biology had provided alternative methods for the development of vaccines.

There are many approaches to design vaccines against a microbe. These choices are typically based on fundamental information available about the microbe, such as the mechanism of infection to host cells and response of immune system to it. In addition, other practical considerations such as parts of the world where the vaccine is ought to be used are also reviewed. Following are some of the options that a researcher may pursue in course of development of vaccines:

- live, attenuated vaccines,
- inactivated vaccines,
- subunit vaccines,
- toxoid vaccines, and
- conjugate vaccines.

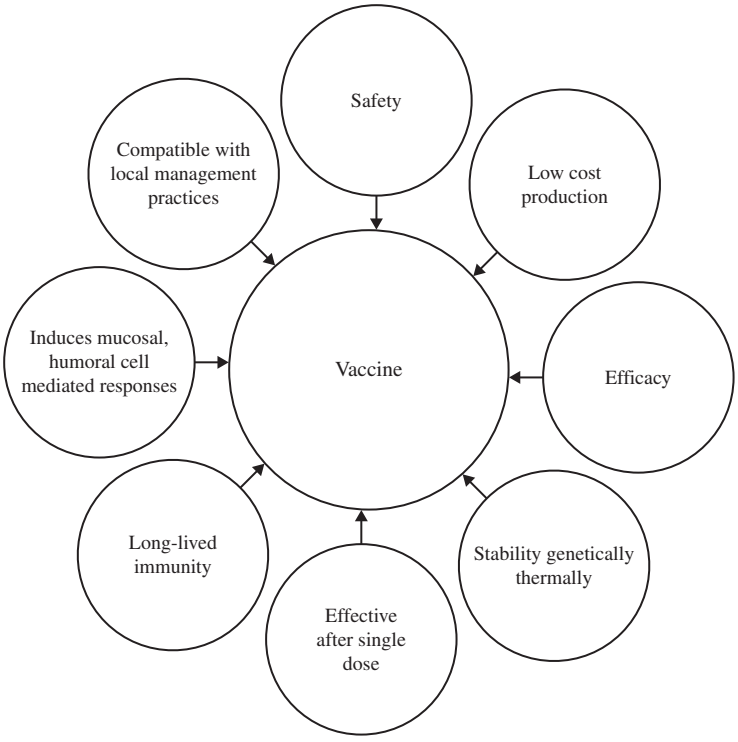


FIGURE 26.1 Properties of a vaccine.

Two additional types of vaccines currently being developed are as follows:

- DNA vaccines and
- recombinant vector vaccines.

Live, attenuated vaccines

Methodology

Live, attenuated vaccines contain a version of the living pathogenic microbe that has been attenuated or weakened by growing it in a in the lab so that it has lost its significant pathogenicity. This is accomplished by serial passage of pathogenic form of microbe in an unnatural host (tissue culture, embryonated eggs, and live animals for multiple generations). This extended passaging introduces one or more mutations into the new host. The mutated pathogen is significantly different from the original pathogenic form so that it cannot cause disease in the original host, but can effectively induce the immune response. After repeated passages, the virus is administered to the natural host. Majority of classical vaccines used in humans and animals have been developed by passaging the pathogen in unnatural host, for example, 17D strain of yellow fever was developed by passage in mice and then in chick embryos. Polioviruses were passaged in monkey kidney cells and measles in chick embryo fibroblasts. Human diploid cells WI-38 and MRC-5 are now widely used for attenuation.

Live, attenuated virus vaccines are prepared from attenuated strains that are almost or completely devoid of pathogenicity but are capable of inducing a protective immune response. They multiply in the human body and provide continuous antigenic stimulation over a period of time. Several methods have been used to attenuate viruses for vaccine production.

Live attenuated vaccines stimulate protective immune responses when they replicate in the host. The viral proteins produced within the host are released into the extracellular space surrounding the infected cells and are then acquired, internalized, and digested by antigen-presenting cells (APCs) that circulate throughout the body. These APCs include macrophages, dendritic cells, and B cells, which work together to expand immune response. The APCs recirculate and display fragments of the processed major histocompatibility complex (MHC) class II antigens on their cell surface. This complex of processed antigen peptides constitute part of the specific signal with which APCs along with the MHC peptide complex triggers the activation of T helper (T_H) lymphocytes. The second part of the activation signal comes from the APCs themselves, which displays costimulatory molecules along with MHC–antigen complexes on their cell surface. Both drive T cell expansion and activation through interaction with their respective ligands, the T cell receptor complex and the costimulatory receptors CD28/CTLA4, present on the T cell surface. Activated T cells secrete molecules that act as a powerful activator of immune cells. Viral peptides produced within the host cells are processed through proteasome degradation pathway. Small parts of these processed intracellular proteins associate with MHC class I and are displayed on the cell surface. These complexes together are recognized by a second class of T cells, killer or cytotoxic cells. This recognition, along with other stimulation by APCs and production of cytokine stimulated T cells, is responsible for the development of mature cytotoxic T cells (CTLs) capable of destroying infected cells. In most instances, live infection induces lifelong immunity. Evidences favor the fundamental role of cytokines in the differentiation of memory cells. The T_H cell–regulated B cell immunity progresses in an ordered cascade of cellular development that culminates in the production of antigen-specific memory B cells. The recognition of peptide MHC class II complexes on activated APCs is critical for effective T_H cell selection, clonal expansion, and effector T_H cell function development. Cognate effectors T_H cell–B cell interactions then promote the development of either short-lived plasma cells or germinal centers (GC). These GCs expand, diversify, and select high-affinity variants of antigen-specific B cells for entry into the long-lived memory B cell compartment. On antigen re-challenge, memory B cells rapidly expand and differentiate into plasma cells under the cognate control of memory T_H cells. Although live attenuated preparations are the vaccines of choice, they do pose the risk of reversion to their pathogenic form, causing infection.

Use of a related virus from another animal: The earliest example was the use of cowpox to prevent smallpox.

Administration of pathogenic or partially attenuated virus by an unnatural route: Virulence of the virus is often reduced when administered by an unnatural route. This principle is used in the immunization of military recruits against adult respiratory distress syndrome using enterically coated live adenovirus type 4, 7, and 21.

Examples of currently available live attenuated vaccines against viral infections include MMR, cowpox, yellow fever, influenza (FluMist intranasal vaccine), and oral polio vaccine. Live attenuated bacterial vaccines include tuberculosis, BCG, and oral typhoid vaccine (Table 26.1).

Today, it is likely that regulatory agencies would require an understanding of the basis of attenuation. Therefore, the development of any new attenuated form of *Mycobacterium* for use as a candidate vaccine is likely to include the introduction of one or more specific mutations into the genome of the pathogen. Likely candidates include mutations that interfere with synthesis of an amino acid or nucleic acid component essential

TABLE 26.1 Bacterial and viral vaccines currently used.

Type	Current	Under trial
Bacterial vaccines		
Live, attenuated	BCG	*Vibrio cholerae* *Salmonella typhi*
Inactivated	*V. cholerae* *Bacillus pertussis* *S. typhi*	*V. cholerae* plus subunit A *Mycobacterium leprae*
Subunit	*Haemophilus influenzae* *Neisseria meningitidis* *Streptococcus pneumoniae*	*S. typhi* *H. influenza* DT conjugate
Toxoid	Tetanus, diphtheria	
Viral vaccines		
Live, attenuated	Vaccinia Measles Yellow fever Mumps Polio A Adenovirus Rubella Varicella zoster	Cytomegalovirus Hepatitis A Influenza Dengue Rotavirus Parainfluenza Japanese encephalitis Polio A
Inactivated	Polio Influenza Rabies Japanese encephalitis	Hepatitis A
Subunit	Hepatitis B, influenza	

Adapted from Roitt, I.M., Delves, P.J. (Eds.), 1992. Encyclopedia of Immunology. Academic Press, San Diego, CA.

for the pathogenesis of the organism. Development of temperature-sensitive mutants may be used in conjunction with the aforementioned method. Live attenuated vaccines have been prepared and evaluated in preclinical trials (Table 26.1). The rBCG30 is a recombinant form of BCG.

Advantages of live, attenuated vaccines

Live, attenuated vaccines are relatively easy to create for certain viruses. Vaccines against measles, mumps, and chicken pox, for example, are made by this method. Live, attenuated vaccines are more difficult to create for bacteria. Bacteria have thousands of genes and thus are much harder to control. Scientists working on a live vaccine for a bacterium, however, might be able to use recombinant DNA technology to remove several key genes. This approach has been used to create a vaccine against *Vibrio cholera* that causes cholera. However, the live cholera vaccine has not been licensed in the United States.

Since a live attenuated vaccine is closest event to a natural infection, these vaccines appropriately activate the immune system: The attenuated vaccines elicit strong immunoprotective cellular and antibody responses and often confer lifelong immunity with only one or two doses.

Disadvantages of live, attenuated vaccines

Despite the advantages of live, attenuated vaccines, there are some disadvantages as well. It is the nature of living things to change or mutate, and the organisms used in live, attenuated vaccines are not different. The major disadvantage of the attenuated vaccine is that secondary mutations might lead to the reversion of virulence and cause disease. There is another possibility of interference by related viruses as is suspected in the case of oral polio vaccine in developing countries. People with immunocompromised, damaged, or weakened immune systems due to chemotherapy, HIV infection, or pregnancy cannot receive live vaccines. Another limitation is that live, attenuated vaccines usually need the cold chain to stay potent and skilled health care workers to handle them. It adds the extra cost that hampers the mass immunization programs in developing countries that lack widespread refrigeration, and the limited skilled health care workers restrict it widespread use. The possibility of contaminating alien viruses in cultured cells exasperates the vulnerability of live attenuated vaccines.

Inactivated whole virus vaccines

Considering the disadvantages of attenuated vaccines, another approach was used to develop vaccines. In this approach, the whole causative pathogen is killed or inactivated.

Methodology

The inactivation of antigens is done typically by heat or chemicals such as formaldehyde or β-propiolactane or radiation. The chemical treatment destroys multiplication ability of the pathogen, but keeps the immunogenic structure intact in its natural form. It is very crucial to ensure the structural integrity of antigenic epitopes of surface antigens. Thereby, inactivated whole pathogen vaccines provide protection by directly generating cell-mediated and humoral immune responses against the natural pathogen. To be effective, nonreplicating virus vaccines must contain much more antigen than live vaccines that are able to replicate in host. In the absence of cellular production of antigen, these vaccines usually lack the ability to induce significant cytotoxic T lymphocytes (CTL) responses. The immunity induced by inactivated killed vaccines frequently decreases during the life of host and may require additional boosters to achieve lifelong immunity. However, killed vaccines offer some important advantages over live vaccines: they are produced outside the host, and they can be designed to

contain only specific antigenic target of pathogen that is involved in the development of protective immunity and exclude all other viral components.

Examples of the currently available inactivated vaccines are limited to inactivated whole viral vaccines against influenza, polio, rabies, and hepatitis A (Table 26.1). Whole inactivated bacterial vaccines include pertussis, typhoid, cholera, and plague. "Fractional" vaccines include subunits (hepatitis B, influenza, and acellular pertussis) and toxoids (diphtheria and tetanus).

Advantages of inactivated whole virus vaccines

Inactivated vaccines are more stable and safer than live vaccines as dead microbes cannot mutate back to their pathogenic state. Such vaccines usually do not require refrigeration and can be easily stored and transported in a freeze-dried form, thus making them cheaper and easily accessible to people in the developing countries.

Disadvantages of inactivated whole virus vaccines

Most inactivated vaccines stimulate weaker immune response than live vaccines. So, they require multiple booster doses to maintain their protective immunity. This could be a limiting factor for the use of inactivated vaccines in areas where people do not have regular access to health care and cannot get booster shots on time. Also, excessive treatment for inactivation of pathogen may destroy immunogenicity, whereas insufficient treatment can leave infectious virus capable of causing disease. In addition, there is an increased risk of allergic reactions due to the presence of large amounts of unrelated structural antigens of microbes. Table 26.2 shows the comparison of live, attenuated vaccine and inactivated whole virus vaccines.

Toxoid vaccines

Toxoids are bacterial toxins, usually exotoxins, secreted by pathogens that produce many disease symptoms after infection.

TABLE 26.2 Comparison between live, attenuated vaccine and inactivated whole virus vaccine.

Features	Live	Dead
Dose	Low	High
No. of doses	Single	Multiple
Need for adjuvant	No	Yes
Duration of immunity	Many years	Short
Antibody response	IgG	IgA IgG
Cell-mediated immunity	Good	Poor
Reversion to virulence	Possible	Not possible

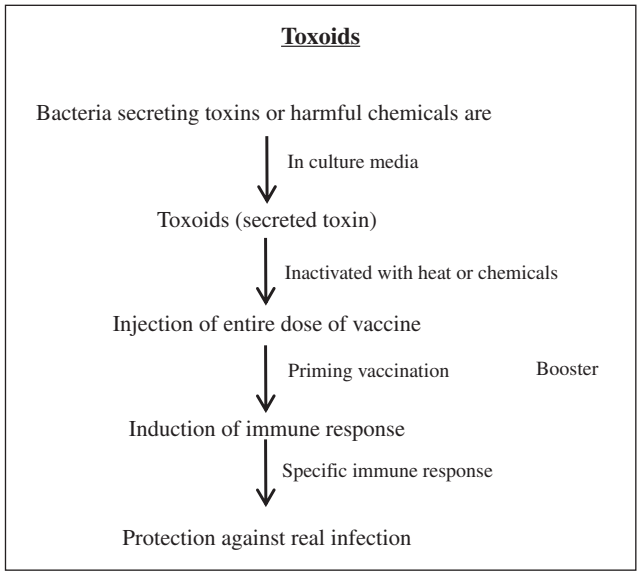
Methodology

Toxoid vaccines (e.g., vaccines for diphtheria and tetanus) are prepared by purifying bacterial exotoxin (Flow Chart 26.1). Toxicity of purified exotoxins is then suppressed or inactivated either by heat or with formaldehyde, while maintaining their immunogenicity. Such detoxified exotoxins are harmless/safe and are used as vaccines. Vaccination with toxoids induces antitoxoid antibodies that have ability to bind with toxin and opsonize/neutralize the deleterious effects of natural exotoxin.

However, procedures for the production of toxoid vaccines ought to be strictly controlled to achieve detoxification/inactivation without excessive structural modification of antigenic epitopes. This technique is reserved for diseases in which secreted toxins are the main cause of illness. Vaccines against diphtheria and tetanus are the best examples of toxoid vaccines.

Subunit vaccines

Originally, nonreplicating vaccines were derived from crude preparations of a virus from animal tissue. As the technologies for growing viruses to high titers in cell cultures advanced, it became possible to purify the viruses and viral antigens. Advancement in biotechnology has made it possible to identify the peptide sites encompassing the major antigenic sites of viral antigens. Hence, instead of using the entire microbe for immunization, subunit components of antigens that can best stimulate immune system are used as vaccine. Subunit vaccines may contain variable antigens ranging from 1 to 20 antigens. Since only the specific antigenic determinants (very specific parts of antigen that antibodies or T cells recognize and bind to) of antigens



FLOW CHART 26.1 Production of toxoid vaccines.

are used for this type of vaccine, the risk of adverse effect is significantly lowered and the chance of reversal of virulence is completely eliminated. Examples of purified subunit vaccines include vaccines for influenza virus *Haemophilus influenza* A and B (HiA and HiB) and hepatitis B surface antigen.

Methodology

Identification of immunoprotective antigen and its antigenic epitopes is imperative for the development of a subunit vaccine (Flow Chart 26.2). However, identification of the best antigens that induce the immune system is empirical and time-consuming process; yet after identification, subunit vaccines can be easily made adopting either of the following procedures:

- By growing microbe in laboratory, and using chemicals, it is break apart, followed by purification of important antigens, and then such antigens are uses as subunit vaccines.
- By generating antigen molecules from microbe using recombinant DNA technology. Vaccines produced by using such antigens are called recombinant subunit vaccines.

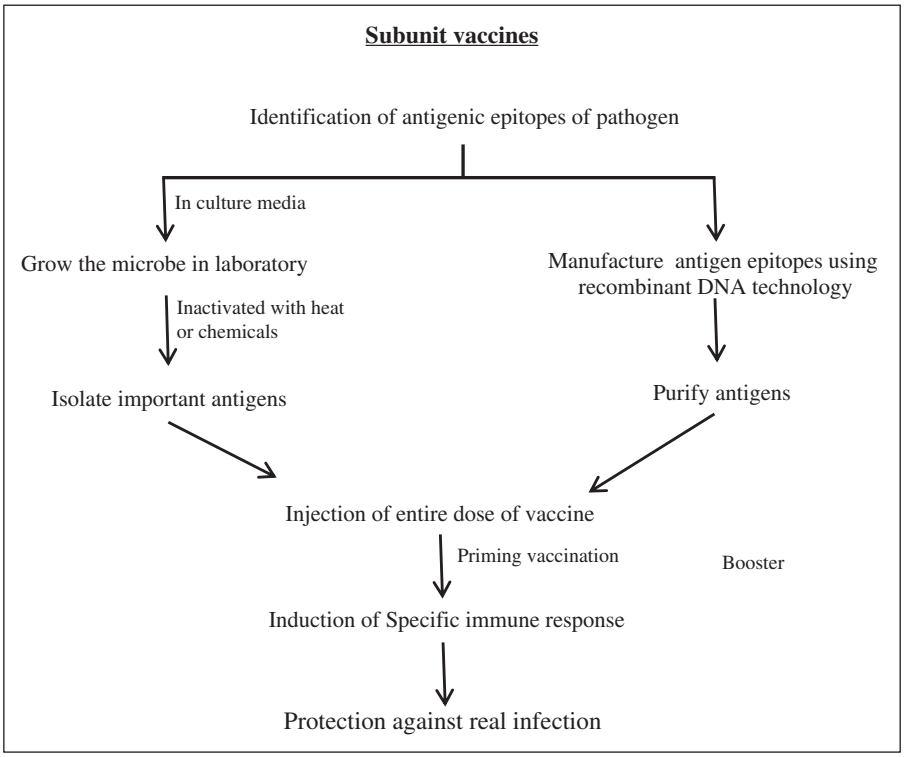
Currently, following subunit vaccines are in use.

1. Hepatitis B vaccines produced by insertion of a segment of hepatitis B virus (HBV) gene into a gene of yeast cell. These recombinant yeast cells produce pure hepatitis B surface antigen.
2. Human papillomavirus vaccines produced by inserting genes encoding viral coat protein into either yeast or insect cell lines. Recombinant yeast cells/insect cell lines produce virus-like particles, which when used as vaccine, induce immunoprotective response.
3. A live oral typhoid vaccine (Ty21a), produced by genetic modification of causal bacterium, *Salmonella typhi*, does not cause illness.
4. Live attenuated influenza vaccine, produced by genetic engineering of causal agent can replicate effectively in the mucosa of nasopharynx, but not in the lungs, thus bestow improved efficacy to the vaccine.

However, increased purification of subunit component may lead to decreased in immunogenicity of subunit vaccines and limit its use. This decrease can be modified by the coupling of subunit vaccines to an adjuvant.

Conjugate vaccines

Some harmful bacteria possess an outer polysaccharide coating. Such polysaccharide coatings of bacterial pathogens mimic human polysaccharides, so immature immune system of infants and younger children is unable to recognize or respond to them (Maiden, 2013). Therefore, conjugate vaccines were developed by



FLOW CHART 26.2 Production of subunit vaccines.

chemical conjugation of the polysaccharide to a stronger T-cell-stimulating antigen, like diphtheria or tetanus toxoids. It leads to costimulation of the immature immune system against the linked protein and linked polysaccharide providing defense against disease-causing bacterium. Some common examples of conjugate vaccines include influenza vaccine *Haemophilus influenza* B (HiB), pneumococcal (Prevnar), and meningococcal (MenC, MenAfriVac).

DNA vaccines

DNA vaccination is a relatively recent development in vaccine methodology. It involves direct introduction of a plasmid into the appropriate tissue containing complete expression cassette that independently encodes antigens against which immune response is sought (Koprowski and Weiner, 1998). DNA immunization is a novel technique used to efficiently stimulate humoral and cellular immune responses to protein antigens. Wolff et al. (1990) demonstrated direct transfer of plasmid DNA into mouse muscle without any special delivery system. Plasmid DNA encoding reporter gene was induced for protein expression within muscle cells providing evidence that naked DNA could be delivered in vivo for direct protein expression. DNA vaccines usually consist of bacterial plasmid vectors containing heterologous genes expressed under the control of eukaryotic promoter, thus allowing protein expression in mammalian cells (Davis, 1997). Genes encoding highly specific antigen are expressed, and gene products then undergo glycosylation and post-translational modifications similar to natural infections (Hasson et al., 2015).

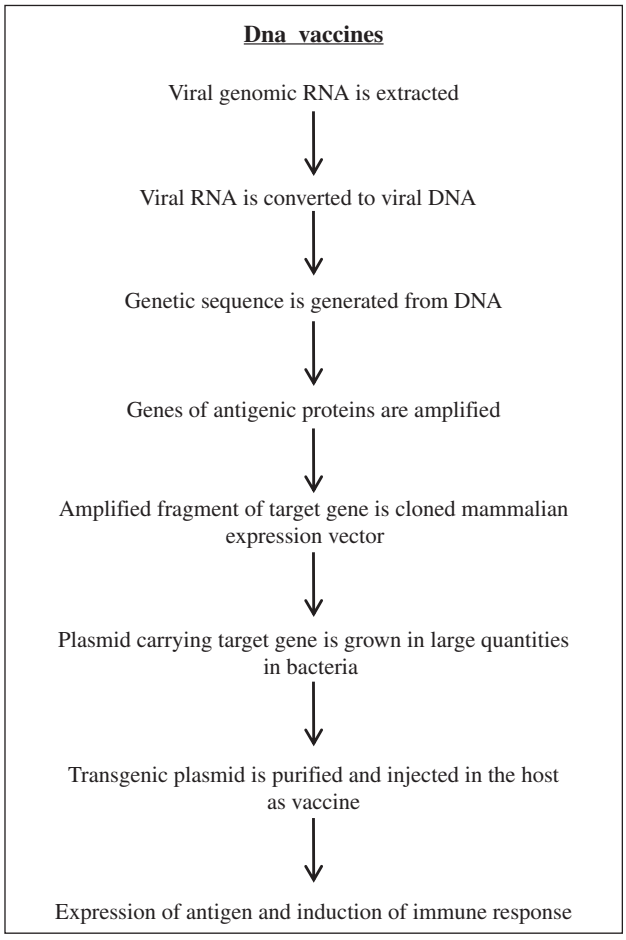
The use of genetic material to deliver genes for therapeutic purposes has been practiced for many years. Experiments outlining the transfer of DNA into cells of living animals were reported as early as 1950. Initial experiments revealed that direct gene injection resulted in the expression of gene in host in the absence of viral vectors. Additional experiments extended the earlier findings to recombinant DNA molecules, illustrating that purified nucleic acids could be directly delivered into host and transfected host cell produces the antigen of interest. Genetic immunization with complete expression cassette containing human growth hormone gene produced detectable levels of growth hormone in mice. Immunized mice developed antibodies against human growth hormone. It describes the ability of inoculated genes to act as an individual immunogen (Koprowski and Weiner, 1998).

DNA-based immunization has become a novel approach for vaccine development. Direct injection of naked plasmid DNA induces strong immune responses to the antigen encoded by gene vaccine. Once the plasmid DNA construct is transfected, host cells take up the foreign DNA, express the viral gene, and produce corresponding viral antigen inside host cell. Such antigen presentation and processing induce both MHC class I and class II mediated cellular and humoral immune responses (Encke et al., 1999).

These vaccines show promising response, and several are being tested on humans. DNA vaccines are third-generation vaccines that took immunization process to a new technological level. DNA vaccines use genes that encode for nearly all important antigens.

Methodology

Construction of DNA vaccine DNA vaccines are composed of bacterial plasmids. Expression plasmids used in DNA-based vaccination normally contain two units, (1) antigen expression unit composed of promoter/enhancer sequences, followed by antigen-encoding and polyadenylation sequences; and (2) production unit composed of bacterial sequences necessary for plasmid amplification and selection. Construction of bacterial plasmids with antigen coding DNA sequence is accomplished using recombinant DNA technology (Flow Chart 26.3). Once constructed,



FLOW CHART 26.3 Production of conjugate vaccines.

DNA vaccine plasmid is used to transform bacteria, and bacterial growth in culture medium allows the production of multiple copies of plasmid vaccine. The plasmid DNA vaccine is then purified from bacteria culture by separating circular plasmid DNA from much larger circular bacterial genomic DNA and other bacterial impurities. The purified plasmid DNA so obtained is then used as DNA vaccine (Fig. 26.2). DNA vaccine against a pathogenic microbe would evoke a strong antibody response to antigens secreted by cells, and vaccine would also stimulate a strong cellular response against microbial antigens displayed on cell surfaces. The biggest advantage of the DNA vaccines is that it cannot cause disease as they contain only copies of a few genes of pathogen and not the whole microbe. In addition, DNA vaccines are relatively easy and inexpensive to design and produce.

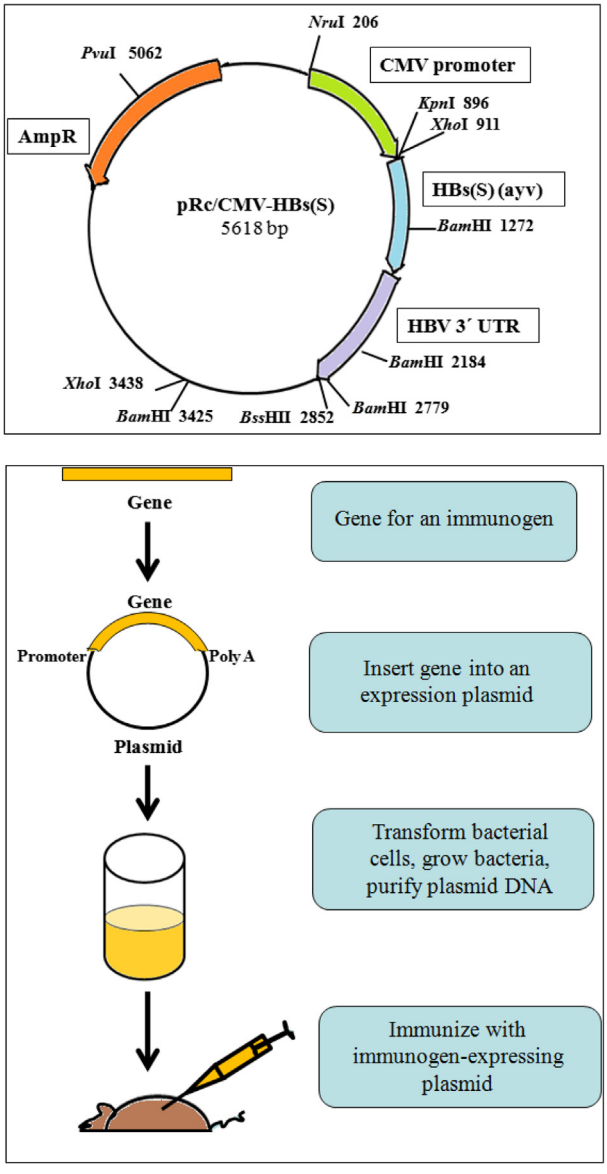


FIGURE 26.2 Steps involved in DNA vaccine development.

The naked DNA vaccines consist of DNA that is administered directly into the body. Such vaccines can be administered with a needle and syringe or with a needleless device that uses high-pressure gas to shoot microscopic gold particles coated with DNA vaccine directly into cells. Sometimes even DNA vaccine is mixed with molecules that facilitate their uptake by the cells of host. Naked DNA vaccines against influenza and herpes viruses are now being tested in humans.

Action mechanisms of DNA vaccines

A plasmid vector that expresses the protein of interest (e.g., viral protein) under the control of an appropriate promoter is injected under the skin or in the muscle of the host (Fig. 26.3). After uptake of plasmid, the protein is produced endogenously and processed intracellularly into small antigenic peptides by the host proteases. The peptides then enter the lumen of endoplasmic reticulum (ER) by membrane-associated transporters. In ER, peptides bind to MHC class I molecules. These peptides are then presented on the cell surface in context of MHC class I. Subsequently, CD8 + CTLs are stimulated and thus, they evoke cell-mediated immunity. CTLs inhibit viruses both through cytolysis of infected cells and noncytolytic mechanisms such as cytokine production (Encke et al., 1999).

The foreign protein can also be presented by MHC class II pathway by APCs, which elicit helper T cells (CD4 +) responses. These CD4 + cells are able to recognize the peptides generated from exogenous proteins that were endocytosed or phagocytosed by APC and then degraded to peptide fragments that were loaded onto MHC class II molecules. Depending on the type of CD4 + cell that binds to the complex, B cells are stimulated, which in turn stimulate antibody production. The mechanism of action of DNA vaccine is similar to the traditional vaccines (Schirmbeck and Reimann, 2001).

Advantages of DNA vaccines

DNA immunization offers many advantages over traditional forms of vaccination. It is able to induce the expression of antigens that resemble native viral epitopes more closely than standard vaccines do, as live attenuated and killed vaccines often alter protein structure and antigenicity. Plasmid vectors can be constructed and produced quickly, and the coding sequence can be manipulated in many ways. DNA vaccines encoding several antigens can be delivered to host in a single dose, which only requires a microgram of plasmids to induce immune responses. Rapid and large-scale production of DNA vaccine is possible at considerably lower costs than traditional vaccines. These vaccines are also temperature stable and thus, their

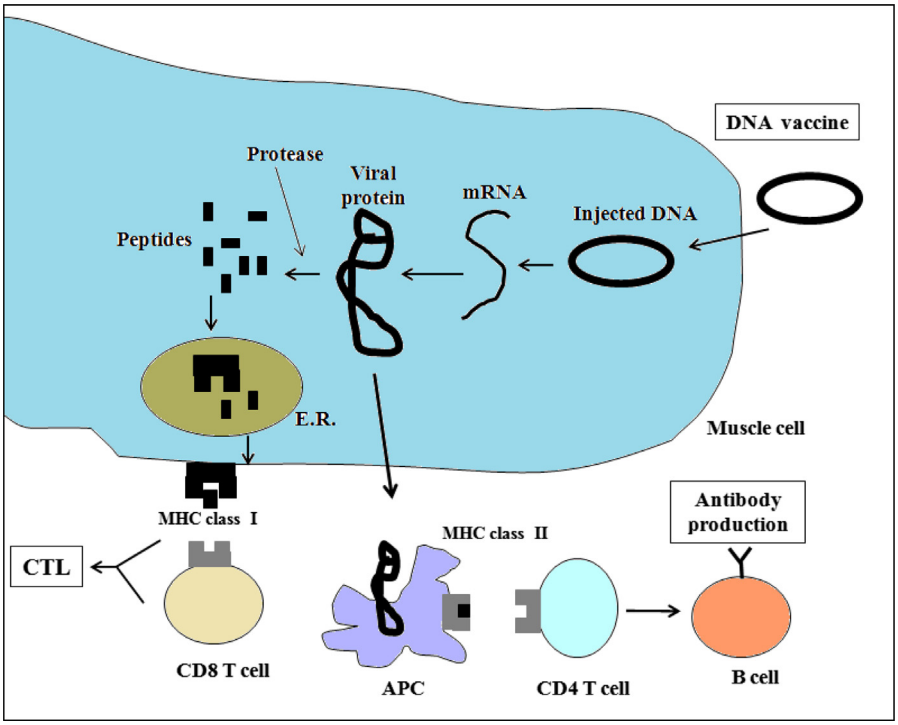


FIGURE 26.3 Action mechanism of DNA vaccine.

storage and transport is much easier than traditional vaccines. Another important advantage of genetic vaccines is their therapeutic potential for ongoing chronic viral infections. DNA vaccination may provide an important tool for stimulating an immune response in HBV, hepatitis C virus, and HIV patients. Continuous expression of the viral antigen caused by DNA vaccination creates an environment containing many APCs that may promote successful therapeutic immune response, which otherwise cannot be obtained by traditional vaccines (Encke et al., 1999). Thus, such characteristic features of DNA vaccines have generated a lot of interest in this area in the past decade.

Limitations of DNA vaccines

Although DNA can be used to raise immune responses against pathogenic proteins, certain microbes have outer capsules made up of polysaccharides. This limits the use of DNA vaccines for such specific cases as genes do not code for polysaccharides. Hence, DNA vaccines cannot serve as a substitute to polysaccharide-based subunit vaccines.

Future of DNA vaccines

Recently, it has been discovered that the transfection of myocytes can be amplified by pretreatment with local anesthetics or with cardiotoxin, which induce local tissue damage and initiate myoblast regeneration. Gaining a full understanding of this mechanism of DNA uptake could prove helpful in improving applications of gene vaccination. Both improved expression and better engineering of DNA plasmids may enhance antibody response to the gene products and expand the applications of gene vaccines.

Recombinant vector vaccines

Recombinant vector vaccines are experimental vaccines that are quiet similar to DNA vaccines. But unlike DNA vaccine, these vaccines use an attenuated virus or bacterium for the introduction of microbial DNA into host. Hence, "vector" here in particular refers to the virus or bacterium being used as a carrier.

In nature, viruses attach on to host cell surface and inject their genetic material. Scientists have taken advantage of this natural process to figure out the utilization of large genomes of certain harmless or attenuated viruses for inserting portions of pathogenic microbial genetic material encoding antigen proteins. The carrier viruses are then used to ferry such recombinant viral DNA with microbial genes into host cells. Therefore, recombinant vector vaccines closely mimic a natural infection and stimulate the immune system.

Attenuated bacteria can also be used as vectors. In such cases, inserted genetic material causes display of antigenic epitopes of other microbes on the surface of carrier bacterium. Consequently, the harmless bacterium mimics a harmful microbe, provoking immune response.

Researchers are working on both bacterial and viral-based recombinant vector vaccines for HIV, rabies, and measles.

Molecular farming using plants as bioreactor

Plants have been used as herbal drugs for millennia; they also play an important role in modern medicine. Recent advances in molecular biology techniques helped in the development of new strategies for the production of subunit vaccines comprising proteins derived from pathogenic viruses, bacteria, or parasites. Although mammals, their tissues, and cell lines are currently utilized for commercial production of vaccines, these systems are expensive and their scale-up is an arduous task (Larrick and Thomas, 2001; Houdebine, 2009). Plants have emerged as a promising system to express and manufacture a wide range of functionally active pharmaceutical proteins (Daniell et al., 2009) of high value to health industry with advantage over traditional bioreactors (Fig. 26.4). Technological advancements have played a vital role in establishing the use of plants as "surrogate production organisms" (Yadav et al., 2013). Fig. 26.5 presents the general procedure to develop and characterize the plant-made injectable, oral, or edible vaccine (Davoodi-Semiromi et al., 2009). One or more immunoprotective antigens of pathogens can be produced in plants (Ashraf et al., 2005) by the expression of gene(s) encoding protein(s). Plant-based novel production systems aimed at developing "edible" or "oral" vaccines have been discussed in detail (Ma et al., 2003; 2005; Koprowski, 2005; Lal et al., 2007; Mishra et al., 2006; Houdebine, 2009; Rybicki, 2010). Compared to traditional vaccines, edible vaccines offer simplicity of use, lower cost, convenient storage, economic delivery, and mucosal immune response.

The original concept of edible vaccines implied that transgenic fruit or vegetable expressing an antigen from virus or bacteria can be eaten raw without any previous processing and act as a vaccine for launching sufficient protective immune response against a particular disease. Currently, it is widely accepted that this original concept was rather naive mainly because of two reasons: first, different fruits from the same plant express different levels of antigens, and therefore, it is crucial to make plant-derived vaccine by using pool of fruits with homogeneous antigen concentration (vaccine dose). In general, at least a minimum processing, pooling, and freeze-drying of fruits from the same or different plants will be required before incorporation into formulations or capsules for oral vaccination. Second, it is important to ensure complete separation of fruits or vegetables intended for human or animal consumption from fruits or vegetables intended for pharmaceutical purposes like vaccines.

Hence, the approach of "edible vaccines" has been replaced with "plant-derived vaccine antigens." Antigen expression in plant tissue opens an important alternative to meet the global demand for cheaper, safer, and quality vaccines. Topographic compartmentalization of vaccine antigens in plant cell, tissue, or organ is a kind of encapsulation and provides a protective covering by protecting them from in vivo proteolytic degradation. Such edible encapsulation protects the antigen from degradation in mucosal and gut systems and allows its efficient absorption. This can facilitate transportation without

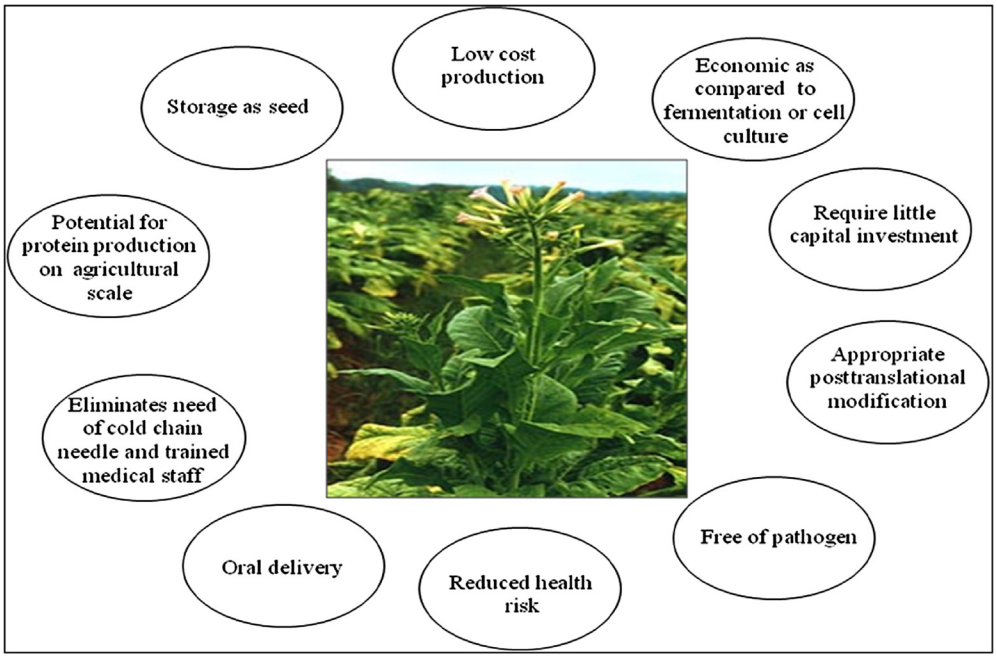


FIGURE 26.4 Advantages of using plants as a bioreactor.

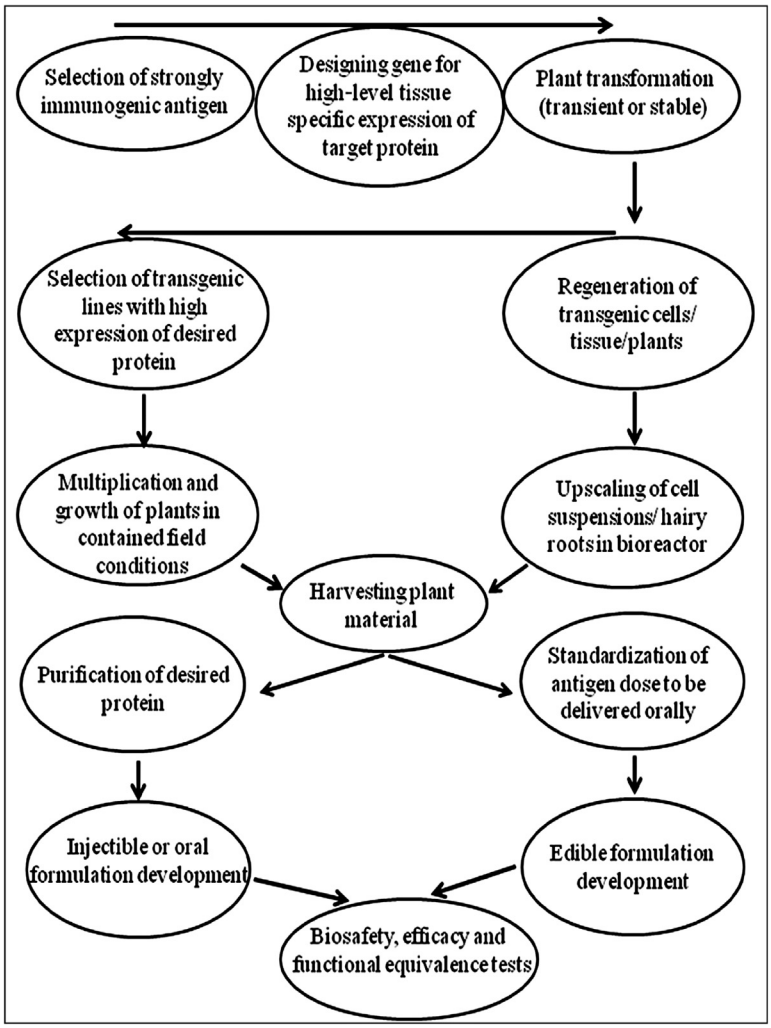


FIGURE 26.5 Steps involved in the production of plant-derived vaccines.

expensive infrastructure to the manufacturing site where dose standardization and packaging may be undertaken.

Advancement in vaccine adjuvants

The word "adjuvant" is acquired from the Latin word "adjuvare," meaning "to help" or "to aid." Adjuvants are the agents supplemented to vaccine formulations to sustain and direct the immunogenicity and induce appropriate protective immune response against the infection. Following are the potential benefits of adjuvants (Lee and Nguyen, 2015):

- Decrease the dose of antigen needed (dose sparing).
- Decrease the number of vaccine doses needed.
- Enhance vaccine efficacy in infants, elderly, and immunocompromised people.
- Increase the functional antibody titer.
- Induce more rapid and long-lasting immune responses.
- Induce robust cell-mediated immunity.
- Provide broad protection (cross-reactivity).
- Facilitate mucosal immunity.
- Overcome antigen competition in multivalent vaccines.

Traditional live attenuated or inactivated vaccines elicit robust protective immune responses without adjuvant because those vaccines contain naturally occurring adjuvants. Modern recombinant vaccines, particularly the highly purified or synthetic antigens, require adjuvants to induce a protective and long-lasting immune response. New vaccine targets will require not only strong antibody responses but also robust cell-mediated immunity including T_H and CTL cells. The use of appropriate adjuvants will allow vaccine formulations that selectively trigger innate immunity and/or adaptive immunity to obtain a desired

type of antigen-specific immune responses. Thus, adjuvants are necessary to help these proteins to become effective vaccines by inducing strong and long-lasting protective immune responses. Even though aluminum salts are the most commonly used adjuvant for human vaccines, they are weak adjuvant having complex mechanisms favoring induction of antibodies and are poor inducer of T-cell response. New forms of adjuvants have been proposed for various vaccines, which include oil-based emulsions; bacterial products [cholera toxin B (CTB) subunit, heat labile enterotoxin B (LTB) subunit]; viral products (virus-like particles); plant products (saponin derivatives); biodegradable particles (liposomes); molecular adjuvants; and synthetic adjuvants (Reed et al., 2009). Adjuvant mechanisms include depot effects, recruitment of innate immunity−specific targeting mechanisms, and carrier functions that hold antigen in an appropriate conformation. The safety of proposed adjuvants is a primary consideration. Therefore, it is often necessary to devise methods to reduce or eliminate the reactive effects of an adjuvant while preserving the efficacy. The most effective use of adjuvants for certain types of vaccines, particularly for stimulating mucosal immunity, may be to combine adjuvant with a particular mode of delivery, such as oral, intranasal, or transcutaneous immunization. Carriers that carry and combine both adjuvant and antigen in a single formulation can serve as the basis for creation of important formulations for improved vaccines. The enhancement of immunogenicity of antigenic protein is an important aspect if vaccine antigens, expressed at a modest level in edible plant part, have to succeed in mounting sufficiently high immune response after passage through the mucosal and intestinal routes. General immune stimulators (adjuvants) and better targeting to immune system might compensate in part for low delivery of antigen. One of the targeting strategies involves linking antigens to molecules that bind well to immune system components such as villiousM cells in the intestinal lining. VilliousM cells, found in follicle-associated epithelium of Peyer's patches, take up antigens that enter the small intestine and pass them to other cells of immune system. If white blood cells (helper T lymphocytes) recognize the fragments as foreign, they induce B lymphocytes (B cells) to secrete neutralizing antibodies and initiate other strategies against the perceived enemy. The CTB and LTB are potent mucosal immunogens and adjuvants. They both bind directly to the GM1-ganglioside receptor molecules on M cells by fusing antigens from other pathogens to any of these subunits (Cuatrecasas, 1973; Mishra et al., 2006). Thus, it ushers foreign antigen into the M cells. By fusing antigens to this subunit, it is possible to improve uptake of antigens by M cells and enhance immune response. The carrier molecules also modulate immune response against watery diarrhea (Yasuda et al., 2003). Tiwari et al. (2009) reported the use of CTB as an adjuvant, fused with rabies glycoprotein antigen. The transgenic tobacco seeds showed a high expression of the functional fusion of CTB-rabies glycoprotein.

Adjuvants licensed for human use: Adjuvants that have been approved and licensed for human vaccines use are listed in Table 26.3.

Alum salts

Aluminum salts are the most widely used adjuvants in human vaccination for >80 years and substantially enhances the T_{h2} type [IgG1, IgE, interleukin (IL)-4, IL-5, and eosinophil] immune response. Until recent alum was the only adjuvant licensed for human use. Alum is a component of licensed human vaccines such as hepatitis A virus (HAV), HBV, human papillomavirus (HPV), diphtheria, tetanus, *Haemophilus influenzae* type b (Hib), and meningococcal. Despite the wide use of alum as adjuvant, its mechanism of action is not well understood. It is shown to be highly safe, stabilizes antigen, and augments high and stable antibody titer. The limitation of alum as an adjuvant is that it does not elicit CTL response, and vaccines supplemented with alum cannot be frozen, lyophilized, or sterilized by filtration.

TABLE 26.3 Approved and licensed vaccines adjuvants for human use (Lee and Nguyen, 2015).

Adjuvant	Year	Class	Description
Alum	1926	Mineral salt	Improves HI and Th2 responses, antigen stability, used in >80% human vaccines
MF59	1997	Oil-in-water emulsion	Improves HI and CMI responses, used in influenza vaccines
Virosomes	2000	Liposome	Improves HI and CMI responses, used in influenza and hepatitis A vaccines
AS03	2009	Oil-in-water emulsion	Improves HI and CMI responses, used in H1N1 pandemic
AS04		Alum-adsorbed TLR4 agonist	Improves HI and CMI responses, used for HPV and HBV vaccines

MF59 and AS03

Emulsions are amorphous two-phase systems containing two immiscible liquids, mixed with a surfactant for stabilization. The major advantages of emulsion-based adjuvants are the antigen dose sparing and facilitating production of high antibody titer. The MF59 and AS03 are squalene (a natural 30-carbon compound obtained from shark liver)-based oil-in-water emulsions developed by Novartis and Glaxo Smithkline, respectively. MF59 has been used in H5N1 pandemic influenza vaccine and also for the H1N1 influenza vaccine. The AS03 and MF59 have been assayed with herpes simplex virus, HIV, HBV, and cytomegalovirus vaccine trials. MF59 facilitates the local secretion of chemokine that activates the monocytes and macrophages at the site of vaccine administration. It also augments the antigen uptake by surveillancing dendritic cells, which in turn activate CD4 + T cells.

Virosomes

A virosome is a reconstituted viral capsid that contains a unilamellar phospholipid membrane and viral spike glycoproteins but lacks the viral genetic material. Virosomes resemble the viral particle and facilitates its fusion with target cells, but not able to infect it. The virosome vaccine for influenza virus (Inflexal V) and HAV (Epaxal) is being used in different parts of the world. Inflexal V is the only virosomal adjuvant-supplemented influenza vaccine licensed for all age groups. The major advantage of a virosomal adjuvant vaccine is that it processes the antigen through both MHC class I and II and are able to induce both humoral immunity and cell-mediated immunity. Moreover, it provides high and long-lasting antibody titer, protection of antigen from degradation, slow opsonization, high safety, and suitability to infants, elders, and immunocompromised patients.

Toll-like receptors agonist

Toll-like receptor (TLR) agonists are natural ligands that initiate the TLRs that recognize the patterns of the antigens and connect innate and adaptive immunity. The TLR 3, 5, and 9 are more significant adjuvant candidates amid TLR agonists. Monophosphoryl lipid A (MPL) is a TLR agonist, which increases the initiation of proinflammatory cytokines, namely, interferon-γ and IL-2, and results in robust induction of T_{H1} immune response. Adjuvant system is a combination of classical adjuvants like alum, oil−water emulsion, and liposomes to formulate the adaptive immune response. AS04 is a combination of MPL adsorbed to alum, while AS03 is a combination of α-tocopherol and squalene. AS03 and AS04 have been approved for several human vaccine uses/trials and appear as attractive candidate adjuvants.

Immunostimulating complexes

Immunostimulating complex (ISCOM) is an efficient mucosal delivery system for respiratory viruses to envelop antigens. It allows selective incorporation of viral envelop proteins by hydrophobic interaction into a defined supramolecular structure of *Quillaja saponins*. In this, a mixture of the plant glycoside saponin, cholesterol, and phosphatidylcholine provides a vehicle for presentation of several copies of the protein on a cage-like structure. Such a multimeric presentation mimics the natural situation of antigens on microorganisms. These in-built *Q. saponins* have strong adjuvant activity. Antigens are arranged in an accessible, multimeric physically well-defined cage-like liposomal structure of 40 nm diameter. Recent studies have also shown that the immunogenicity of envelop proteins of respiratory viruses and CTB when incorporated in ISCOM are greatly enhanced after mucosal administration, leading to potent mucosal IgA and systemic immune response. Furthermore, it was reported that ISCOMs containing antigens from *Echinococcus granulosus* efficiently induced humoral and cell-mediated immune responses against carbohydrate antigens by intranasal administration. It shows a great promise for the presentation of genetically engineered proteins.

Similar considerations apply to the presentation of peptides. The building of eight lysine-residue framework peptide has been shown to induce the immune response of much greater magnitude. A novel approach involves the presentation of the peptide in a polymeric form combined with T cell epitopes. The sequence coding for the foot and mouth disease virus peptide was expressed as part of a fusion protein with the gene coding for the hepatitis B core protein. The hybrid protein, which forms spherical particles 22 nm in diameter, elicited increased levels of neutralizing antibodies against foot and mouth disease virus that were at least a hundred times greater than those produced by the monomeric peptide.

Future challenges in vaccine development

Discovery of vaccination by Edward Jenner ranks as one of the most important medical advances of all time. Millions of lives are saved each year by vaccination against a range of diseases. It essentially works on the same principles that were established by Edward Jenner more than 200 years ago and has a huge impact on public health. However, the detailed immunological

relationship of protective immunity imparted by successful conventional vaccines against most infections pathogens is not at all clearly understood.

Foremost infectious disease problems

Despite the best efforts of researchers, medical professionals, and public health officials, a number of infectious diseases continue to pose significant public health problems. Diseases of concern in the developed world are strikingly different from diseases in developing nations deal with, in part because of the success of vaccination efforts in richer nations. Also, some diseases are limited geographically for ecological reasons due to the need for a suitable vector or appropriate habitats. The agents listed in Table 26.4 are noteworthy for their high infection rates, for exhibiting high morbidity or mortality, or for their economic impacts in both developed and developing nations and for which vaccines are not available.

Infectious disease threats

A number of infectious agents that are relatively rare today are poised for an upsurge in incidence either by natural or terrorism-related means. The natural threats are led by the influenza strain H5N1, which, like many of the other natural threats, is a zoonotic organism, an infectious agent that crossed over from animals to humans. Originally found in poultry, the virus has sickened humans in Asia twice in the past decade. Zoonoses may quickly become serious problems for human health if the agents adapt to humans by mutation, recombination, reassortment, acquisition of new genes, plasmid or phage interchange of genetic material, or geographical advance of their vector.

Human pathogens that could be employed for the terrorist purposes must be easily deliverable, but they need not be extremely virulent. Infectious diseases must only be severe enough to instill panic and disrupt civil life to be of use to a terrorist group. Many agents could be enhanced through engineering to either make the disease they cause more severe or to enable the agent to escape vaccines or treatment.

TABLE 26.4 Infectious agents posing significant human health concerns.

	Availability of licensed vaccine
Sexually transmitted diseases	
Human immunodeficiency virus	No
Human papillomavirus	No
Herpes simplex virus	No
Chlamydia trachomatis	No
Neisseria gonorrhoeae	No
Treponema pallidum	No
Respiratory agents	
Respiratory syncytial virus	No
Parainfluenza virus	No
Human metapneumovirus	No
Group of streptococci	No
Chlamydia pneumonia	No
Meningococcous B	No
Enteric agents	
Salmonella species	No
Shigella species	No
Escherichia coli (ETEC, EHEC, EPEC)	No
Helicobacter pylori	No
Noroviruses	No
Vector-borne agents	
Plasmodium falciparum	No
Dengue fever virus	No
Hantaviruses	No
Borrelia burgdorferi	No
Schistosoma species	No
Leishmania species	No
Hookworm (multiple genera)	No
Others	
Hepatitis C and E viruses	No
Cytomegalovirus	No
Group B Streptococcus	No

Ethical issues

Vaccines are responsible for many public health successes at global levels. Even so, vaccinations have also long been the subject of various ethical controversies. There are real risks associated with vaccination programs (Caplan and Schwartz, 2008). In the past, live attenuated vaccines against respiratory syncytial virus resulted in more severe disease and increased mortality in infants.

In 1976, the vaccination program against swine influenza in the United States was stopped because it was thought to be associated with a concomitant increase in the

Guillain–Barré syndrome. This association could not be confirmed after seasonal influenza vaccination. The whole-cell pertussis vaccine was previously thought to be associated with an increased risk of postvaccination encephalopathy, which lacked evidences for such casual association (Ray et al., 2006).

The key ethical debates related to vaccine regulation, development, and use generally revolve around (1) mandates, (2) research and testing, (3) informed consent, and (4) access disparities.

Mandates

Ethical debates and objections arise due to some individuals and communities disagree with the mandates and/or have religious or philosophical beliefs that conflict with vaccination. For example, in an effort to protect the greatest number of people, public health vaccine regulations may infringe upon individual autonomy and liberty. Tension results when individuals want to exercise their right to protect themselves and/or their children by refusing vaccination, if they do not accept existing medical or safety evidence, or if their ideological beliefs do not support vaccination. Many scientific and medical research studies have found that individuals who exercise religious and/or philosophical exemptions are at a greater risk of contracting infections and put themselves and their communities at risk (Feikin et al., 2000; Salmon and Omar, 2006). Thus, medical and public health supporters often struggle to balance the ethics of protecting individual beliefs and health of communities.

Vaccine research and testing

Ethical discussions also surround the research and testing of vaccines, including discussions about vaccine development and study, design, population, and trial location. Licensing of vaccines go through many years of research and must pass rigorous safety and efficacy standards. The vaccine development and research processes involve experts from diverse disciplines, namely, scientific, social field, public health, epidemiology, immunology, statistics, and from pharmaceutical industries. These stakeholders may have conflicting priorities and motives, which initiates various ethical discussions. Moreover, it is important to understand the safety and efficacy of a vaccine in various populations. But, testing a vaccine in vulnerable populations, such as children, also raises ethical concerns. Researchers must balance the need to protect the safety of children once the vaccine is administered with the need to adequately understand the performance of a vaccine. Similarly, it is important to understand the effect of vaccines administration on the people in developing countries. Yet, conducting vaccine research in developing countries includes a list of ethical concerns such as provision of necessary screening or treatment if diseases are detected; meaningful involvement of local communities in the research design process; assurance of the trial of vaccines be supervised by local ethical review panels; and to ensure that the participants understand consent (Snider, 2000). Ethical discussions are a key component of HIV vaccine research and development because HIV vaccines pose numerous unique ethical challenges (Celada et al., 2011). For example, AIDS stigma may put vaccine trial participants at psychological risk if they encounter discrimination. In addition, researchers have to figure out the methods to provide appropriate and adequate medical care and protection from stigma for participants screened as HIV positive. Also, researchers have to consider that participants do not misunderstand the trial and consider themselves protected from the virus and put themselves at risk. The complexity of these issues places ethics analyses at the forefront of HIV vaccine research.

Informed consent

Ethical debates also surround vaccine implementation and delivery, such as those concerning informed consent. Although federal guidelines do not require consent before vaccination, the National Childhood Vaccine Injury Act of 1986 requires that doctors have to provide a Vaccine Information Statement (VIS) to the vaccine recipients or their parents or legal representatives. The VIS provides basic information about vaccine risks and benefits and is designed to provide the information a patient or parent needs, to make an informed decision.

Access issues

Many vaccine-related ethical debates center on the evidence that access to vaccination depends to some extent on socioeconomic and racial ethnic minority status. These discussions implicit the question of whether all lives are of equal value and equally deserving of opportunities to be protected by vaccination. Global disparities signal the need for continued efforts to ensure equal opportunities to people to be benefited from vaccination.

Translational significance

New science, new technologies, and an ever more sophisticated understanding of immunology have yet to make a significant impact on worldwide vaccination. Novel and improved vaccines for the threatened

agents are currently under development. The struggle to develop a vaccine for HIV has been ongoing since the virus was first discovered in 1982, and although a great deal of progress has been made, an effective and safe vaccine is still elusive. Nearly, all vaccine designs and vectors have been tried in the effort to build an HIV vaccine. Some of the promising formulations today include a prime-boost vaccine (named so, because of two-pronged strategy of administering a primer vaccine and following up later with a booster vaccine), which is being tested in Thailand, and a vaccine based on three recombination adenovirus constructs, each expressing a different HIV gene.

The excellent work of academic community in development of early vaccine and in antigen discovery is exciting, but a wide variety and complexity of vaccine products and their production processes require technologies, expertise, production infrastructure and regulatory insights, which are widely unavailable. Identification of trained individuals in translational research and vaccine development could build bridges to carry innovation from lab to the actual field or market place.

There is a growing demand for vaccine safety, fueled in by antivaccination groups. As disease recedes, the need for vaccination becomes less evident to the public, and more people opt out to be vaccinated, depending instead on the herd immunity of surrounding vaccinated persons. Of course, herd immunity will fail if too many refuse to be vaccinated. But some real safety problems are associated with vaccines, such as paralysis after oral polio vaccine and disseminated infection after Bacille Calmette–Guerin vaccine. For this reason, older vaccines need to be re-examined for their safety, and if necessary, improvement should be forced; this was done for whole-cell pertussis vaccine, which is now made in cell culture instead of in the brain of animal. In the near future, Jenner's vaccinia will be replaced by further attenuated vaccinia and Bacille Calmette–Guerin by engineered vaccines for tuberculosis. Indeed, one of the advantages of newer molecular technologies is improved safety. As risk-benefit ratios become more controversial when disease presence declines, it will be important to reduce vaccine-associated reactions to the minimum.

World Wide Web resources

http://www.dnavaccine.com/: This interesting website contains references on the production and administration of a wide variety of vaccines.

http://www.cdc.gov/vaccines: This website provides information related to vaccines, immunization against preventable diseases, vaccine safety, and their side effects.

http://www.vaccines.org: This website provides access to up-to-the-minute news about vaccines and an annotated database of vaccine resources on the Internet.

https://www.historyofvaccines.org/timeline/all: This web resource is by The College of Physicians of Philadelphia and is a very interactive link, and in 2014, it was certified by WHO as a reliable, accurate provider of information on vaccine safety topics.

Protocols

Protocol for the development of vaccines

Following is the protocol explaining the development of a candidate vaccine using traditional or modern recombinant DNA technology:

1. Establishment of need for a vaccine required against a specific infection or disease.
2. Identification of causative agent of the disease, its isolation, and growing it in the laboratory under in vivo/in vitro.
3. In an attempt to develop a traditional vaccine, pathogens are attenuated or chemically inactivated in the laboratory.
4. However, aiming to develop the recombinant vaccine, the immunoresponsive antigen that promotes immunoprotective antibodies are identified. Candidate immunoprotective antigen is genetically or proteomically modified and proliferated in the suitable expression system.
5. Appropriate methods of purification are used to obtained traditional or recombinant immunoprotective candidate vaccine.
6. Depending on the type of disease, an animal model (e.g., mice, ferrets, rabbits, Rhesus monkeys, and chimpanzees) are chosen to test the candidate vaccine in animal models for its success in protecting against live infection or disease.
7. Determination of methods to measure the immune response against the candidate vaccine and to identify the immune response necessary to reach a level of protection.
8. Development of the clinical trial protocols necessary to test the candidate vaccine in human volunteers to establish its success in the final target. In phase 1 trial, the putative vaccine is tested for safety, immunogenicity, and the efficacy of different dosages. In phase 2 trials, investigators test safety and immunogenicity with a larger group of volunteers. Phase 3 trials study the candidate vaccines efficacy and safety among an even larger group, often in the thousands. After successful

phase 3 trials, vaccine is approved and recommended for safe immunization programs.

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Glossary

Attenuated vaccine A weakened pathogen used as vaccine to stimulate protective immunity, for example, BCG, MMR, cowpox, oral polio vaccine.

DNA vaccine A vaccine where a purified DNA carrying sequences encoding for the expression of subunit antigen of interest under the control of eukaryotic promoter, for example, DNA expressing rabies glycoprotein used as vaccine for eliciting protective immunity; many DNA vaccines are in clinical and preclinical trials including vaccines for influenza, malaria, tuberculosis, Ebola, herpes, human immunodeficiency virus (HIV), hepatitis.

Molecular farming Application of biotechnology to crop plants to use them as bioreactors for production of valuable plant-made pharmaceuticals.

Reverse vaccinology Application of genomics for determination of genes encoding candidate antigenic proteins and their subsequent in vitro and in vivo screening for vaccine production.

Subunit vaccine A preparation of pathogenic constituent (instead of whole pathogen), which can be administered to stimulate protective immunity, for example, purified hepatitis B surface antigen heterologously expressed in yeast cells and used as vaccine to elicit protective immunity.

Vaccine A preparation of pathogenic agent or their constituent, which can be administered to stimulate protective immunity, for example, cowpox and chicken pox vaccines.

Variolation Smearing of a skin fissures with smallpox skin crust to confer immunity to smallpox or variola.

Long answer questions

1. What should be properties of an ideal vaccine?
2. Explain the advantages and disadvantages of an attenuated vaccine.
3. Why cannot we follow the classical paradigm to develop a vaccine against HIV infection?
4. What is the major concern with DNA vaccine?
5. Why attenuated vaccines are more likely to induce cell-mediated immunity than killed vaccines?

Short answer questions

1. Is it possible to make vaccine against all diseases?
2. Why DNA vaccines cannot be prepared against all antigens?
3. What is the basic advantage of edible vaccines?
4. Explain whether HIV virions can be used to develop a killed vaccine.

Answers to short answer questions

1. It is not possible to develop vaccine against all diseases because the antigenicity of the causative agent/molecule is extremely important to induce the humoral and cell-mediated immunity. Thus, the chemical nature of antigen determines the possibility of making the vaccine against the disease.
2. DNA vaccines can be prepared only against the proteinaceous antigens. As DNA molecule alone can determine the translation of proteins. Hence, DNA vaccine cannot be prepared against sugar and lipid antigens.
3. Basic advantage of edible vaccines is the low cost of vaccination programs. It significantly reduces the need of cold chain, injection, adjuvants, and skilled worker to administer the vaccine.
4. Inactivated or attenuated HIV virus loses its antigenicity; hence, the use of killed HIV virions cannot be used as a vaccine. Furthermore, vaccines protect against disease, but not against infections and a very high rate of mutation in HIV virions evade the developed immunity.

Yes/no type questions

1. Do CD_4 antigen is present exclusively on the cell surface of T_H cells?
2. Do infection has any impact on the rate hematopoiesis?
3. Do all lymphoid cells have antigen-binding receptors on their cell surface?
4. Do vaccination against any pathogen is 100% effective?
5. Do live attenuated vaccines induce cell-mediated immunity?
6. DNA vaccines do not generate sufficient memory cells.
7. Is the use of adjuvants is always necessary in vaccination?
8. Do the human trail of vaccines need informed consent of subjects before vaccination?
9. Do IgG is more advantageous than IgM?
10. Do toxoid vaccines can only prepared against bacterial diseases?

Answers to yes/no type questions

1. Yes—CD_4 antigen is present exclusively on the cell surface of T_H cells and helps in binding with class II MHC molecules.
2. Yes—Infections induce the activation of T_H and macrophages and secretion of lymphokine-induced hematopoiesis for expansion of lymphocytes.
3. No—Only B and T lymphocytes have antigen-binding receptors on their cell surface and while natural killer cells do not.
4. No—Effect of vaccination is variable and depends on age, gender, and genetics of the recipient.
5. Yes—Live attenuated vaccines induce cell-mediated immunity because antigenically it is similar to natural pathogen and immune system has extended exposure to it but do not cause pathogenicity.
6. No—DNA vaccines generate sufficient memory cells due to prolonged exposure to constitutively synthesized antigen in the host's cell.
7. No—Use of adjuvants depends on the type of vaccine use for vaccination. Traditional vaccines possess natural adjuvants, while modern vaccines, particularly highly purified subunit vaccines, require adjuvants.

8. Yes—The National Childhood Vaccine Injury Act, 1986, suggests that the vaccine recipient should have informed consent regarding the trail.
9. Yes—IgG is more advantageous than IgM because it can cross placenta; its serum concentration is very high and its smaller in size helps it to diffuse in intercellular fluids.
10. Yes—Because toxoid vaccines are prepared by inactivation of secreted toxins by the bacterial cells.

CHAPTER

27

Perspectives on the human genome

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Summary

This chapter describes salient features of human genome beginning with a brief history of the Human Genome Project. With the advent of next generation of sequencing, technology leading to drastic reduction in the cost of sequencing has almost made the DNA sequences as a routine diagnostic tool for identifying the genes involved in complex diseases. Proliferation of human genome sequences during the last 10 years and application of genome-wide association studies are revolutionizing the study of biology and medicine. This has led to new perspectives and paradigm shift in thinking in disease diagnosis, gene therapy, and personalized medicine, also called precision medicine.

What you can expect to know

Human genome sequencing has been one of the greatest endeavors in biology. Because of the efforts of publically funded Human Genome Project (HGP), the DNA sequence is freely available to the public, which has contributed to significant discoveries worldwide. This chapter concentrates on understanding the architecture of the human genome in the light of human genome sequencing, and how this knowledge has revolutionized the study of biology and medicine. This chapter briefly describes the HGP carried out by a publicly funded international consortium and a private company. The major part of this chapter elucidates various findings of the human genome and the outcomes and implications of this information. DNA sequence of the human genome obtained through Human Genome Project is serving as a reference genome for alignments of sequences obtained using next generation of sequencing technology. Cost-effectiveness of sequencing and in turn proliferation of human genome sequences are making personalized medicine or precision medicine and individualized drug therapy almost a reality.

History and methods

Introduction

Humans suffer from a plethora of diseases caused by infectious agents, nutritional deficiency, or genetic factors. Changes in an individual's genome can result in a genetic disorder. Alterations in nucleotide sequences not only cause known genetic diseases such as hemophilia, sickle-cell anemia, cystic fibrosis, etc., but also influence how a person responds to a drug or an infectious agent and perhaps even our mood swings and behavior. Complex diseases such as cancer, diabetes, asthma, cardiovascular diseases, mental illness, etc. have also been shown to have some degree of genetic predisposition. The need to sequence the human genome arose from an urge to understand the genetic basis underlying diseases and response of an individual to a particular treatment. Sequencing of the human genome has helped in understanding these genetic factors and in many cases leading to improved methods of disease diagnosis, better prevention, and risk assessment in terms of predisposition of individuals to diseases or drug toxicity (and consequently their treatment). Understanding the sequence of the human genome also has tremendous potential in biology. The human genome project was initiated with this optimism that knowledge gained from understanding the human genome sequence would immensely accelerate the pace of biomedical research. The premise and optimism have more or less been justified. The development of new generation of sequencing technology has

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led to drastic reduction in the cost of DNA sequencing, a proliferation of sequences, and genome-wide association (GWA) studies for identification of candidate genes associated with many complex diseases. In fact, cost reduction in DNA sequencing has ensured that individual genome sequences have almost become diagnostic tools. The human genome is around 3200 million base pairs (Mb). It constitutes roughly 30% genes or gene-related sequences, comprising ~20,000 genes. Over 45% of the genome consists of repeated sequences including transposable elements.

Human genome sequencing project

History

In 1985, scientists met at the University of California to explore the prospect of sequencing the entire human genome. The HGP was initiated by the United States Department of Energy (DOE) in 1987 and was later joined by the National Institutes of Health (NIH). In 1990, James Watson was nominated as a director of the NIH component, which later became the National Human Genome Research Institute (NHGRI). In 1992, Watson resigned, and the position was taken over by Francis Collins, who successfully led the project to completion. Major funding for the HGP came from the US DOE and the NIH. Several other countries also became associated with the project, and the International Human Genome Sequencing Consortium (IHGSC), an open collaboration involving 20 centers in 6 countries, was formed. Outside the United States, the UK Medical Research Council and the Wellcome Trust supported the genomic research in the United Kingdom. Additional contributions came from France, Japan, Germany, and China. The finished sequence covering 99% of the genome was completed in 2003 on the 50th anniversary of elucidation of the DNA double helical structure (IHGSC, 2001, 2004). At the time, the HGP was being undertaken by the DOE and NIH, and a private company named Celera Genomics under the direction of Craig Venter also initiated sequencing of the human genome (Venter et al., 2001). The company intended to sequence the human genome within 3 years. This provided the drive for the HGP, as it was believed that the human genome sequence should be freely available. The HGP proposed new goals for 1998–2003. The major goal was to release a "working draft" by the end of 2001 and a complete sequence of the human genome by 2003. By the end of 1999, full-scale sequencing of the human genome began. The sequence of chromosome 22, the first human chromosome to be sequenced entirely, was published in *Nature* (Dunham et al., 1999). The second human chromosome to be sequenced by HGP scientists was chromosome 21, which is involved in Down's syndrome, Alzheimer's, and cancer (Hattori et al., 2000). By June 2000, a working draft of the human genome, covering more than 90% of the genome, was made available, followed by the publication of the finished sequence, covering 99% of the genome, in 2003; this marked the 50th anniversary of elucidation of the DNA double helix structure (IHGSC, 2001, 2004). For more information, visit www.genome.gov and www.yourgenome.org. Both public (IHGSC) and private enterprises (Celera Genomics) had completed their respective draft genome sequences. The IHGSC had used hierarchical or map-based or bacterial artificial chromosome (BAC)-based approach to achieve its goal, while Celera Genomics sequencing was accomplished by a whole-genome shotgun method, skipping the mapping phase (described later in the chapter). With the publication of the human genome sequence, it was expected that scientists would be able to understand the molecular basis of many genetic diseases. With the advent of next-generation sequencing (NGS) technology, understanding the molecular intricacies of complex human diseases is in the realm of possibility. There was a general euphoria when the draft sequence of the human genome was announced in 2000 simultaneously in Washington, D.C. by the President of the United States and in London by the Prime Minister of England. In the media, this landmark achievement was compared to landing a man on the moon. This was the beginning of the "postgenomic era."

Some major bottlenecks encountered in the publicly funded HGP were nonavailability of high-density genetic and physical maps. These maps are used in assembling the sequence of the whole genome of complex organisms like humans. Keeping in view the small cloning capacity of the then cloning vehicles, there was a need to look for better alternatives that made cloning of large inserts possible. The time taken and cost of sequencing using existing technologies at that time were high. Also, there was a need to store and analyze large amounts of sequencing data, and hence, there was a need to develop better algorithms for computing (e.g., development of software packages to analyze the sequence data). Practical difficulties such as cloning bias and the presence of a large quantity of repeat sequences in the human genome all provided hurdles to sequencing and assembly of human genome sequences. As a result, the first draft of the human genome had many gaps. Notwithstanding, the HGP was aided by several breakthroughs, some of which have been described as follows:

- Sanger's method of sequencing: its automation and miniaturization, use of fluorescent dNTPs, and

development of capillary-based sequencing machines greatly accelerated the speed of sequencing.
- DNA-based genetic markers helped in the construction of high-density genetic, and physical maps greatly helped in the assembly of YAC/BAC clones and hence the sequence assembly.
- Polymerase chain reaction, which was invented in 1983, also propelled genetic research.
- Development of large-insert cloning systems such as *E. coli*-based BACs, which can carry large segments of DNA, contributed to HGP's success.
- Development of software packages such as PHRED and PHRAP for sequence analysis, etc.

Human genome: organization and perspective

Sequencing of the human genome represents a significant milestone in the history of biomedical science. The efforts of the HGP have provided detailed information about the structure, organization, and function of the human genome. To understand human genetics and diseases, it is important to understand the structural and functional complexities of the human genome. The human cell consists of two different genomes: nuclear and mitochondrial. The size of the nuclear genome is ~3200 Mb and is organized into 24 (22 + X + Y) different chromosomes; when one talks about the human genome, it invariably means the nuclear genome. The mitochondrial genome is 16,569 bp long and is described later in this chapter.

The human genome sequences are split into 24 chromosomes. There are 22 autosomes, 1 X chromosome, and 1 Y chromosome. Each chromosome has multiple domains: (1) the centromeric region required for chromosomal separation during cell division, (2) the telomeric region required for maintaining the structural integrity of chromosomal DNA during DNA replication, and (3) the chromosomal arms designated the short arm (p) and the long arm (q). Human chromosomes are composed of euchromatic and heterochromatic regions. Most genes are located in the gene-rich, transcriptionally active regions of the chromosome known as euchromatin. The other parts of the chromosome constitute heterochromatin, which is composed of highly condensed and transcriptionally inactive regions.

Complexity of human genome

The human genome is a very large and complex genome. Unlike prokaryotes, which have compact genomes, the human genome is not constrained. It contains several different kinds of sequences. Only ~30% of the genome contains genes or gene-related sequences. The remaining ~70% of the sequence constitutes various kinds of repeats, pseudogenes, transposable elements, and many other uncharacterized sequences.

Gene content

Knowledge of a complete set of genes and protein is integral to the study of human biology and medicine. However, this task has remained elusive, and their numbers have been fluctuating. A "gene" is defined as the part of nucleotide sequence that is necessary for the synthesis of a functional polypeptide or RNA molecule. It includes all the sequences required for the production of a particular RNA transcript and involves distinct regulatory and coding regions. The end product of a gene could be a polypepeptide (protein-coding genes) or RNA (RNA coding genes). Evolution has defined the type and the complexity of transcriptional machinery and genes. In prokaryotes, a single RNA polymerase transcribes both RNA and protein-coding genes and has DNA-binding activity (i.e., it recognizes specific nucleotide sequences within the regulatory region of the gene). Prokaryotic protein-coding genes are often polycistronic, and their transcripts are colinear with the amino acid sequence of the polypeptides they encode. Eukaryotes, on the other hand, have at least three different transcriptional machineries to transcribe different categories of genes [i.e., *rRNA* genes (class I genes), protein-coding genes (class II genes), and *tRNA* genes (class III genes)]. None of the eukaryotic RNA polymerases has DNA-binding activity, implying that they do not recognize specific nucleotide sequences in the regulatory region to initiate transcription. The transcription unit of eukaryotic protein-coding genes can be split into exons and introns (split genes).

There are over 20,000 protein-coding genes in the human genome. As given in Table 27.1, human chromosomes vary in size. They are arranged in order from longest to shortest (1−22), chromosomes 19 and 21 being exceptions (since the karyotyping preceded the sequencing). The largest chromosome is chromosome 1, which is ~249 Mb and contains ~2012 known protein-coding genes. The smallest is chromosome 21 (~48 Mb) with 225 genes. The X chromosome is ~155 Mb with 815 genes, and the Y chromosome is one of the smallest, with a size similar to chromosome 19 (~59 Mb); it has the least number of genes (only 45). The gene density of chromosomes varies. Gene density is defined as number of genes per million base pairs. For example, chromosome 2 is ~243 Mb long and contains ~1203 genes, whereas chromosome 11

TABLE 27.1 Ensemble database version 68.37.

Chromosome	Length (Mb)	Protein-coding genes	rRNA genes	Pseudogenes
1	249.25	2012	66	1130
2	243.19	1203	40	948
3	198.02	1040	29	719
4	191.15	718	24	698
5	180.92	849	25	676
6	171.12	1002	26	731
7	159.14	866	24	803
8	146.36	659	28	568
9	141.21	785	19	714
10	135.53	745	32	500
11	135.01	1258	24	775
12	133.85	1003	27	582
13	115.17	318	16	323
14	107.35	601	10	472
15	102.53	562	13	473
16	90.35	805	32	429
17	81.19	1158	15	300
18	78.08	268	13	59
19	59.13	1399	13	181
20	63.02	533	15	213
21	48.13	225	5	150
22	51.30	431	5	308
X	155.27	815	22	780
Y	59.37	45	7	327

(~135 Mb) contains ~1258 genes. Chromosome 19, on the other hand, is only ~59 Mb, but carries about 1399 genes. Thus, the highest gene density is on chromosome 19. Chromosome 17, which is about ~81 Mb, has 1158 genes and also has a high gene density (Table 27.1).

Many human genes have exons separated by long introns and are located outside the heterochromatic region as expected. There is considerable variation in the overall gene and intron sizes. Usually, longer genes are the result of very long introns and not the result of coding for longer products. Some human genes are exceedingly large. For example, the largest known human gene, "dystrophin," which is associated with Duchenne/Becker muscular dystrophy, is approximately 2.4 Mb, but the transcript is only 14,000 nucleotides long. Apparently the large size is due to intronic sequences. The gene encoding the muscle protein "titin" has the longest coding sequence (~27,000 amino acids), the longest single exon (17 kb), and also the largest number of exons (178). The gene density varies from region to region. The GC-rich regions tend to be gene-dense, with many compact genes, whereas genes with large introns are found in AT-rich regions (IHGSC, 2001).

Noncoding genes: As explained earlier, of about 3.2 billion bp of the human genome sequence, less than one-third, are gene or gene-related sequences. More than two-thirds of the genome includes a variety of repeat sequences, both tandem and dispersed repeats. Some of the repeats are in a very high copy number, repeated more than a million times. A section of the genome is transcribed, but these transcripts do not code for protein and are known as long noncoding

RNAs (lncRNAs). This has been one of the great surprises of the postgenomic era. Certain genes encoding RNAs have been known for a while, but they have been small RNAs besides ribosomal RNAs (rRNAs) and transfer RNAs (tRNAs) that have been involved in information transfer process. There are two categories of noncoding RNAs (ncRNAs): (1) microRNAs (miRNAs) involved in silencing genes (RNA interference), thereby regulating their expression and (2) a large number of sequences that encode RNA (lncRNA) of up to several thousand nucleotides. The latter category, untranslated lncRNAs, has been implicated in a variety of biochemical processes and possibly has a regulatory role in gene expression. This realization has led to an entire field of inquiry called RNA biology.

In recent times, there has been a complete rethinking of the role of RNAs. Other untranslated RNA molecules that are involved in a variety of functions, including gene regulation, RNA processing, protein synthesis, etc., have been identified. Whole-genome or high-throughput transcripts analyses have revealed that the human genome is pervasively transcribed, and a greater part of the euchromatic region is represented in the primary transcripts. Many of these transcripts overlap with protein-coding genes or are found in the region believed to be transcriptionally silent (The ENCODE Project Consortium, 2012). The ENCyclopedia Of DNA Elements (ENCODE) project is an International Research Consortium that aims to identify all functional DNA elements in the human genome. These data obtained through the ENCODE project show that around 80% of the human genome displays some functionality in at least one cell type. Many of the regulatory elements identified are physically associated with one another possibly interrelated in distinct as well as overlapping functionality. These elements are also related to sequence variants associated with diseases or traits. The findings of the ENCODE project provide us new insights into the organization and regulation of genes and genome and serve as useful resources for analyzing issues related to human health and disease (Qu and Fang, 2013).

Some of ncRNAs are described as follows (data and information adapted from IHGSC, 2001).

Transfer RNAs: tRNAs are adapters that decode the nucleotide sequence of mRNA into an amino acid sequence. tRNAs are small RNA molecules of about 80 nucleotides. They play an important role in protein synthesis. The dispersal of tRNA throughout the genome is nonrandom, with *tRNA* genes seen in clusters throughout the genome. In fact, chromosomes 1 and 6 contain almost half of the *tRNA* genes. Chromosome 6 harbors the largest *tRNA* gene clusters in a region of about 4 Mb. This region contains multiple sets of tRNAs, representing 36 of 49 anticodons. Only tRNAs coding for Asn, Cys, Glu, and selenocysteine are missing. Chromosome 7 carries 18 of 30 Cys tRNAs in a region of about 0.5 Mb, while chromosomes 22 and Y carry no functional tRNA genes.

Ribosomal RNAs: rRNAs are components of ribosomes that are involved in the process of translation. Ribosomes are protein-synthesizing machinery of the cell consisting of complexes of RNAs and proteins. Each ribosome has two subunits: large (60S) and small (40S). The large subunit is made up of proteins and 28S, 5.8S, and 5S rRNAs, while the small subunit is composed of 18S rRNA complexed with proteins. Ribosomal genes are present in clusters on the short arms of five human acrocentric chromosome pairs (13, 14, 15, 21, and 22) in tandem arrays. Each repeat produces one precursor transcript that is processed to produce three types of rRNAs. The 5S *rRNA* genes also occur in tandem repeats. Chromosome 1 harbors the largest cluster of 5S *rRNA* genes. Since *rRNA* genes are present in arrays of tandem repeats, they are not sequenced in their entirety and are thus underrepresented in the human genome draft sequence. Nevertheless, several rDNA-derived sequences are dispersed throughout the genome. The number of ribosomal genes on each chromosome is listed in Table 27.1.

Small nuclear RNAs: Several small RNA molecules have been identified that play a role in gene expression, mostly at the level of posttranscriptional processing. Some of these are described below:

Spliceosomal small nuclear RNAs: Eukaryotic pre-mRNA undergoes posttranscriptional processing (splicing), in which introns are removed to form mature mRNA. Some introns are self-splicing, and others require a spliceosome; a large ribonucleoprotein is made up of over 200 proteins and five small nuclear RNAs called U1, U2, U4, U5, and U6. These RNAs form part of a major or U2-dependent spliceosome and process GU/AG splice sites. U11, U-12, U4atac, and U6atac, together with U5, form a minor spliceosome. A number of genes (~1944) coding for snRNAs have been identified throughout the genome. Some of these RNAs are present in clusters; for example, U2 RNAs are clustered in tandem arrays at q21–q22. Thirty U1 RNA genes are present in a nonuniformly organized locus at 1p36.1.

Small nucleolar RNA genes: rRNAs are synthesized in the nucleolus, and small nucleolar RNA (snoRNAs) are extensively involved in processing and modification of rRNA in the nucleolus. These RNAs modify regions of rRNA (e.g., the peptidyl transferase center and the mRNA–decoding center). In addition, many other targets, including snRNA, are being identified. A total of 1521 *snoRNA* genes distributed among different chromosomes have been identified.

Besides these, an increasing number of other ncRNAs that are involved in a plethora of functions

have been identified or are being identified. Table 27.2 lists other types of ncRNAs. For more information, refer to Matera et al. (2007), Ghildiyal and Zamore (2009), Carthew and Sontheimer (2009), Taft et al. (2010), Kaikkonen et al. (2011), Wright and Bruford (2011), Zhang et al. (2012), Strachan and Read (2011), or visit www.noncode.org (NONCODE, 2005).

MicroRNA: miRNAs are ~19–25 nucleotides long, evolutionarily conserved, small single-stranded molecules that regulate gene activity at the posttranscriptional level by a phenomenon related to *RNA interference*. They are involved in regulation of various cellular activities including pathological conditions. The primary transcript of miRNA has a 5′ cap, 3′ polyA tail, and an inverted repeat that can base pair to form a hairpin structure. A nuclear RNase III complex (Drosha) processes this transcript to release a typical hairpin loop precursor miRNA (pre-miRNA) that moves to the cytoplasm where it is cleaved by the enzyme dicer, a cytoplasmic RNase III, to release mature miRNA. In the cytoplasm, an effector complex called RNA-induced silencing complex (RISC; contains argonaute RNase) degrades one strand of the miRNA to leave a mature single-stranded miRNA (guide strand) bound to argonaute. This complex then selectively base pairs with target RNA that has the sequence complementary to the guide strand. The binding usually occurs at the 3′-untranslated regions (3′-UTRs) of the target mRNA sequence, but in some cases, 5′-UTRs are also involved. Thus, an miRNA can target several gene transcripts. Depending on the extent of similarity, the target RNA is degraded, destabilized, or prevented from being translated. A total of 1913 miRNA genes have been identified in the human genome. Chromosome 1 has 156 *miRNA* genes, while Y chromosome has 4 (www.mirbase.org). Table 27.3 lists the chromosomal-wise distribution of miRNA genes in the human genome. miRNAs are small but critical regulators of posttranscriptional gene expression that have been linked to various cellular processes and are associated with a number of diseased conditions. Many human diseases have been identified that may be due to mutation or dysfunction of ncRNAs; for example, Prader–Willi Syndrome, cancer, central nervous disorders, and cardiovascular diseases (Davis-Dusenbery and Hata, 2010; Deiters, 2010; Taft et al., 2010). A full catalog of human miRNAs can be found at www.mirbase.org. However, after more than 20 years since the discovery of the first animal miRNA, the exact number of sequences coding

TABLE 27.2 Types of noncoding RNAs.

Name	Function
Small Cajal body RNA (scaRNA)	Modification of snRNA in Cajal bodies
RNA ribonucleases	An endoribonuclease that cleaves pre-tRNA in nucleus
RNase P	Cleaves rRNA in nucleolus
RNase MRP	
Small cytoplasmic RNAs	Component of signal recognition particle
7SL RNA	
TERC (telomerase RNA component)	RNA component of the telomerase that extend telomeres or ends of chromosomes
Vault RNA	Found in ribonucleoprotein complex that is believed to be involved in drug resistance
Long transcripts Xist	Involved in X chromosome inactivation and dosage compensation
Tsix RNA	An antisense to Xist
scAluRNA	Transcribed from Alu repeats in primates
PCGEM1 RNA	Involved in prostate cell biology and tumorigenesis
microRNAs	Several miRNAs have been identified that regulate gene expression
Endogenous short interfering RNA (endo-siRNA)	Involved in posttranscriptional regulation of transcripts and transposons
Piwi-binding RNA (pi-RNA)	Involved in regulating transposon activity in germline cells
Promoter-associated RNAs (PARs)	Regulate gene expression
Transcription-initiation RNAs (tiRNA)	Regulate gene expression
Centrosome-associated RNAs (casiRNAs)	Guide local chromatin modifications

TABLE 27.3 Distribution of miRNA genes in the human genome.

Chromosome	No. of miRNA genes	Chromosome	No. of miRNA genes
1	156	13	40
2	117	14	99
3	96	15	71
4	62	16	82
5	76	17	110
6	71	18	35
7	82	19	143
8	90	20	48
9	86	21	30
10	69	22	46
11	102	X	118
12	80	Y	4

for these regulatory molecules continues to perplex scientists.

Genes and diseases: Genetics plays an important role in disease occurrence and progression. There are many gene mutations that have been known to cause diseases/disorders. The diseases may be classified as monogenic (involving a single-gene) or polygenic or complex diseases involving multiple genes. Monogenic diseases are due to mutations in a single gene. These diseases can be followed by pedigree analysis of families. They can be dominant or recessive, autosomal, or sex-linked. Examples include hemophilia, sickle-cell anemia, cystic fibrosis, etc. Information about these can be accessed from the Online Mendelian Inheritance in Man (OMIM) database on the NCBI website (http://www.ncbi.nlm.nih.gov/omim). Attempts have been made to understand the underlying molecular basis of these diseases. Many of the diseases are caused by point mutations, whereas others have deletion or frameshift mutations. A class of neuromuscular diseases such as Hungtington's disease, Fragile X chromosome, and myotonic dystrophy is caused due to expansion of trinucleotide repeats. The Hungtington's disease is due to the repeats code for the polyglutamine stretch in the protein product. These repeats may be present in 5′- or 3′-UTR, intron, or promoter region where they can cause loss or gain of function. This leads to non-Mendelian-type of inheritance called anticipation, which is an increase in the probability of onset and severity of the disease as it passes through generations (Siyanova and Mirkin, 2001).

Polygenic disorders (or complex diseases) on the other hand are caused due to mutations in multiple genes. Examples include heart disease, Alzheimer's disease, diabetes, cancer, obesity, etc. Since in such complex diseases there are contributions from many genes, and they are often influenced by environmental factors, there is high degree of epigenetic modulation. These diseases are challenging with respect to decoding their molecular scenario and developing an animal model system. However, whole-genome sequencing has revealed the association of certain mutations to the disease conditions.

Changes in the number or structure of chromosomes, which are the carriers of genetic material, also result in genetic disorders. For example, Down's syndrome or trisomy of chromosome 21 occurs due to the presence of an extra chromosome 21. Similarly, Turner syndrome (XO, 45) is due to the loss of one X chromosome, and Klinefelter syndrome is due to the presence of an extra X chromosome (XXY, 47). Changes in chromosomal structure due to translocation (Cri du cat syndrome), inversion, or deletion (Sotos syndrome) are also associated with abnormalities. Mutations in mitochondrial DNA (mtDNA) also result in several diseases (described later in the text).

Understanding the human genome: The availability of the human genome sequence has also provided several interesting insights, some of which are discussed below (adapted from IHGSC, 2001, 2004). Such knowledge was possible only after the sequencing of the whole genome.

GC content: The presence of GC-rich and GC-poor regions in the human genome sequence had prompted the idea that these regions might have different biological characteristics, such as gene density, repeat sequences, etc. Availability of the genome sequence made possible analysis of GC content of the human genome at a global level. The expected GC content is $41 \pm \sqrt{((41)(59)/n)}\%$. However, there are regions in the genome that show extreme variations in GC content. Long-range variation in the GC content is seen throughout the genome. For example, the distal 48 Mb of chromosome 1p and the 40 Mb region of chromosome 13 have average GC content of 47.1% and 36%, respectively. Cytogenetic analyses have revealed significant association between large GC-poor regions and "dark G" (Giemsa) bands and the lightest G-bands with high GC content.

CpG islands: Dinucleotides CpG are extensively underrepresented in the human genome. The reason for this is that most CpG dinucleotides are methylated at cytosine residues, and spontaneous deamination of methylated cytosine gives rise to thymine. As a consequence, over long evolutionary periods, CpG readily and gradually mutated to TpG dinucleotides, making

them rare. However, there are "CpG" islands in the genome, which are found at the 5′-end of genes and play a role in many processes, such as gene silencing, genomic imprinting, etc., thus putting these regions under functional constraint. The availability of the whole-genome sequence enables one to have a global look at the CpG content and its distribution. Most of the CpG islands have 60%–70% GC content. The majority of them (about 75%) are less than 850 bp. The longest CpG island is 36,619 bp long and is present on chromosome 10. Most chromosomes have 5–15 islands per Mb. Chromosome Y has an unusually low 2.9 islands per Mb, while chromosome 19 has 43 islands per Mb. The number of CpG islands is found to be associated with the number of genes on the chromosome.

Repeat content of the human genome: The majority of the human genome is made up of repetitive DNA elements. The human genome has a much greater portion (~50%) of these repeat sequences than the *Arabidopsis*, the nematode worm (*Caenorhabitis elegans*), and the fruit fly (*Drosophila*) (Table 27.4). These repeats fall into five main categories:

1. Transposable element-derived repeats (also called interspersed repeats)
2. Inactive (partially) retroposed copies of cellular genes (including protein-coding genes and small structural RNAs), also referred to as processed pseudogenes (PPs)
3. Simple sequence repeats (SSR; including microsatellites and minisatellites)
4. Segmental (interchromosomal and intrachromosomal) duplications
5. Tandemly repeated sequences such as at centromeres, telomeres, and ribosomal gene clusters

Transposable element-derived repeats: Transposable elements are ubiquitous component of all the genomes studied so far. They are mobile DNA sequences that have potential to move from one location to another within the genome. On the basis of their structure and mechanism of transposition, they are classified into two main categories: type I and type II elements. Type I elements (retrotransposons) are thought to be derived from retroviruses, and their transposition requires a reverse-transcription step that is mediated via an RNA intermediate by a mechanism called "copy-and-paste." The copy-and-paste mechanism implies that a copy of the element transposes, leaving the original element. Thus, due to transposition, their copy number is likely to increase. Previously named as "junk DNA," selfish, or parasitic elements, these sequences have contributed in evolution of many genes and have been involved in genome restructuring and possibly expansion or shrinkage of genomes. They are also known to be involved in regulating other cellular and genetic activities. They serve as epigenetic regulators of the genome and are involved in many epigenetic mechanisms, such as imprinting, X-inactivation, position effect variegation, etc. (Slotkin and Martienssen, 2007). These sequences have played an important role in influencing the genome by causing rearrangements, chromosomal breakage, illegitimate recombination, modification, and reshuffling of existing genes, thus creating new genes and modulating the overall GC content.

Long-terminal repeat retrotransposons: Retrotransposons are subdivided into two categories based on the presence or absence of long-terminal repeats (LTRs): LTR and non-LTR elements. They are flanked by LTRs that carry transcriptional regulatory elements. The LTR retroelements contain *gag* and *pol* (protease, reverse transcriptase, RNase H, and integrase) domains and closely related to retroviral proteins. Transposition occurs through reverse transcription of an RNA intermediate. On the basis of internal arrangement of subdomains in the *pol* region, they are classified as *copia* or *gypsy* family of retroelements. The mammalian genomes also have vertebrate-specific endogenous retroviruses, which seem to be active in the mammalian genome.

Long interspersed nuclear elements: The non-LTR retrotransposons are LINEs (Long Interspersed Nuclear

TABLE 27.4 Mobile elements in genomes of various organisms.

Organism	Genome size (Mb)	Number of genes	Fraction TE
Saccharomyces cerevisae	13.5	~6000	~2.4
Drosophila	180	~13,600	~30
Human	~3200	~20,000	~45
Arabidopsis	125	~25,600	~14
Rice	389	~37,550	~33
Maize	2300	~32,540	~84.2

Elements) and SINEs (Short Interspersed Nuclear Elements). LINEs are the most primitive autonomous mobile elements found in the human genome. In humans, three distantly related LINE families are present: LINE1, LINE2, and LINE3, of which only LINE1 is still active. LINE machinery has resulted in reverse transcription in the human genome (including those of SINE and PPs). These transposons are about 6 kb long, encoding two open reading frames (ORFs) and possess a polymerase II promoter. Transposition of LINE occurs via RNA that assembles with encoded proteins and moves back to the nucleus. LINE transposition occurs preferably within AT-rich regions. The preference of LINEs for AT-rich sequences could be explained by the fact that these regions are poor in genes, and as a result, these elements do not encounter a functional selection pressure. The LINE endonuclease cleaves at TTTT/A in the DNA sequence where the LINE element is inserted into the genome.

Short interspersed nuclear elements: Unlike LINEs, SINEs are nonautonomous retrotransposons. They cannot transpose independently and are believed to use the LINE machinery for transposition. Nevertheless, they have a very high copy number within the mammalian genome. They share their 3′-end with the LINE element. They are 100–400 bp long, encode no protein, but possess an internal polymerase III promoter. There are three distinct monophyletic families of SINEs: the active Alu, the inactive mammalian-wide interspersed repeat (MIR), and Ther2/MIR3. The promoter of all SINEs but one is derived from *tRNA* genes. The Alu elements are the only active class of SINEs that have derived a promoter from signal recognition particle component 7SL. The Alu family is the most abundant sequence in the human genome occurring more than once in 3 kb on an average. They are of comparatively recent evolutionary origin. An Alu repeat is about 280 bp long and consists of two asymmetric tandem repeats, each about 120 bp long followed by a short An/Tn sequence (Strachnan and Read, 2011). Again, unlike LINEs, which are preferentially located in AT-rich regions, Alu repeats are found in gene-rich, high GC-rich regions, indicating their positive role in genome function. The fact that Alu uses LINE machinery for transposition, but is found in a GC-rich region, indicates that its distribution is influenced by evolutionary forces. Some Alu sequences are transcribed, and it is believed that these elements may have been involved in the evolutionary dynamics of the human genome.

DNA transposons: The type II elements are DNA transposons similar to bacterial transposons, and their movement is mediated by a DNA intermediate by a mechanism called "cut and paste." They contain terminal inverted repeats and a gene for transpose. Transposons that can transpose independently are called autonomous, and others that require the presence of a cognate element for transposition are called nonautonomous (Slotkin and Martienssen, 2007). For transposition of autonomous elements, structural integrity of terminal inverted repeats and transposase is essential. This protein, transposase, recognizes the terminal inverted repeats and mediates transposition by excising the transposon out of the donor position and integrating it into the new acceptor site. The transposition may occur through a cut-and-paste mechanism in which the donor site is repaired by the removal of the transposon or filled with a copy of the transposon by gap repair. They can be autonomous or nonautonomous type. The latter type lacks the transposase enzyme and is usually a deletion derivative of the cognate autonomous elements. There are seven major classes of DNA transposons that can be further subdivided into many families with independent origins (RepBase, www.girinst.org/repbase/update/index.html). Some examples of DNA transposons are MER1-Charlie, MER2-Tigger, Mariner, PiggyBac-like, etc.; DNA transposons have a short lifespan within a species and have not been as successful as LINEs. The transposase cannot differentiate between an active and an inactive copy of a DNA transposon in the nucleus; as a result, the transposon activity declines because of accumulation of inactive copies in the genome. Many human repeat sequences are derived from transposable elements. About 45% of the genome is composed of potentially mobile elements (Table 27.5).

Pseudogenes: Pseudogenes are the nonfunctional copies of normal genes with which they share sequence homology. They are of two types: duplicated pseudogenes and processed pseudogenes (PPs). Pseudogenes that are derived from duplication of genomic DNA are called duplicated pseudogenes, as they possess characteristic exon–intron structure. Such pseudogenes are derived by tandem duplication, and as a result, they

TABLE 27.5 Summary of mobile elements in the human genome (IHGSC, 2001).

Element	Fraction TE (%)	Copy number
L1(LINE)	16.9	0.5×10^6
Alu (SINE)	10.6	1.1×10^6
L2 (LINE)	3.2	0.3×10^6
MIR (SINE)	2.5	0.46×10^6
LTR Elements	8.3	0.3×10^6
DNA Elements	2.8	0.3×10^6
Processed pseudogenes	<1.0	$1–2 \times 10^4$
Total	~ 45	$\sim 3 \times 10^6$

are located close to the functional copy of the gene. In contrast, "retrotransposed" or PPs are possibly derived from reverse transcription of processed transcripts that integrate into the genomic DNA. Therefore, they are devoid of introns and promoter sequences. They also possess polyA tracts and direct repeats at either end of the pseudogenes. There are about 14,427 pseudogenes in the human genome. Prevalence of pseudogenes in the human genome has been problematic for genome annotation. Availability of a near-complete sequence has made the analysis of pseudogenes possible. The highly expressed housekeeping genes have multiple PPs. For example, ribosomal proteins account for ~20% of human PPs. Protein-coding pseudogenes are more easily identified than RNA pseudogenes (Pink et al., 2011).

Pseudogenes were believed to be nonfunctional, but in the postgenomic era, this view is changing. Evidence shows that many pseudogenes (2%–20%) are transcribed into RNA. Examples include pseudogenes for tumor suppressor PTEN, glutamine synthetase, glyceraldehyde-3-phosphate dehydrogenase, etc. Many pseudogenes have been found to have tissue-specific expression, and some have an expression pattern that is different from the parent gene. Alteration in pseudogene expression has been seen under different physiological conditions, such as diabetes and cancer. Some pseudogenes have been implicated in gene regulation in mice via siRNA production (Pink et al., 2011). For more information, visit www.pseudogene.org.

Simple sequence repeats: SSRs are another class of repeats represented in the human genome. SSRs, which are 1–13 bases long, are called microsatellites, whereas minisatellites have longer repeat units (14–500 bases). SSRs comprise about 3% of the human genome, of which the greatest contribution comes from dinucleotide repeats. There is about one SSR per 2 kb. The most commonly present SSRs are AC and AT, followed by AG, AAT, and AAC. Trinucleotides are less frequent than dinucleotides.

Segmental duplications: These duplications represent duplication of 1–200 kb of genomic sequence and transfer from one location to another. Segmental duplications are regions ≥1 kb in length and have sequence identity of ≥90%. These duplications appear to have arisen recently in terms of evolutionary time scale, as indicated by high similarity in their sequences, and their absence in closely related species. Sequence divergence in terms of base substitutions from the original sequence may provide possible time of duplication events. There are a high proportion of large duplications in the human genome as compared to *Drosophila* and *C. elegans*. Particularly, the pericentromeric and subtelocentromeric regions of chromosomes largely have recent segmental duplications. Approximately 5.3% of the euchromatic genome is covered by segmental duplications (IHGSC, 2004). Segmental duplications are both interchromosomal and intrachromosomal. Interchromosomal duplications involve transfer of a region between nonhomologous chromosomes; for example, the 9.5 kb region of the adrenoleukodystrophy locus from Xq28 is found on chromosomes 2, 10, 16, and 22. Intrachromosomal duplications occur within a chromosome or a chromosome arm and include low copy number repeats. The Y chromosome is peculiar since >25% of its total length carries segmental duplications as large as 1.4 Mb of 99.97% similarity.

Mitochondrial genome: As mentioned earlier, human cells have another genome that is present in mitochondria. Mitochondria are the major energy generator of the cell and are involved in cellular respiration. They also carry their own DNA, which is small in size. The human mitochondrial genome is 16,569 bp long. It is a circular molecule that is present in multiple copies.

Because of its smaller size, the mitochondrial genome was the first to be sequenced. The complete sequence of the mitochondrial genome was available in 1981 by Sanger and colleagues (Anderson et al., 1981). The mitochondrial genome codes for 37 genes that include 22 tRNAs, 2 rRNAs, and 13 protein-coding genes. The entire mtDNA is transcribed and contains very small noncoding regions. The gene density of mtDNA is 1 per 0.45 kb. All 37 mitochondrial genes lack introns. Protein-coding genes encode proteins involved in the electron transport chain and oxidative phosphorylation. Mitochondrial genetic maps are also available at www.mitomap.org. The majority of mitochondrial proteins are encoded by nuclear DNA. The mitochondrial genetic code has 60 codons and 4 stop codons. The stop codons include UAA and UAG (which are universal stop codons) and AGA and AGG (which specify arginine in the universal genetic code). The stop codon UGA codes for tryptophan in mitochondria, and AUA codes for methionine instead of isoleucine. Thus, the mitochondrial genome has a unique genome organization and is different from the nuclear genome (www.mitomap.org).

Mutations in mtDNA have been implicated in a broad spectrum of degenerative diseases involving the central nervous system, heart, muscle, kidneys, eyes, etc., as well as aging. These mutations are inherited from mothers' side or through accumulation of mutations with age in somatic cells. Because mitochondria are inherited from the maternal side, the affected mothers can have affected progeny, but affected fathers will not transmit the disease (maternal inheritance). The other characteristic of diseases that are coded by mitochondria is recurrent heteroplasmy. This is because there are several copies of mtDNA in a eukaryotic cell. Mutations may be present in all

the copies (homoplasmy) or only a fraction of them (heteroplasmy). As they replicate and segregate randomly to daughter cells, their ratio changes with each replication. Depending on the mutation, heteroplasmy, and tissue affected, there is wide diversity in disorders/diseases caused by mtDNA abnormalities. Mitochondrial myopathies are neuromuscular diseases caused due to mutation in the mitochondrial genome. Some common mitochondrial myopathies include myoclonus epilepsy, Kearns–Sayre syndrome, mitochondrial encephalopathy, etc. It is suspected that many other diseases such as diabetes mellitus, Alzheimer's, and Parkinson's diseases, etc., are due to dysfunction of mitochondria (Crimi and Rigolio, 2008). Variants such as point mutations, in the coding or noncoding region of protein-coding genes of mtDNA have been identified that correlates with a disease. For example, base substitutions in the protein-coding genes (~42) have been confirmed to cause diseases such as Leber hereditary optic neuropathy, MELAS, Leigh syndrome, etc. (for more information, visit mitomap.org).

There is also interest in mitochondrial tRNAs, as several pathological point mutations associated with neuromuscular disorders and diverse clinical phenotypes have been identified. For example, point mutations (~45) in the *tRNA* genes have been confirmed to be linked to various diseases including mitochondrial myopathy, MELAS and MERRF, Progressive encephalopathy, etc. For more information, visit MamittRNAdb (mamit-trna.u-strasbg.fr) and the mitomap database (www.mitomap.org). MITOMAP reports the published data on human mtDNA variations. By the end of December 2018, the total number of nondisease variants are 13,691 extracted from 47,412 full-length mtDNA sequences.

Development of next-generation sequencing technology

Development of automation of DNA sequencing based on the Sanger sequencing had revolutionized the study of molecular genetics. With the introduction of next generation of sequencing, technology in 2005 has dramatically decreased the cost of sequencing by massive quantity of data generated. Major developments have occurred in this field, which has abridged the time and cost involved in sequencing the whole genome. The cost of sequencing has been reduced from $5,292.39 per Mb in September 2001 to $0.012 per Mb in July 2017 as estimated by *National Human Genome Research Institute*. The entire genome sequencing cost is below $1000 in 2018. Besides, the cost of whole-genome is also dependent on the quality of sequences, that is, draft sequence (covering ~90% of the genome at ~99.99% accuracy) versus finished sequence (covering ~95% of the genome at ~99.99% accuracy). The finished sequence is much more labor intensive leading to higher cost. Therefore, most of the human sequences produced today are draft sequences. Since the human genome constitutes a large proportion of repeat sequences as well as intronic sequences, an alternative to whole-genome sequencing has emerged just to sequence only the portion of the genome involved in protein coding, that is, exons, called exome. The exome constitutes approximately 1.5% of the total human genome. Thus, the cost of exome sequencing is just a fraction of whole-genome sequencing.

This has been possible through the development of "massively parallel" sequencing technologies developed independently but significantly differ from capillary sequencing methods. These sequencing technologies called NGS technologies, which exclude the bacterial cloning step and use different methods to produce sequence information of millions of DNA molecules simultaneously. One of the major limitations of Sanger's sequencing was that only reaction per capillary tube could be analyzed at a time necessitating labor-intensive multiple steps in sample preparation, which included preparation of individual template fragments by randomly cloning the DNA fragments into cloning vectors, etc. as described in the earlier section leading to very high cost of sequencing. These sequencing techniques have completely been overtaken by the development of NGS technologies beginning with 2004. However, the sequence obtained by the HGP serves as a reference human genome sequence.

The NGS Technologies include the Roche/454 FLX pyrosequencer (introduced in 2004), the Solexa/Illumina genome analyzer (2006), and Applied Biosystems' SOLiD sequencer (2007), Helios Helioscope (2008), Ion Torrent PGM and Pacific Biosciences SMRT (2010), Oxford Nanospore MinION (2014), and Qiagen Gene Reader (2015) (Mardis 2013, 2017; Alekseyev et al., 2018). The first commercial NGS technology was introduced in 2004 by 454 Life Sciences (later purchased by Roche). This technology utilized luminescent detection of a pyrophosphate released upon incorporation of a correct nucleotide during sequence by synthesis (SBS) and produced relatively long sequences. This technology was used to sequence the genome of James Watson and the price dropped from $10 million with Sanger sequencing to about $2 million in just 2 months. It took 13 years to obtain the first human genome sequence. Thus, these massively parallel sequencing technologies reduce the time and cost involved in sequencing. More recently, Illumina introduced two new instruments, the HiSeq X and NextSeq 500. The first is able to sequence a human genome at

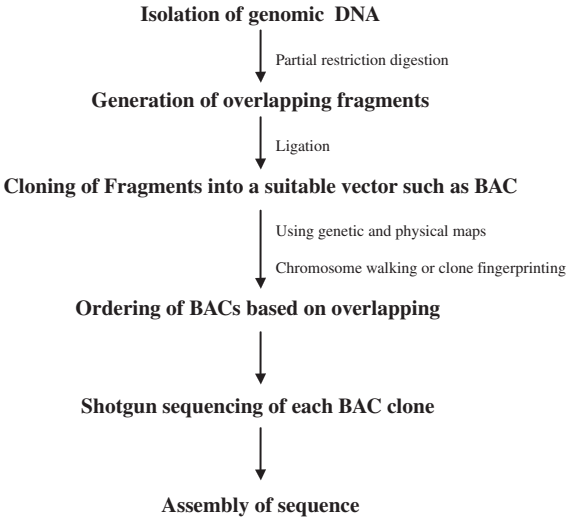
30× coverage for less than $1000, and the latter does the same for a slightly higher price but in less than 20 hours. Moreover, a new series of instruments introduced in 2017 (NovaSeq) should reduce the costs by almost another order of magnitude. A conceptually different sequencing platform called IonTorrent was introduced in 2011. This SBS technology detects the minute changes in pH caused by H^+ ions released during the incorporation of the correct nucleotide in the microenvironment around the beads with the attached clonally amplified DNA template molecules. Consequently, it does not require fluorescently labeled nucleotides and expensive optics to detect the fluorescence. Currently, the most popular applications from this company (now part of ThermoFisher) are targeted disease panels used in clinical settings including cancer (Alekseyev et al., 2018). Several companies now advertise to sequence the human genome for less than $1000. These sequencing technologies are also contributing to the detection of variants, including rare, copy number, or structural variants, single-nucleotide polymorphisms (SNPs), metagenomics, and transcriptomics studies such as gene expression, small RNA, or novel RNA discovery (Mardis, 2013, 2017; Alekseyev et al., 2018).

With the advancement of DNA sequencing technologies and consequent drastic reduction in cost of sequencing led to the establishment of several DNA testing companies. In 2007, 23andMe became the first company to begin offering a DNA testing for ancestry, which all other major companies now use. Its saliva-based direct-to-consumer (DTC) genetic testing business was named "Invention of the Year" by Time magazine in 2008. In September 2016, an ancestry-only version was being offered at a price of $99 with an option to upgrade to include the health component for an additional $125 later. The health component includes the predisposition to several genetic conditions. In April 2017, the Food and Drug Administration approved the applications for 10 predisposition tests: late-onset Alzheimer's disease, Parkinson's disease, celiac disease, hereditary thrombophilia, alpha-1 antitrypsin deficiency, glucose-6-phosphate dehydrogenase deficiency, early-onset of dystonia, factor XI deficiency, and Gaucher's disease for a DTC test for three specific *BRCA* mutations that are the most common *BRCA* mutations in the people of Ashkenazi descent. The rapid reduction in costs have already helped in clinical research and sequencing being used as a diagnostic tool for genetic diseases. While a large number of clinics and researchers have been able to access genetic data in cancer research and other diseases, consumer interest in genetic research has also encouraged by companies like 23andMe and AncestryDNA.

Principle and history of human genome sequencing

The major objectives of the HGP were to sequence the entire human genome, to identify all 20,000–22,000 genes, and to provide high-quality data of human genome sequences as a free resource. This was by far the most complex project of biology undertaken by mankind. In addition, the human genome has greater than 50% of repeat elements with regions of genomes that are duplicated or present in tandem or dispersed across the genome. It was therefore anticipated that without the development of genetic and physical maps, it would not be feasible to construct the human genome, as the presence of repetitive DNA would lead to mis-assemblies. Hence, a strategy was used by IHGSC; it was known as a "hierarchical," "clone-by-clone," "map-based," and "BAC"-based approach (Flow Chart 27.1) (IHGSC, 2001; www.genome.org). This approach required construction of a genetic map, a physical map, and a comprehensive library of large-insert DNA clones prior to human genome sequencing. Consequently, the initial major focus of HGP was construction of high-density genetic and physical maps. The development of DNA-based markers, restriction

Hierarchicalor clone by clone, map-based, BAC-based approach



FLOW CHART 27.1 Hierarchical or clone by clone, map based, BAC-based approach (IHGSC, 2001).

1. The DNA is isolated from samples provided by anonymous donors.
2. Next step is to generate overlapping fragments of desirable size into BAC vectors, which have high cloning capacity.
3. The BAC library thus obtained is used to prepare an ordered map with help of use of genetic and physical maps.
4. Each BAC clone is further fragmented into smaller size and sequenced via shotgun sequencing.
5. The genome sequence is assembled using computer programs.

fragment length polymorphism (RFLP), and later PCR-based marker simple sequence length polymorphism (SSLPs) that could detect DNA polymorphism, greatly enhanced the construction of a high-density linkage map. Physical mapping was also carried out at the same time. Another PCR-based DNA marker, called a sequence-tagged site (STS), was very useful in the construction of physical maps (Olson et al., 1989). STSs are short unique sequences of known DNA that occur only once in the genome. These can be derived from expressed sequence tags, microsatellites (SSLPs), or random genomic sequences (Brown, 2007). STS derived from SSLPs are particularly useful as they provide a direct connection between the physical and genetic maps. These molecular markers were very useful for ordering large segments of DNA. Hence, once the genetic and physical maps were made, and the next step was to sequence each BAC clone by shotgun sequencing.

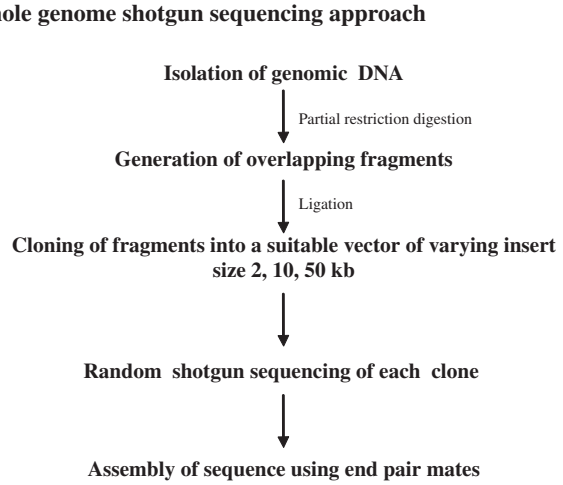
Prior to human genome sequencing, sequencing of more modest-sized genomes of model organisms such *as E. coli*, yeast, fruit fly, and *C. elegans* was carried out. Of these, *C. elegans* was the first animal to be completely sequenced. It has about 16.5% repeat elements in its genome. This provided the assurance that large-scale genome sequencing of complex multicellular organisms is possible. Sequencing of fruit fly and worm genomes also contributed to the development of software required to assemble and sequence annotations.

An alternative strategy of whole-genome shotgun sequencing was used by Celera Genomics. The feasibility of this method was demonstrated by sequencing the *Haemophilus influenza* genome (Fleischman et al., 1995). This strategy was first proposed by Weber and Myers and later by Craig Venter of Celera Genomics, as a rapid way to sequence the human genome. Unlike the strategy used for the publically funded genome sequencing, this strategy involves generation of small fragments of the genome, random shotgun sequencing to approximately fivefold coverage, and using software to obtain sequence assembly without prior ordering of BACs. It was argued that the human genome is too complex to be sequenced by this method. To test the hypothesis, the *Drosophila* genome was sequenced over a span of 1 year by this method. This project demonstrated that the chromosomal assembly is possible with high accuracy, order, and with <10-fold coverage (Venter et al., 2001).

Methodology used for initial Human Genome Project

In a hierarchical method (adapted from IHGSC, 2001) (Flow Chart 27.1), DNA was obtained from anonymous human volunteers of diverse backgrounds. All samples were stripped of their labels and were given random labels such that the identity of donors for the library could not be ascertained (in accordance with US Federal Regulation). The genome was fragmented into overlapping DNA molecules of high molecular weight (~100–200 kb) by partial restriction digestion. Please note sequencing a large genome such as the human genome requires vectors such as YAC or BAC, which has the capacity to clone large DNA molecules. A library of clones is thus obtained. Each clone has a contiguous stretch of genomic DNA, and it is expected that all the clones together comprise the complete genome. The DNA cloned in BACs is then ordered, covering the genome. Ordering of BACs is on the basis of the way in which they overlap, which shows that they contain overlapping segments of the genome. This can be done in several ways: chromosome walking, or clone fingerprinting and BAC end alignment. The existing genetic and physical maps were used for this purpose. Once this is done, the next step is to sequence them. The genomic fragment cloned in a BAC is too large to be sequenced. Hence, it must be sheared into smaller fragments before being sequenced by shotgun method. To increase efficiency and reduce price, it is desirable to cover the whole genome using a minimum number of clones and only sequence them. The selected clones were subjected to shotgun sequencing. The sequencing data obtained from various centers could be processed and assembled using PHRED and PHRAP software packages. The PHRED software package made use of "base-quality control," which is helpful in monitoring raw data quality. The PHRAP computer program assembled the sequence into sequence contigs on the basis of base-quality scores and assigned "assembly-quality scores." Assembled contigs of more than 2 kb were deposited in a public database within 24 hours of assembly and were freely available to the public as per the principles of HGP.

The hierarchical approach is technically demanding, slow, and costly because it requires construction of a genetic and physical map before sequencing is undertaken. There is also a possibility of rearrangements of large-insert clones, which can cause complications during genome assembly. This can be prevented using quality control measures. Nevertheless, this approach greatly reduces the challenge of assembling the finished sequence of complex and outbred organisms, like humans, who have a high percentage of repetitive DNA. Because the relative order of BACs is known, the possibility of long-range mis-assembly is reduced. This approach ensures complete coverage of the genome and helps reduce sequencing redundancy. It was believed that this strategy would be able to identify regions that showed cloning biases, which could



FLOW CHART 27.2 Whole-genome shotgun sequencing approach (Venter et al., 2001).

1. The DNA is isolated from samples provided by anonymous donors.
2. Next step is to generate overlapping fragments of desirable sizes.
3. This is followed by cloning of DNA fragments in appropriate vectors.
4. Random shotgun sequencing is carried simultaneously for all the clones.
5. The genome sequence is assembled using computer programs.

be then focused for additional sequencing. As the IHGSC was a collaboration of different countries, the work and responsibility could be easily disseminated among its members using this strategy (IHGSC, 2001).

Whole-genome shotgun sequencing: For HGP, after obtaining a certificate to protect the privacy of individuals, the DNA from five individuals—one African-American, one Asian-Chinese, one Hispanic-Mexican, and two of Caucasians—were selected for genome sequencing (Flow Chart 27.2). This method involves breaking DNA into smaller size fragments and requires generation of a high-quality plasmid library. Clones are randomly sequenced, and a master sequence is obtained based on overlapping stretches of sequences using a computer program. The central feature of this strategy is to obtain paired-end reads (mate pairs), which are 500–600 bp in length from both ends of the inserts. The end sequences of the BACs provide a connection between the continuous sequences across the genome, which allows simultaneous mapping. As a result, the time taken by this method to produce a whole-genome sequence is greatly reduced (Venter et al., 2001).

Examples with applications

The availability of a reference human genome sequence has given several new insights into biology and has also enabled a broad range of scientific advances, some of which are discussed below:

Identification of disease-related genes: Even before sequencing of the human genome began, linkage maps prepared by pedigree analyses were used to identify genes responsible for causing disease. Mapping genes using RFLP helped in defining gene positions. This helped in positional cloning of genes. Huntington disease was the first genetic disease mapped on chromosome 4 (using a DNA polymorphism). The first human disease gene to be positionally cloned was chronic granulomatous disease, followed by Duchenne muscular dystrophy and retinoblastoma. The availability of genetic and physical maps (and later the human genome) allowed rapid identification of candidate genes in silico. Mendelian disorders are now readily identified in short timespans, aided by the whole-genome sequence. More than 6000 Mendelian diseases have been catalogued (http://www.ncbi.nlm.nih.gov/omim). Several genes of biomedical importance have been identified using resources and information. Phenotypes include (1) single-gene Mendelian disorders and traits; (1) susceptibilities to cancer and complex diseases (e.g., BRCA1 and familial breast-ovarian cancer susceptibility and Complement Factor H (CFH) and macular degeneration); (3) variations that lead to abnormal but benign laboratory test values ("nondiseases") and blood groups (e.g., lactate dehydrogenase B deficiency, and ABO blood group system); and (4) select somatic cell genetic disease (e.g., GNAS and McCune-Albright syndrome, and IDH1 and glioblastoma multiforme). The total number of phenotypes for which the molecular basis is known are 6309, and total number of genes with phenotype-causing mutation are 4001 (http://www.ncbi.nlm.nih.gov/omim). Table 27.6 lists various disorder entries in the OMIM database.

TABLE 27.6 OMIM morbid scorecard (updated November 21, 2018).

Class of phenotype	Phenotype	Gene[a]
Single-gene disorders and traits	5261	3621
Susceptibility to complex disease or infection	700	506
"Nondiseases"	146	115
Somatic cell genetic diseases	219	122

[a]*Some genes may be counted more than once because mutations in a gene may cause more than one phenotype and the phenotypes may be of different classes (e.g., activating somatic BRAF mutation underlying cancer and germline BRAF mutation in Noonan syndrome).*

The 1000 Genomes Project

The 1000 Genomes Project ran between 2008 and 2015, creating the largest public catalog of sequence

variations and genotype data. The goal of the 1000 Genomes Project was to find most genetic variants with frequencies of at least 1% in the populations studied. The 1000 Genomes Project took advantage of developments in sequencing technology, which sharply reduced the cost of sequencing. It was the first project to sequence the genomes of a large number of people and to provide a comprehensive resource on human genetic variation. Data from the 1000 Genomes Project were quickly made available to the worldwide scientific community through freely accessible public databases. The final data set contains data for 2504 individuals from 26 populations (The 1000 Genomes Project Consortium, 2015; Sudmant et al., 2015).

Genome-wide association studies

With the drastic reduction in cost of sequencing, the genome sequencing of large cohorts has become routine. The basic premise of the whole-genome sequencing was to sequence the genomes of a large number of individuals diagnosed with the same ailment especially the complex diseases such as diabetes, cardiovascular diseases, neurological disorders, and certain categories of cancer. Then, it compares their sequences with those of the healthy individuals to detect possible sequence variants to predict the association of the variant with disease. This could possibly predict the possible predisposition of individuals to that disease. With the availability of the human genome, the next logical step was to identify genetic variants that predispose some individuals to common diseases such as cancer, diabetes, depression, and heart diseases. Unlike Mendelian diseases, these common diseases are caused by multiple genetic and environmental factors working together. To understand the genetic factors involved, one can compare the genomes of healthy and disease-carrying individuals to look for the association of genetic factors with that particular disease. Genome-wide association (GWA) studies have been defined as "any studies of common genetic variation across the entire human genome designed to identify genetic associations with observable traits" (Manolio and Collins, 2009). Such genetic analysis in humans requires variations or polymorphisms. The International HapMap Project was conceived in 2002 with the aim of providing data on human genetic variation that could be used for gene identification associated with common diseases. The International HapMap project is an extensive collaborative effort between Canada, China, Japan, Nigeria, the United States, and the United Kingdom. Its goal is to create a database of human genetic variations, particularly SNPs, which are present once in every 300 bp of the human genome. In addition to their abundance, SNPs are easy to genotype on large scale, are present throughout the genome, and hence are useful in GWA studies (http://www.hapmap.org and http://hapmap.ncbi.nlm.nih.gov/). The NCBI has since decommissioned this site by stating that "the number of novel variants is constantly increasing and many believe that the 1000 Genomes Project could potentially overshadow the utility of HapMap," and this information can now be accessed through https://www.ncbi.nlm.nih.gov/variation/tools/1000genomes/.

Even before the complete human genome sequence was available, more than 1.4 million SNPs had been identified, and thereafter, their numbers have been increasing (https://www.ncbi.nlm.nih.gov/snp). If a particular SNP or group of SNPs is more common among people carrying the disease/trait. SNP is more likely to be associated with and can be used to locate genes involved in the phenotype. Thus, HapMap made possible GWA studies, in which numerous SNPs are tested for their association with a condition or disease. These studies have provided valuable insight into the physiopathology of diseases. Such studies are now possible, and they are less time consuming and cheaper because of the availability of the human genome sequence and cost-effective genotyping technologies. For more information, an updated catalog of published GWA studies can be obtained from *European Bioinformatics Institute (EMBL-EBI)* at http://www.ebi.ac.uk/gwas or MacArthur et al. (2017).

Some examples of GWA collaborations are the following: Genetic Association Information Network (GAIN), which studies attention deficit hyperactivity disorder (ADHD), bipolar 1 disorder, schizophrenia, major depressive disorder, etc., and Cancer Genetic Markers of Susceptibility (CGEMS), which studies prostrate, breast, lung, bladder, renal, pancreatic cancers, etc. (Manolio and Collins, 2009; Manolio et al., 2008). Such studies have implicated previously unknown mechanisms in certain conditions. For example, in age-related macular degeneration, a complement-mediated inflammation is involved. Cell-cycle regulators (CDKN2A and CDKN2B) have been shown to be associated with coronary diseases and CDKAL1 in type 2 diabetes (Manolio, 2010). The ultimate goal of the GWA studies is to provide information so that better decisions can be made in terms of risk assessment, diagnosis, and drug therapies. Information from such studies will be useful in identifying new therapeutic targets and in pharmacogenetic and pharmacogenomc studies (Zhang et al., 2008; Green et al., 2011). The use of genomic approaches in understanding the molecular basis of diseases is best depicted by the study of a gastrointestinal disorder: Crohn's disease. GWA studies have revealed the role of autophagy, innate immunity, and interleukin signaling in this ailment. Thus, this genomics approach can lead to better understanding of diseases, identify new

variations, or biological mechanisms that will contribute to development of new treatments, early detection, or prevention. GWAS have also been used for link between variation and response to a drug (favorable or unfavorable). For more information, visit (https://www.pgrn.org/riken-gwas-statistics.html). Thus, the information gained through GWAS will also contribute toward personalized or precision medicine

Ethical issues

The bright side of whole-genome sequence information is that it has tremendous potential for use in medicine, biology, genetic counseling, etc. However, access to this information also has a potential dark side because it can impact individuals in various ethical, legal, and social ways. One of the goals of HGP was to address the ethical, legal, and social issues that could arise as a consequence of genomic research (resources from www.genome.gov). With the whole-genome sequences and subsequent data freely available to the public, concerns were raised. The information can be misused by schools, courts, adoption agencies, employers, and in particular, health insurers, who may discriminate on the basis of an individual's genetic makeup. For example, if a person is a carrier or has a predisposition to certain diseases that can be determined by genetic tests, he or she could be unfairly refused employment by a company. A medical insurance agency might not cover such an individual, or might charge hefty insurance premiums. This is discrimination based on a person's genetic information and could be exploited by unscrupulous companies. Social issue concerns, such as stigmatization of an individual based on race or ethnicity and their genetic differences, could have a profound psychological impact or could create social disparity. To prevent misuse of genomic information, the Genetic Information Non-Discrimination Act was passed in the United States (Hudson et al., 2008). This law prohibits discrimination of individuals by insurance companies and employers based on information from genetic tests.

Translational significance

Development of pharmaceutical drugs to treat disorders and diseases requires understanding of suitable drug targets. Information about the complete set of human genes and proteins has increased the search for suitable drug targets. In addition, knowledge about paralogs has provided an opportunity for new therapeutic interventions. Already, several new paralogs of common drug targets have been identified in silico that could represent new drug target candidates. Some examples include calcitonin (CALCA), dopamine receptor, D1-α, insulin-like growth factor-1 receptor, etc. (IHGSC, 2001).

As mentioned earlier, GWAS is helping to understand the molecular basis of complex diseases, which in turn is pinpointing or identifying new or candidate targets for drug development. In addition, Hapmap and GWAS have allowed identification of clinically important genetic polymorphisms that have been linked to differences in response to drugs by different individuals. Consequently, the field of pharmacogenomics (pharmacology + genomics) has evolved in the postgenome era; it is based on the fact that the response of different individuals to a drug varies due to variations in their DNA sequences. These could be variations in drug receptors, drug transporters, or drug-metabolizing enzymes (Nwanguma, 2003). Completion of HGP, one can identify people who can or cannot efficiently metabolize a drug. This information can also be used to distinguish individuals who show an adverse response to a drug. Such studies will assist in providing patients and physicians with individualized drug therapy or personalized medicine; this will make it possible to decide which drug and what dosage should be prescribed based on a patient's genetic predispositions. Clinical testing for genetic disorders is already available; this will have implications for carrier screening, prenatal diagnosis, preimplantation genetic diagnostics, presymptomatic testing, etc. Results based on these tests will be useful for discussing the probability of having genetic defects in progeny and hence may be valuable in marriage counseling, in vitro fertilization, etc.

Precision medicine

One of the outcomes of the human genome sequencing has been a realization that 99.9% of genome sequences of any two individuals are same. The differences among the individuals reside in 0.1% of the sequence. Undoubtedly 0.1% of ~3.2 billion bp are sequences amounting to up to 3 million bp. Genetic differences among the individuals have been known even before the human genome sequencing. Two examples would suffice to highlight these variations in humans: (1) two different individuals suffering from the same disease and treat with the same drug may not respond in a similar way and (2) the manifestation of AIDS following infection with HIV in different individuals is not identical. Some manifest the disease earlier and others keep on harboring the virus for years together without apparently manifesting the disease. Now after the genome sequencing it has become

evident that these variations, in large part, could be due to SNPs. It was conjectured that if these variations could be identified and delineated by sequencing, a particular drug based on this genetic information might be designed. This led to the concept of individual-based therapy or precision medicine.

The tremendous advancement in sequencing technologies and drastic reduction in cost of sequencing have led to the realization that an era of precision medicine has begun. The beginning of personalized genomics was probably started with the Harvard 1000-Personal Genome Project. Ginsberg and Philips (2018) make a conceptual distinction between personalized and precision medicine as follows: "*Personalized medicine* refers to an approach to patients that considers their genetic makeup but with attention to their preferences, beliefs, attitudes, knowledge and social context, whereas *precision medicine* describes a model for healthcare delivery that relies heavily on data, analytics, and information." Though both personalized and precision medicine are based on use of genomics/DNA sequencing for healthcare in an overlapping way. Now DNA sequences could be used as pediatrics clinical genomics especially for (1) prenatal genetic testing for detection of disease risk in a developing fetus by sequencing fetal DNA or once technology is developed, using small amount of fetal DNA circulating in the maternal bloodstream, and (2) newborn screening for diseases as well as for predisposition to diseases. In his 2015 State of the Union address, US President Obama announced that he is launching the Precision Medicine Initiative—a bold new research effort to revolutionize how we improve health and treat disease. Until now, most medical treatments have been designed for the "average patient." As a result of this "one-size-fits-all" approach, treatments can be very successful for some patients but not for others. Precision medicine, on the other hand, is an innovative approach that takes into account individual differences in people's genes, environments, and lifestyles. It gives medical professionals the resources they need to target the specific treatments of the illnesses we encounter, further develops our scientific and medical research, and keeps our families healthier. Precision medicine based on sequencing of targeted gene panels from a large number of cancer patients may have applications. However, so far the results have been mixed and potential benefits debatable (Kumar-Sinha and Chinnaiyan, 2018). However, the precision medicine or personalized medicine holds a great potential. It already started transforming the way we can treat diseases such as cancer: Patients with breast, lung, and colorectal cancers, as well as melanomas and leukemias, for instance, routinely undergo molecular testing as part of patient care, enabling physicians to select treatments that improve chances of survival and reduce exposure to adverse effects.

DNA encode project: Short DNA sequences located in the upstream region of genes have been known to regulate the expression of genes, by transcriptional activation of genes, upregulation or downregulation, or modulation of gene expression. There are a variety of elements known to be present throughout the genome. The goal of the pilot phase of the Encode Project (2003−07) was to comprehensively study the structural and functional elements encoded in 1% of the human genome. The objective was to map a variety of sequences involving genes (protein-coding and noncoding exons), regulatory regions such as promoters, enhancers, termination sites, RNA transcripts, binding sites of transcription factors, epigenetic changes such as DNA methylation, chromatin accessibility, etc. All this information has further advanced our understanding of the function of the human genome. More information can be obtained from their website (http://genome.ucsc.edu/ENCODE). With the success of the pilot project, the focus is now on the remaining 99% of the genome (The ENCODE Project Consortium, 2007; The ENCODE Project Consortium, 2012). Such studies would not have been feasible on a large scale without the decoding of the human genome.

Comparative genomics: A complete version of the human genome has also significantly enhanced comparative genomics. The human genome can now be compared to that of a mouse, rat, fly, etc., to understand human genetic makeup and function. Laboratory experiments on these model organisms can reveal the functions of genes and thus their homologs in humans. Several other genomes of vertebrates and nonvertebrates have been sequenced or are being sequenced, such as zebrafish, pufferfish, sea urchin, honeybee, platypus, dog, chicken, and bovine, as well as primates such as chimpanzee, rhesus macaque, etc. The chimp genome sequence is the first nonhuman primate sequence available and provides an important tool to study our genome with respect to a closely related species. The human and chimp genomes are 99% identical, with 10 times more genetic differences than between 2 humans and 60 times less than between mouse and human (resources from www.genome.gov). Such information will be useful in comparative genomics and will allow biologists to elucidate gene functions as well as do evolutionary studies. Such studies will also identify key developments that led to vertebrate and nonvertebrate lineages.

Animal biotechnology has made significant contributions to HGP and holds the potential to contribute much more. Animal model systems not only allow us to study the molecular basis of the pathophysiology of human diseases but also provide systems for developing and testing new therapies. Of these, the mouse has succeeded as the

best human disease model system so far [e.g., Duchenne muscular dystrophy and cancer]. The mouse has a similar set of genes to humans and consequently is the best model for understanding the function of genes. Knock-in, constitutive knockout, conditional knockout, and knockdown mice, in combination with RNA interference technology, provide useful tools to study gene functions (Chaible et al., 2010). More than 1000 mouse knockout mutations are available, which will help in understanding the functions of disrupted genes, especially those of biomedical importance.

World Wide Web resources

http://asia.ensembl.org/Homo_sapiens/Info/Index: Provides open access to HGP and other sequencing projects.

http://www.ncbi.nlm.nih.gov/omim: Maintains a catalog and information about Mendelian diseases.

http://www.genome.gov/GWAStudies: Contains a catalog of the genetic associations between diseases and genomic loci.

http://genome.gov/encode: Has a catalog of functional elements in the human genome.

http://www.1000genomes.org: A database that stores information about DNA variations.

http://hapmap.ncbi.nlm.nih.gov; https://www.ncbi.nlm.nih.gov/snp : A database of SNPs.

http://www.genome.gov: Provides information regarding human genome project.

http://www.noncode.org: Describes and catalogs ncRNA.

http://www.mitomap.org: A database focused on the mitochondrial genome.

https://www.pgrn.org/riken-gwas-statistics.html: Database regarding GWAS studies related to drug response.

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Glossary

Bacterial artificial chromosomes *Escherichia coli* F-plasmid-based DNA cloning vector with capability to clone large DNA fragments.
Clone fingerprinting A technique to identify overlapping clones by generating a DNA fragment profile.
Contig Is a set of overlapping DNA sequences.
CpG islands Regions of genome that are rich in GC content and usually found at the 5′ end of the genes. C in the CpG is a potential site for cytosine methylation.
Dicer Dibonuclease that plays a central role in RNA interference.
DNA Deoxyribonucleic acid is a genetic material.
Euchromatin Is the decondensed form of chromatin that stains lightly in G banding and contains transcriptionally actively genes.
Finished sequencs A sequence of DNA that is no less than 99.99% accurate and ideally has no gaps. In case gaps cannot be closed, size of the gap is indicated.
Genome A complete set of DNA information of an organism.
Heterochromatin The condensed form of DNA that appears as darkly stained bands in G banding.
Human Genome Project A publically funded project that was initiated with the goal of sequencing the entire human genome and to identify all the genes and other features encoded by it.
Noncoding RNA A plethora of RNA molecule that do not code for any protein and are involved in variety of functions.
PCR A molecular biology technique to amplify exponentially a segment of DNA in a vial.
Positional cloning Also called map-based cloning is a procedure by which a gene is cloned based on information about its position on a physical/genetic map.
Pseudogene A nonfunctional copy of a gene.
Repetitive DNA A DNA sequence that is present many times in a genome.
Restriction fragment length polymorphism (RFLP) A class of molecular markers that detects DNA polymorphism by use of restriction enzymes and change in size of restriction fragment, generated due to change in a nucleotide in the recognition site of the restriction enzyme.
Retrotransposon Transposable elements that replicate via RNA intermediate, possibly originated from retroviruses.
RNA interference (RNAi) A term used for the process of gene silencing mediated by miRNA.
Sequence-tagged site (STS) Is a defined unique sequence present only once on a genome.
Shotgun approach Method of sequencing in which DNA is randomly broken into fragments and sequenced.
Simple sequence repeats (SSR) Are short tandem repeats (2–6 bp) of DNA, also called microsatellites.
Simple sequence length polymorphism (SSLP) Another type of DNA polymorphism that detects variation in length of repeat sequences between individuals.
Single-nucleotide polymorphism (SNP; pronounced as snip or plural snips) A DNA sequence variation occurring due to a single nucleotide (A, T, G, or C) in chromosomes.
Transposons Mobile DNA sequences that can move from one region to another region of the genome.
Transfer RNA Adaptor molecules that decode genetic code of RNA into amino acid sequence.
Whole-genome shotgun approach Strategy used by Celera genomics to sequence human genome by random shotgun sequencing.
Yeast artificial chromosomes (YAC) Yeast-based cloning vector that can clone very large DNA inserts.

Abbreviations

BAC Bacterial artificial chromosome
DOE Department of Energy, United States
dNTP Deoxyribonucleotide triphosphate
EnCode Encyclopedia of DNA elements
GWAS Genome-wide association studies
HGP Human Genome Project
IHGSC International Human Genome Sequencing Consortium
LINE Long interspersed nuclear element
LTR Long-terminal repeat
miRNA microRNA
ncRNA Noncoding RNA
NHGRI National Human Genome Research Institute, United States
NIH National Institute of Health, United States
OMIM Online Mendelian Inheritance in Man
PCR Polymerase chain reaction
PP Processed pseudogene
RFLP Restriction fragment length polymorphism
RISC RNA-induced silencing complex
RNAi RNA interference
rRNA Ribosomal RNA
SINE Short interspersed nuclear element
SNP Single-nucleotide polymorphism
snRNA Spliceosomal small nuclear RNA
SnoRNA Small nucleolar RNA
SSLP Simple sequence length polymorphism
SSR Simple sequence repeat
STS Sequence tagged site
UTR Untranslated region
YAC Yeast artificial chromosome

Long answer questions

1. Describe various strategies used for sequencing the human genome. Explain advantages and disadvantages of each.
2. What are repetitive DNA elements? Describe them with respect to the human genome.
3. Describe noncoding RNAs.
4. Elaborate on human genome organization.
5. Describe how information from HGP contributed to the welfare of human society as a whole.

Short answer questions

1. Write short notes on the following:
   a. miRNA.
   b. Pseudogenes.
   c. LINE.
   d. SINE.
2. Write a short note on the mitochondrial genome.
3. What are the advantages of using a hierarchical method for the sequencing of complex eukaryotic genomes?
4. List major bottlenecks encountered by the publically funded genome project and breakthroughs that helped to achieve its goal.
5. Discuss various ethical, legal, and social issues associated with sequencing the human genome.

Answers to short answer questions

1a. miRNA are a class of noncoding RNAs. They are ~19–25 nucleotides long, evolutionarily conserved, small, single-stranded molecules that regulate gene activity at the posttranscriptional level by a phenomenon related to RNA interference. The primary transcript of miRNA has a 5′-cap, 3′-polyA tail and an inverted repeat that can base pair to form a hairpin structure. A nuclear RNase III complex (Drosha) processes this transcript to release an atypical hairpin loop precursor miRNA (pre-miRNA), which moves to the cytoplasm where it is cleaved by the enzyme Dicer, a cytoplasmic RNase III, to release mature miRNA. In the cytoplasm, an effector complex called RNA-induced silencing complex (RISC) containing argonaute RNase degrades one strand of the miRNA to leave a mature single-stranded miRNA (guide strand) bound to the argonaute. This complex then selectively base pairs with the target RNA with the sequence, complementary to the guide strand. The binding usually occurs at the 3′-untranslated regions (3′-UTRs) of the target mRNA sequence, but in some cases, 5′-UTRs are also involved. Thus, an miRNA can target several gene transcripts. Depending on the extent of similarity, target RNA is degraded, destabilized, or prevented from being translated. A total of 1756 miRNA genes have been identified in the human genome. Chromosome 1 has 134 miRNA genes, while the Y chromosome has the least (~15). Chromosome 19 has 110 miRNA genes. ncRNAs seem to have occupied a center stage because of their involvement in many human diseases. Many human diseases have been identified that may be due to mutations or dysfunction of ncRNAs. For example, Prader–Willi syndrome, cancer, central nervous disorders, cardiovascular diseases. Utilizing the properties of miRNAs and RNA interference mechanisms, they have great potential as therapeutic agents in terms of downregulating the expression of specific genes.

1b. Pseudogenes are the nonfunctional copies of normal genes with which they share sequence homology. They can be of two types: duplicated pseudogenes and processed pseudogenes (PPs).

Pseudogenes derived from the duplication of genomic DNA are called duplicated pseudogenes, as they possess characteristic exon−intron structure. Such pseudogenes are derived by tandem duplication, and as a result, they are located close to the functional copy. In contrast, "retrotransposed," or PPs, are derived from reverse transcription of processed transcripts that integrate into the genomic DNA. Therefore, they are devoid of introns. They also possess polyA tracts and direct repeats at either ends of the pseudogenes. Lack of a promoter usually results in an inactive gene copy. There are about 14,427 pseudogenes. The prevalence of pseudogenes in the human genome has been problematic for genome annotation. The highly expressed housekeeping genes have multiple PPs. For example, ribosomal proteins account for ~20% of human PPs. Protein-coding pseudogenes are more easily identified than RNA pseudogenes.

Pseudogenes were believed to be nonfunctional, but evidence has shown that many pseudogenes (2%−20%) are transcribed into RNA. Examples include pseudogenes for tumor suppressor PTEN, glutamine synthetase, glyceraldehyde-3-phosphate dehydrogenase, etc. Many pseudogenes have been found to have tissue-specific expression, and some have an expression pattern that is different from the parent gene. Alteration in pseudogene expression has been seen under different physiological conditions, such as diabetes and cancer. Some pseudogenes have been implicated in gene regulation in mice via siRNAs production.

1c. LINEs are a type of non-LTR retrotransposon that replicate via an RNA intermediate. They are the most primitive autonomous mobile elements found in the human genome. In humans, three distantly related LINE families are present: LINE1, LINE2, and LINE3, of which only LINE1 is still active. LINE machinery has resulted in reverse transcription in the human genome (including those of SINE and PPs). These retrotransposons are about 6 kb long, encoding two ORFs and possess a polymerase II promoter. Transposition of LINE occurs via RNA that assembles with encoded proteins and moves back to the nucleus. LINE transposition preferably occurs in AT-rich regions. The preference of LINEs for AT-rich DNA could be explained by the fact that these regions are poor in genes, and as a result, these elements do not encounter a functional selection pressure. The LINE endonuclease cleaves at TTTT/A in the DNA sequence where the LINE element is inserted into the genome.

1d. SINE is a type of non-LTR retrotransposon that replicate via an RNA intermediate. Unlike LINEs, SINEs are nonautonomous retrotransposons. They cannot transpose independently, and are believed to use the LINE machinery for transposition. Nevertheless, they have a very high copy number within the mammalian genome. They share their 3′-end with the LINE element. They are 100−400 bp long, encode no protein, but possess an internal polymerase III promoter. There are three distinct monophyletic families of SINEs: the active Alu, and the inactive MIR, and Ther2/MIR3. There is a promoter of all SINEs, but one is derived from *tRNA* genes. The Alu elements are the only active class of SINEs that have a derived promoter from the signal recognition particle component 7SL. The Alu family is the most abundant sequence in the human genome, occurring more than once in 3 kb on an average. They are of comparatively recent evolutionary origin. An Alu repeat is about 280 bp long and consists of two asymmetric tandem repeats, each about 120 bp long followed by a short A_n/T_n sequence. Again, unlike LINEs, which are preferentially located in AT-rich regions, Alu repeats are found in gene-rich, high GC-rich regions, indicating their positive role in genome function. The fact that Alu sequences use LINE machinery for transposition (but are found in GC-rich regions), indicates that their distribution is reshaped by evolutionary forces. Some Alu sequences are transcribed, and it is believed that these elements may have been involved in the evolutionary dynamics of the human genome.

2. The human mitochondrial genome is small in size (16,569 bp). It is a circular molecule present in multiple copies. Being small in size, the mitochondrial genome was the first to be sequenced. The complete sequence of the mitochondrial genome was made available in 1981 by Sanger and colleagues. The mitochondrial genome has 37 genes that include 22 tRNAs, 2rRNAs, and 13 protein-coding genes. The entire mtDNA is transcribed, and it contains small noncoding regions. The gene density of mtDNA is 1 per 0.45 kb. All 37 mitochondrial genes lack introns. Protein-coding genes encode proteins involved in the electron transport chain and oxidative phosphorylation. In fact, a majority of proteins present in mitochondria are encoded by nuclear genes. The mitochondrial genetic code has 60 codons and 4 stop codons. The stop codons include UAA and UAG, which are universal stop codons,

and AGA and AGG, which specify arginine in the nuclear genetic code. The universal stop codon (UGA) codes for tryptophan in mitochondria, and AUA codes for methionine. Thus, the mitochondrial genome has its unique genome organization and is different from the nuclear genome.

3. A hierarchical approach greatly reduces the challenge of assembling the finished sequence of complex and outbred organisms (like humans) that have a high percentage of repetitive DNA. Because the relative order of BAC is known, the possibility of long-range mis-assembly is reduced. This approach also ensures complete coverage of the genome and helps to reduce the sequencing redundancy. In addition, it was believed that this strategy would be able to identify regions that show cloning biases, which could then be focused for additional sequencing. As the IHGSC was a collaboration of different countries, the work and responsibility could be easily disseminated among its members by this strategy.

4a. Some major bottlenecks encountered in the publicly funded Human Genome Project were the following:

- **i.** The nonavailability of high-density genetic and physical maps. These maps are used in assembling the sequence of the whole genome of complex organisms like humans.
- **ii.** Keeping in view the small cloning capacity of the then-current cloning vehicles, there was a need to look for better alternatives, which made cloning of large inserts possible.
- **iii.** The time taken and cost of sequencing using existing technologies at that time were very high.
- **iv.** There was a need to store and analyze large amounts of sequencing data, and hence, there was a need to develop better algorithms for computations (e.g., development of software packages to analyze sequence data).
- **v.** Practical difficulties such as cloning bias and the presence of a large quantity of repeat sequences in the human genome all provided a hurdle to sequencing and assembly of the human genome sequence.

4b. The Human Genome Project was aided by several breakthroughs, some of which have been described as follows:

- **i.** Sanger's method of sequencing, and its automation and miniaturization, use of fluorescent dNTPs, and development of capillary-based sequencing machines, greatly accelerated the speed of sequencing.
- **ii.** DNA-based genetic markers that helped in the construction of high-density genetic and physical maps, greatly helped in assembly of YAC/BAC clones, and hence the sequence assembly.
- **iii.** Polymerase chain reaction, which was invented in 1983, also propelled genetic research.
- **iv.** Development of large-insert cloning systems such as *E. coli*-based bacterial artificial chromosomes (BAC), which can carry large segments of DNA, contributed to the success.

5. With the whole-genome sequence and subsequent data freely available to the public, concerns were raised as to how this knowledge would impact an individual or society as a whole. There was apprehension that this information would be misused by schools, courts, adoption agencies, employers, and particularly health insurers, who would discriminate on the basis of an individual's genetic makeup. For example, if a person is a carrier or has predisposition to certain diseases that can be determined by genetic tests, he or she may not be hired by a company. A medical insurance agency might not cover such an individual, or might charge hefty insurance premiums. This would be discrimination based on genetic information of a person and could be exploited by greedy companies. Social issue concerns, such as stigmatization of an individual based on race, ethnicity, or genetic differences, could have a profound psychological impact or disparity. To prevent misuse of genomic information, the Genetic Information Non-Discrimination Act was passed in the United States. (Hudson et al., 2008). This law prohibits discrimination of individuals by insurance companies and employers based on information from genetic tests.

Yes/no type questions

1. In the HGP, the method of sequencing used was next-generation sequencing.
2. A "BAC library" is a collection of entire human genome cloned in a BAC vector.
3. In HGP, YACs were preferred over BACs.
4. In the methodology used by IHGSC, physical maps were not a requisite for genome sequencing.
5. "*Personalized medicine* refers to an approach to patients that considers their genetic makeup but with attention to their preferences, beliefs, attitudes, knowledge and social context."
6. Fredrick Sanger proposed "whole-genome shotgun sequencing."

7. The "Draft genome," which was released in 2001, covered 90% of the human genome.
8. Next generation of sequencing technology has dramatically decreased the cost of sequencing as well as made possible generation of massive quantity of data in a small time.
9. Human genome has very less percentage of repetitive DNA elements.
10. Transposable elements are DNA sequences that change their location within the genome.

Answers to yes/no type questions

1. No—The method of sequencing used was automated DNA sequencing method.
2. Yes.
3. No—BACs were preferred due to problems associated with the YACs.
4. No—Physical maps were generated before sequencing began on large scale.
5. Yes.
6. No—It was proposed by Craig Venter of Celera genomics.
7. Yes—It had many gaps.
8. Yes.
9. No—It has a very high percentage repetitive DNA.
10. Yes—They are of different types, also found in human genome.

CHAPTER

28

Marine resources and animals in modern biotechnology

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Summary

Marine environment is the widespread untapped, least-explored reservoir of the flora and fauna. Elucidation of recent advances in biotechnological exploitation of marine animals and novel bio-active products for human welfare is the goal of this chapter. Exploring the diversity, their sustainable application for food, pharmaceuticals, ornamental purposes, and many more in association with their genomics is the focus of the chapter.

What can you expect to know

Marine world represents a large untapped reservoir for biotechnological applications as least-explored diverse microorganisms and other floral and faunal diversity reside in it. Marine organisms are of enormous scientific interest as they possess wide diversity, unique structures, metabolic pathways, reproductive systems, sensory and defense mechanisms due to their adaptation to the extreme environmental conditions unparalleled to their terrestrial counterparts. Marine biotechnology has received huge attention nowadays due to its vast majority of applications in the field of food industry, pharmaceuticals, ornamental industry, and many more. However, the knowledge in marine genomics needs to be broadened for a better output from the above-mentioned industries.

Introduction

Oceans are the largest ecosystem on the planet encompassing around 70% of the living organisms. Owing to extremes of temperature, salinity, light and pressure conditions, oceans harbor huge marine biological diversity at various zones and niches including hydrothermal vents, continental shelves, cold seeps, asphalt volcanoes, oceanic trenches, and submarine canyons (UNEP, 2006). Marine organisms play an indispensible role in maintaining the biogeochemical cycle and balancing water carbonate chemistry. Apart from generating economy through tourism, recreation and transportation sectors, oceans serve as an essential source of seafood and pharmaceuticals (Kijjoa and Swangwong, 2004).

Marine biotechnology is poised to speculate and exploit the untapped reservoir of marine life with huge biotechnological potential. Ever since the discovery of early oceanic expeditions, oceans have been exploited for novel bioactive compounds, enzymes, pharmaceuticals, and important source of raw materials for industrial applications (Fig. 28.1). Additionally, with the advent of the sophisticated sampling techniques, underwater robots, lasers, sonars, and deep sea submersibles, there have been increased opportunities for marine researchers to venture into the realm of marine biotechnology and decipher its unexplored applications (Thakur and Thakur, 2006).

Marine biological diversity

Historical background

According to the Greek mythology, oceans were considered as the source of food and means of transportation. Ever since the emergence of human race on earth, marine life became the primary source of food, trade, and warfare for the early man who stayed

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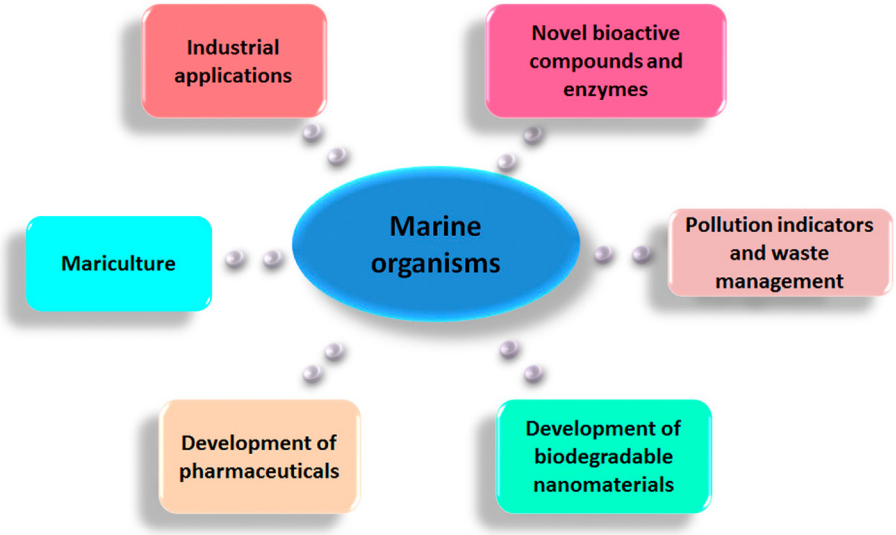

FIGURE 28.1 Overview of biotechnological applications of marine organisms.

TABLE 28.1 Average depths and total areas of four major ocean basins (Castro and Huber, 2003).

	Area		Average depth	
Ocean	Millions of km^2	Millions of mile^2	Meters	Feet
Pacific	166.2	64.2	4188	13,741
Atlantic	86.5	33.4	3736	12,258
Indian	73.4	28.3	3872	12,704
Arctic	9.5	3.7	1330	4,360

confined to the shores. The discovery of stone tools, piles of shells, remains of ancient "clambakes," by the archeologists emphasizes that early man possessed capabilities of exploiting oceans to their benefit (Castro and Huber, 2003). However, the landmark in the history of the marine biology could be traced back in the 1200 BC when Phoenicians and Greeks were encouraged to take ocean voyages due to the development of navigation strategies. The first recorded observations regarding the habitat and diversity of marine life (Crustaceans, mollusks, echinoderms, fish, and cetaceans) were made by a Greek philosopher, Aristotle, as evident from his writings in early 384 BC (Levinton, 2008). This was followed by the modern day early expeditions by Captain James Cook, who circumnavigated the world and described many plant and animal species. It was then, that a lot of more researches like Charles Darwin and Charles Wyville Thomson were driven into the realm of exploring the untapped potential of marine life and made revolutionary contributions during their independent expeditions.

The oceans cover 71% of the earth's surface and are distributed unequally with respect to the Equator. About two-thirds of the earth's land area is found in the Northern Hemisphere. About 61% of ocean lies in Northern Hemisphere, whereas Southern Hemisphere comprises of 80% of ocean. These vast water bodies are traditionally classified into four large basins (Table 28.1). The Pacific is the deepest and largest ocean, almost as large as all the others combined. The Atlantic Ocean is little larger than the Indian Ocean, but the two are similar in average depth. The Arctic is the smallest and shallowest ocean. Connected or marginal to the main ocean basins are various shallow seas, such as the Mediterranean Sea, the Gulf of Mexico, the South China Sea, Bay of Bengal, and Arabian Sea. Maine animals which are found in these oceans, seas, and bays have shown wide diversity in terms of biology and chemical ecology. Some of the most important characteristics of the major animal phyla of marine origin have been listed in Table 28.2.

Biotechnologically important marine animals

1. *Sponges*: Sponges belong to the oldest metazoan group (Porifera) and are structurally simplest of all the marine animals. These multicellular organisms

TABLE 28.2 Some of the most important characteristics of the major animal phyla of marine origin.

Phylum	Representative groups	Distinguishing features	General habitat
Porifera (sponges)	Sponges	Collar cells (choanocytes)	Benthic
Cnidaria (cnidarians)	Jellyfishes, sea anemones, corals	Nematocysts	Benthic, pelagic
Ctenophora (comb jellies)	Comb jellies	Ciliary combs, colloblasts	Mostly pelagic
Platyhelminthes (flatworms)	Turbellarians, flukes, tapeworms	Flattened body	Mostly benthic, many parasitic
Nemertea (ribbon worms)	Ribbon worms	Long proboscis	Mostly benthic
Nematoda (nematodes)	Nematodes, roundworms	Body round in cross section	Mostly benthic, many parasitic
Annelida (segmented worms)	Polychaetes, oligochaetes, leeches	Segmentation	Mostly benthic
Sipuncula (peanut worms)	Peanut worms	Retractable, long proboscis	Benthic
Echiura (echiurans)	Echiurans	Non-retractable proboscis	Benthic
Pogonophora (beard worms)	Beard worms, vestimentiferans	No mouth or digestive system	Benthic
Mollusca (molluscs)	Snails, clams, oysters, octopuses	Foot, mantle, radula (absent in some groups)	Benthic, pelagic
Arthropoda (arthropods)	Crustaceans (crabs, shrimps), insects	Exoskeleton, jointed legs	Benthic, pelagic, some parasitic
Ectoprocta (bryozoans)	Bryozoans	Lophophore, lace-like colonies	Benthic
Phoronida (phoronids)	Phoronids	Lophophore, worm-like body	Benthic
Brachiopoda (lamp shells)	Lamp shells	Lophophore, clam-like shells	Benthic
Chaetognatha (arrow worms)	Arrow worms	Transparent body with fins	Mostly pelagic
Echinodermata (echinoderms)	Sea stars, brittle stars, sea urchins, sea cucumbers	Tube feet, five-way radial symmetry, water vascular system	Mostly benthic
Hemichordata (hemichordates)	Acorn worms	Dorsal, hollow (and ventral) nerve cords, gill slits	Benthic
Chordata (chordates)	Tunicates, vertebrates, (fishes, etc.)	Dorsal, hollow nerve cord, gill slits,	Benthic, pelagic

have not developed any tissues or organ system and completely lack any body plan. About 8000 species of sponges have been identified and probably twice of it remains unidentified. These are known to inhabit various niches at all oceanic depths and are of different colors and sizes. Due to lack of any organized body plan, these organisms are sessile and lay fixed to the substratum except for the free swimming larval stage and are known to play crucial role in benthic ecosystem (De Goeij et al., 2013). As the name of the phyla suggests, the members possess tiny pores all over their body which allows them to feed on plankton through their unique filter feeding activity. Additionally, sponges harbor a large amount of microbes on their surface which account for around 35% of sponge biomass, and this unique microbe–sponge association makes them essential candidates for biotechnological research (Hentschel et al., 2012; Egan and Thomas, 2015). From paleontologist, chemist, microbiologist to evolutionary biologist, sponges have attracted a lot attention due to their unique body plan, skeletal framework, defense mechanism, and ability to produce novel chemical compounds.

2. *Jellyfish*: Jellyfish belong to the phylum "Cnidaria" and are otherwise known as "scyphomedusae" as they belong to the class "Schyphozoa" (Cartwright

et al., 2007). As the name suggests, these are no fishes but are actually invertebrates with no backbones and comprise of about 4000 identified species. Jellies are so named due to their peculiar soft-bodied, gelatinous umbrella-shaped structure. Depending on the clade the species belong, these are classified into schyphozoans (swimming jellyfish), staurozoans (stalked jellyfish), hydrozoans, and cubozoans (box jellyfish) (Boero, 2013). Being known as the oldest multicellular organisms, jellyfish are widespread in deep seas to surface of all oceans (FAO, 2014). These organisms are free-swimming and bear characteristic tentacles that aid in capturing food. Besides food, jellyfish also use these unique structures for releasing toxins as a means of defense from predators. Jellyfish involves several developmental phases which include planula (larval stage), polyp (sessile but free-floating), ephyra (free-swimming), and medusa (adult) (Piraino et al., 1996). Few species of this group are important source of food and medicine in China. Due to their fast-growing rates and feeding habits, these organisms are quite popular as keystone predator species as they feed on all types of fish eggs and larvae and thus maintain the population of winner species (Piraino et al., 2002).

3. *Gastropods*: The class includes the largest group of mollusks having the most diverse habitat ranging from deep sea oceans to high mountains and both extremes of latitudes and climatic conditions. Around 150000 species are estimated to be identified till date which include snails, limpets, abalone, whelks, nudibranchs, and slugs (Ruppert et al., 2004). The term Gastropod refers to "stomach foot," as all members of this class move with the help of a muscular foot lying underside their body. Similar to their habitat, these animals also have diversity in shape, size, feeding, and reproductive habits. They may possess a shell made up of exoskeleton which imparts protection from mechanical damage, predators, and even safeguards the muscular appendage below (Giribet et al., 2006). The animals consist of a characteristic radula that is used to drill hole in the shells of other organisms and to eventually feed on them. Most gastropods use their radula to scrape algae from rocks, as found in periwinkles (*Littorina*), limpets (*Fissurella, Lottia*), and abalones (*Haliotis*). Some, like mud snails (*Hydrobia*) are deposit feeders on soft bottoms. Whelks (*Nucella, Buccinum*), oyster drills (*Murex, Urosalpinx*), and cone shells (*Conus*) are carnivores. Several members of the class are source of popular food which includes periwinkle snails and abalone, whereas their shells are commercially popular for developing musical instruments, ornaments, oil lamps, and currency. *Aplysia* species have been extensively studied for understanding nerve conduction, while *Conus* species are used as source of medicine.
4. *Bivalves*: This class of phylum Mollusca consists of more than 15000 species of clams, scallops, mussels, and oysters which unlike gastropods have shells that can be divided into two halves which may be composed of calcite or aragonite and are connected at one side with the help of ligament. These animals have, however, lost radula and head which is typical to almost all classes of Mollusca. In few species like clams, the folds of the mantle tissue have been modified to form long siphons that aid in breathing and feeding. Bivalves consist of a retractable foot which is otherwise known as byssus and a beak at the end of the dorsal region of the shell. Due to their huge size and characteristic filter-feeding activity, these animals are known to filter gallons of water each day and thus keep the ocean water clean. Clams (*Macoma, Mercenaria*) use their shovel-shaped foot to burrow in sand or mud. Mussels (*Mytilus*) secrete strong byssal threads that attach them to rocks and other surfaces. Oysters (*Crassostrea*) cement their left shell to a hard surface, often the shell of another oyster. They have been swallowed by lovers of good food for thousands of years. Pearl oysters (*Pinctada*) are the source of most commercially valuable pearls. Pearls are formed when the oyster secretes shiny layers of calcium carbonate to coat irritating particles or parasites lodged between the mantle and the iridescent inner surface of the shell, which is called mother-of-pearl. Cultured pearls are obtained by carefully inserting a tiny bit of shell or plastic in the mantle. Some scallops (*Pecten*) live unattached and can swim for short distances by rapidly ejecting water from the mantle cavity and clapping the valves. The largest bivalve is the giant clam (*Tridacna*), which grows to more than 1 m (3 ft) in length.
5. *Cephalopods*: This class of Mollusca includes largest invertebrates that possess remarkable degree of organ system development comparable with vertebrates. The members of the class include squids, octopuses, and cuttlefish. For locomotion, these organisms possess tentacles, lateral fins, and mantle cavity. Unlike their mollusk counterparts, these animals consist of well-developed sensory organs and either lack a shell or have reduced it into a size of a pen. In addition to radula these animals also have "chitinous beak" that help in tearing or mincing the prey. The cephalopods are known to produce ink-like secretion as a means of defense from predators which directly disrupts with the olfactory organs of predators. In addition to this,

these are also known to exploit their camouflaging activity for attracting sexual partner or as a defense mechanism to hide from predators. Squids are popular source of seafood and also serve as bait for fishes. Since decades, the members have provided great biotechnological avenues in the field of research and development and cosmetic industry.

6. *Arthropods*: Arthropods (phylum Arthropoda) comprise the largest phylum of animals, with more than a million known species. Of all the animals on earth, three of four are arthropods. They have invaded all types of environments on the earth's surface, including the oceans. Marine arthropods encompass a huge variety of animals such as barnacles, shrimps, lobsters, and crabs, to cite a few. The arthropod body is segmented and bilaterally symmetrical with jointed appendages such as legs and mouthparts. The overwhelming majority of marine arthropods are crustaceans (subphylum Crustacea), a large and extremely diverse group that includes shrimps, crabs, lobsters, and many less familiar animals. With around 10,000 species, the decapods (the term means "ten legs") are the largest group of crustaceans. They include the shrimps, lobsters, and crabs. Decapods feature five pairs of walking legs, the first of which is heavier and usually has claws used for feeding and defense. The carapace is well developed and encloses the part of the body known as the cephalothorax. The rest of the body is called the abdomen. Shrimps and lobsters tend to have laterally compressed bodies with distinct and elongate abdomens, the "tails" we like to eat so much. Shrimps are usually scavengers and feed on bits of dead organic matter on the bottom. Lobsters (*Homarus*) and the clawless spiny lobster (*Panulirus*) are mostly nocturnal and hide during the day in rock or coral crevices. Their feeding habits are almost like those of shrimps; however, they are also known to catch live prey. Other marine Arthropods include Horseshoe Crabs. The horseshoe crabs are the only surviving members of a group (class Merostomata) that is widely represented in the fossil record.
7. *Echinoderms*: The echinoderms include the very successful phylum of all the invertebrates in terms of organ system which comprise of Sea urchins (Echinoidea), Sea cucumbers (Holothuroidea), Brittle stars (Ophiuroidea), Sea stars (Asteroidea), and Sea lilies (Crinoidea). The phylum derives its name from a Greek word meaning "spiny skin," as the members of the phyla are covered with spines, bumps, and spikes which also protect them from predators. They consist of calcareous exoskeleton and, hence, can be fossilized. All echinoderms are exclusively marine, benthic, and radially symmetrical. Another important feature of these marine animals is the presence of tube feet that aid in locomotion through a remarkable vascular system. Another interesting feature of echinoderms is that these animals (sea cucumbers) are capable of regenerating damaged limbs, intestines, and spines.
8. *Chordates*: The phylum Chordata is divided into three major subphyla. All chordates possess at least during part of their lives a dorsal nerve cord, gill slits, a notochord, and a post-anal tail. Fishes were the first vertebrates appeared more than 500 million years ago. The first fishes probably evolved from an invertebrate chordate not much different from the lancelets or the tadpole larvae of sea squirts which still inhabit the oceans. Fishes feed on nearly all types of marine organisms. They are the most economically important marine organisms as they are a vital source of protein for millions of people.

Advances in mariculture

Mariculture is a specialized branch of aquaculture which involves the cultivation of marine organisms for food and other products in the open ocean, ponds, or raceways which are filled with sea water. This technology has rapidly progressed in last two decades due to new technology, greater understanding of biology of farmed species, improvements in formulated feeds, increased water quality in closed farm systems, higher demand for seafood products, farm expansion, and government interest.

Captive rearing technology

Owing to increasing population, there is an increased pressure on the aquaculture industry to produce more seafood in order to meet the global demand. Marine organisms account for the production of 160 million tons of fish generating billions of revenue and claims 16% of the total global animal protein. Captive rearing technology becomes utmost important for marine fishes, as the larvae do not readily accept rotifers as a first feeding organism. In this case, size sorted wild plankton are provided to them when they reach first feeding. Hence, the fish larvae can be developed easily and can grow to adult in captive rearing The successful breeding of marine fishes under captive condition depends on the development of healthy brood stock, knowledge of modes of their reproduction, adequate lighting, water quality, acceptable first live food organisms, healthy environment for metamorphosis, and overall husbandry techniques which predict the possibility of captive breeding and survivability of larvae (Rowe and Hutchings, 2003).

Captive rearing of marine oysters increases the production of it, as it is commonly consumed by humans and can be used for pearl production. During captive breeding of oysters, there are various advantages like oyster changes sex twice during a single season and also have exceptional parenting skills, with young oysters remaining inside the parents shell for most of their larval period. Mussels are used as food by humans since long time as they are the excellent sources of selenium and Vitamin B_{12}. However, the mussels are in the verge of extinction and the necessary care should be taken like captive rearing to increase its productivity and conservation of biodiversity either by keeping the adults alive in captivity, breed them in captivity with considerable difficulties, or by rearing of young in captivity. In captive rearing, some necessary steps should be taken care of as there is an innate rhythm associated with the breeding cycle timing which relates to the temperature of the environment, and the survival of the juvenile pearl mussels is influenced by population density and situation of the adults (Menon and Pillai, 2001).

Feed technology for culture fisheries

There has been considerable improvement in feeding technology in fisheries in recent years which increases the growth of shrimp culture practices. The availability of artificially produced diets to replace cultured live food organisms would alleviate many of the problems currently limiting shrimp hatchery production by (1) reducing the level of technical skill required to operate a hatchery, (2) assuring a reliable supply of a nutritionally balanced larval feed, (3) reducing sources of contamination and larval disease, and (4) simplifying hatchery design and capital cost requirements, thereby facilitating small-scale hatchery development.

The use of microencapsulated feeds has increased the larval survival rates to 70%. Microcoated feed refers to the miniature feed wrapping the micro-coherent feed by the capsule. The food grains are encapsulated along with the flavors which depend on the physical and chemical forms of the food grains. Bio-encapsulation is an easy step that fits into preexisting onsite live food production for marine fishes. It includes the adhesion of biological materials like proteins and microorganisms to be used as probiotics to increase the yield of the feeds. *Bacillus licheniformis*, *B. subtilis*, and *B. circulans* are the most common probiotic organisms used in encapsulation practices.

Marine food and agri-biotech industry

It is well established that marine fisheries and other marine food products are source of 50% of protein intake by humans. In that context, marine biotechnology plays a crucial role in coping up the increasing demand for healthy and high-quality sea food along with maintaining diversity in marine food products (FAO, 2014). As mentioned earlier, the captive marine industry accounts for huge amount of sea food like oysters, fish, caviars, crabs, squids, octopus, lobsters, prawns and shrimps that are relished worldwide.

"Marine Nutraceuticals" as the name suggests refers to substances that can be considered as food or food components beneficial to humans. Marine food industry every year is known to produce huge tons of marine waste that are by-products from fisheries, fish processing, and aquaculture industry. The marine waste includes crustacean shell waste, fins, skin, heads which is potentially exploited for peptide mining (Harnedy and FitzGerald, 2012). Several food components extracted as byproducts like fish oils and fish proteins are used in bakery products. The omega-3 oils produced from fish are well documented to reduce blood pressure, impart protection during cardiovascular disease, diabetes, depression, cancer, and asthma. Other potential marine food byproducts include "Surimi" which is referred to grinded powder of fish bones that is known to cure obesity, bone-associated disorders, premenstrual syndrome, thyroid-related disorder, and even rickets. Essential amino acids like taurine are derived from shark, salmon, tuna, ray, and cod. Similar to omega-3 oils, these amino acids also help in reduction of blood pressure and additionally aid in cholesterol reduction. Chitin and chitosan are important forms of dietary fibers present in the scales of the crabs, shrimps, and other crustaceans. These molecules are known to possess immunomodulatory and wound healing ability (Kapetanakou et al., 2014).

Marine ornamental fish trade

Marine ornamental fishery has a great resource, and the candidate species gain higher prices due to their vibrant colors. The estimated value of marine ornamental trade is 200–330 million US$ per year. Unlike freshwater aquaria species, where 90 per cent of fish species are currently farmed, the great majority of marine aquaria are stocked from wild caught species (Andrews, 1990). According to the data held in Global Marine Aquarium Database, a total of 1,471 species of marine ornamental fishes are traded globally (Table 28.3). Most of these species are associated with coral reefs, although a relatively high number of species are associated with other habitats such as seagrass beds, mangroves, and mudflats. Generally, the ornamental fishes are selected based on their body color (preferably attractive), body shape (unique shape compared to food fishes), and

TABLE 28.3 The dominant fishes targeted for the global marine aquarium trade (Bruckner, 2005).

Fish type	Family	Volume (%)	Value (%)
Damsel/Anemonefish	Pomacentridae	29	13.0
Angelfish	Pomacanthidae	24	46.0
Butterfly fish	Chaetodontidae	11	10.0
Wrasse	Labridae	7	12.0
Blennies/Gobies	Blennidae/Gobidae	5	3.0
Triggerfish/Filefish	Balistidae/Monacanthidae	4	2.5
Hawksfish	Cirrhitidae	2	3.0
Groupers/Basselets	Serranidae	2	1.5
Other	33 families	15	8.0

aquarium suitability. The ornamental coral reef fishery is a multimillion dollar industry that supports thousands of fishers in developing countries and provides aquarium hobbyists with over 1400 species of marine fishes (Das, 2013). In marine ornamental fish culture, the enhancement of the production is mainly obtained nowadays by long-term selective breeding to produce novel traits and varieties and when species are difficult to obtain from the wild. This selective breeding supports the conservation as it eliminates environmental damage and enhances the production of domesticated species (Tlusty, 2002).

The advanced molecular techniques like exploitation of genetic gains by manipulation at zygote, gamete, chromosome, and gene levels, such as chromosome set manipulation, intergeneric and interspecific hybridization, sperm cryopreservation, nuclear transfer, and transgenesis can also be applied to increase the yield and to overcome the age old long practices of selective breeding to obtain an efficient strain (Basrur and King, 2005). There are many advantages of practicing chromosomal manipulations in marine ornamental fish as intergeneric hybrids grow faster than the parental species.

Chromosomal manipulation in marine fish

The chromosomal manipulation in marine fish culture can be used for two different purposes, either to induce polyploidy especially to obtain triploid or tetraploid fish or to reproduce fish by uniparental chromosome inheritance to obtain gynogenetic and androgenetic fish. The techniques of chromosome set manipulation can be applied to complement other techniques like interspecific hybridization, artificial speciation, genetic engineering, sex control, population control, selective breeding, pure line or clone cross-breeding, and sperm cryopreservation. Fish species are generally very tolerant of artificial manipulation of their chromosomes during early development, and this property has been exploited for the production of inbred lines, monosex populations, and the control of ploidy. A variety of different techniques have been applied to obtain polyploids, gynogenetics, and androgenetics, sex-reversed individuals, and transgenics. Methods used include interspecific hybridization or the control of sex by the administration of sex steroids to larvae or juveniles and even surgical or autoimmune castration. The artificial modification of the chromosome set of an organism permits the production of monosex and sterile individuals, while gynogenesis and androgenesis provide methods for the rapid production of inbred populations which can be used in cross-breeding programs (Das, 2013).

Polyploidy

The manipulation of chromosome number to give polyploidy individuals is common in aquaculture. The retention of the second polar body during the second meiotic division in the oocyte results in triploid individuals, which have two chromosome sets from the mother and one from the father. Unlike mammals, where chromosomal rearrangements of this magnitude are usually fatal, many fish with three sets of chromosomes survive quite readily. In addition to triploids, individuals with four sets of chromosomes (tetraploids) or those with both of their chromosome sets derived from a single parent can be produced. Triploid fish are of interest because their sterility is useful in aquaculture and fisheries management. Sterility caused by genetic and physiological factors leads to different characteristics in male and female sexual development.

Male triploid undergo considerable changes in secondary sex characteristics and gonad development at

the time of maturation, affecting the carcass appearance and reducing the meat quality. Female triploids, on the other hand, normally have minimal gonad development and maintain carcass quality throughout the period of maturation of their diploid counterparts. The performance of triploid fish in production situations has been found to be comparable to that of diploid individuals, and the use of triploid females should be considered when they are to be grown past the time of normal sexual maturation.

Polyploidy of penaeid shrimps is still in infancy. However, polyploidy is currently the only known technique that can achieve the dual outcomes of reproductive sterility for genetic protection and skewing sex ratios toward a high proportion of females, which are larger than males in all of the penaeid shrimp (Sellars et al., 2010). Three main categories of polyploidy have been studied in penaeid shrimp: meiosis I triploidy, meiosis II triploidy, and mitotic tetraploidy. Meiosis I triploidy has been studied in *Fenneropenaeus chinensis* and *Metapenaeus japonicus*. Meiosis II triploid larvae have been reported in *F. chinensis*, *Penaeus monodon*, *M. japonicus*, *P. indicus*, and *Litopenaeus vannamei* (see review by Sellars et al., 2010). However, of these species rearing from egg to adult has only been reported (and thus successful) for *F. chinensis* and *M. japonicus*.

Gynogenesis

Gynogenesis is the production of offspring with genes from the mother only. In practice, in fish and some other organisms, it is possible to produce offspring from a mature female with no paternal genetic contribution. The technique for producing gynogenetic individuals requires the inactivation of the male genome and the diploidization of the female genetic material in the zygote, in a process induced by physical or chemical agents. Gynogenesis can be achieved easily in rainbow trout and other species of fish by fertilizing eggs with irradiated sperm and inducing polar body retention through the same type of treatments (pressure or temperature shocks) used to produce triploids. In the same way, if the eggs are irradiated and then fertilized by normal spermatozoa, androgenetic individuals can be produced under certain conditions, with only a set of parental chromosomes. These haploids are then changed to diploids by heat shock treatment before the first cell division.

Gynogenetic individuals are used to produce all-female fish populations. The reasons for inducing gynogenesis range from the production of monosex populations to the development of partially or completely inbred organisms. Partially inbred offspring (from meiotic gynogenesis) may be useful in genome studies for examining the map position of different loci in relation to the centromeres of their chromosomes. If completely inbred animals (from mitotic gynogenesis) survive to maturity, their eggs can be subjected to a second round of gynogenesis, producing true clones in large numbers. Gynogenesis is the development of embryos from eggs without genetic contribution from penetrating sperm. There are two approaches in inducing gynogenesis. They are as follows:

1. *Meiotic gynogenesis*: Ovulated eggs are exposed to the biologically active but genetically 'blank' milt. As a sperm cell activates an egg, intracellular mechanisms are started to eject the second polar body. At this critical point, a physiological shock such as heat, clod, or pressure should be applied to stop the loss of the second polar body. This allows the egg to proceed with normal development by utilizing the two sets of chromosomes, one set from oocyte and the other set from the second polar body. This process is meiotic gynogenesis. The offsprings are thus the products of maternal inheritance. In order to preserve an endangered Chinese paddlefish *Psephurus gladius*, meiotic gynogenesis has been employed by using ultraviolet-irradiated *Acipenser schrenckii* sperm (Zou et al., 2011). Meiotic gynogenesis has also been reported in European sea bass, *Dicentrarchus labrax*, to obtain triploidy (Peruzzi and Chatain 2000). Meiotic gynogenesis has short-term disadvantages but have long-term advantages. As females produce eggs without undergoing meiosis, the eggs become clones that resemble the parthenogenesis which is much more beneficial for an organism.
2. *Mitotic gynogenesis:* This is the second method of gynogenesis. In this method, the irradiated sperm is used to activate the egg cell but the polar bodies are allowed to expel out. This results in activated egg, with a unique 1 N set of chromosomes, provided by the maternal parent. When the haploid egg begins its development through the usual process of mitosis, the normal mitotic process is interrupted by physiological treatment (through the application of heat, cold, or pressure) and transformed into viable 2 N organism. In the case of ornamental fishes like *Cyprinus carpio*, to obtain identical color pattern of commercial interest, mitotic gynogenesis have been employed using microsatellite DNA as markers (Alsaqufi et al., 2012). Mitotic gynogenesis is of upmost importance as it results in fully homozygous offspring (F = 1) and it is achieved by the inhibition of first meiotic cleavage after the duplication of the genome. Using this reproduction method, genetically identical fish (clonal lines) is obtained after two generations. However, mitotic gynogenesis is more difficult to obtain

experimentally, and there is a chance of greater level of inbreeding depressions.

Androgenesis

Androgenesis is the development of embryos without genetic contribution from oocytes. The mechanism associated with androgenesis is the same as that of mitotic gynogenesis. The only difference in practice is the destruction of genetic material of the mature oocyte and not the sperm. Thus, the off-springs are the products of potential inheritance alone. Many androgenic clones occur in nature and can also be generated artificially. Many reports are there to value the androgenesis procedure by heterospecific insemination in fishes that include *Cyprinus carpio*, *C. auratus*, *C. idella*, *Puntius conchonius*, and *Pangasius schwanenfeldii*. In this regard, artificial androgenesis has been limited to commercially important food and ornamental fishes. *C. carpio* is the universal sperm donor and *Oncorhynchus mykiss* is the universal recipient. The major advantage of this technique is the tracing of density and distribution of parental genome from early embryonic stage as well as confirmation of parental origin of haploid androgenotes that scrumble at the embryonic stage. In the case of a mammal model both live donor and recipient are required; however, in fish model, only live recipient is required and donor can be from the sperm of either live or post-mortem-preserved donor (Pandian and Kirankumar 2003).

Cryopreservation of gametes

Cryopreservation is a branch of cryobiology that relates to the long-term preservation and storage of biological material at very low temperature, usually −196°C, the temperature of liquid nitrogen. At this temperature cellular, the viability of gametes can be stored in a genetically stable form in order to make available both the gametes as and when required. This method, therefore, can be successfully used as a fishery management tool. Spermatozoa could be cryopreserved more easily for their structural simplicity and small size. Cryopreservation involves three basic steps—freezing, storage, and thawing which require extenders and cryoprotectants.

1. *Extenders*: Undiluted gametes are not suitable for freezing, and they must be diluted with a suitable extender. An extender is a solution consisting of inorganic and organic chemicals resembling that of blood or seminal plasma in which the viability of spermatozoa can be maintained during in vitro storage. The chemical formulations of the extenders used for cryopreserving spermatozoa vary widely. In general, simpler extenders, some containing only NaCl, $NaHCO_3$, and lecithin, have been shown to be successful.
2. *Cryoprotectants*: Cryoprotectants are added to extenders to minimize the stress on cells during freezing, for example, DMSO, glycerol, methanol and so on (permeating cryoprotectants) and egg yolk, milk and some proteins (non-permeating cryoprotectants). A non-permeating cryoprotectant is often used in conjunction with a permeating cryoprotectant.

Genetic engineering technology

Marine genomics

Understanding the properties and functions of genome is a fundamental task in modern bioscience. Molecular biology has a major role in many aspects of marine biotechnology. Genome studies of different commercially important fish are related to enhance the fishery. Marine sponge is a primitive organism in animal kingdom and the genome analysis of such as primitive organism is of special interest in molecular evolution. Efforts in this area have also proved that the application of molecular biological techniques in ecological studies will be helpful to explore molecular biodiversity, symbiosis, and defense mechanisms.

Genetic analysis of marine life is increasing our understanding of how organisms have evolved and the roles they play in ecosystems, helping scientists analyze the health of the oceans and discover potential pharmaceuticals from the sea. Genome biology applied to marine organisms is known as marine genomics. It is a combined study of how organisms work, evolve, and adapt at the genomic level, the understanding of which can be implicated in the field of human health and disease, food and ornamental practices. Marine genomics can be applied to study the differences within and among the marine populations using various molecular techniques like microarrays, high-throughput sequencing, genotyping, population genetics, and evolutionary analysis. In this regard, the adaptive difference in gene expression pattern and molecular mechanisms to pollution and other toxicological stress play a role in the difference among the individual marine organisms (Whitehead et al., 2010). In another approach, the measurement of the development, organ-specific metabolism, oxygen consumption, enzyme function which can be combined with measures of mRNA and protein expression using microarray or proteomics (Tomanek, 2011).

In order to understand the biotechnological potential of the marine organisms, the assessment of their genetic capabilities, that is, sequencing of their genome and

annotation of the genes is required. Sequencing of the phylogenetically diverse microbial genomics results in the discovery of novel proteins and the trend of discovery is linear which demonstrates the marine environment to be the reservoir of undiscovered proteins (Angly et al., 2006). Besides prokaryotes, the abundance of marine viruses exceeds that of prokaryotes by a factor of at least ten. Therefore, marine viruses are untapped genetic sources of truly marine character which could provide novel proteins, genetic tools and unexpected functions. The genomics of marine eukaryotes is least explored which comprises microalgae, macroalgae, sea weeds, and protozoa. However, the study of metazoan genomics is highly biased toward vertebrates especially mammals due to their medial and economic relevance. Till now, only a few commercially relevant marine invertebrates such as mussels and oysters have been sequenced because of their importance as aquaculture species (Cunningham et al., 2006).

The role of aquaculture in increasing the fish production is well recognized, and in recent years, an appreciable progress has been made in the fields of selection, inbreeding, hybridization, and sex control. One important difference between fish and terrestrial animals for cultivation and genetic improvement is that, usually, fish have higher levels of genetic variation, and hence more scope for selection, than most mammals or birds (Foresti, 2000). The approach to genetic improvement of aquatic organisms that has emerged as a discipline in its own right in recent years is transgenesis, the transfer of new genes into hosts. Transgenic fish (or mollusks or crustaceans) can be defined as possessing within their chromosomal DNA, either directly or through inheritance, genetic constructs which have artificial origins.

Transgenic fish technology has great potential in the aquaculture industry. By introducing desirable genetic traits into fishes, mollusks, and crustaceans, superior transgenic strains can be produced for aquaculture. The development of transgenic fish has undergone intensive research. A foreign gene can be transferred into fish in vivo by introducing DNA either into embryos or directly into somatic tissues of adults. The direct delivery of DNA into fish tissues is a simple approach providing fast results and eliminating the need for screening transgenic individuals and selecting germline carriers. Transgenic fish are being developed for both academic and applied goals, allowing the production of useful model systems as well as new genetic strains with improved characteristics for aquaculture. A variety of genes have now been introduced into fish with the goal of influencing traits such as growth, improved food conversion, maturation, freezing tolerance, flesh quality, tolerance to low oxygen concentration, and disease resistance. Genes responsible for tolerating extreme cold temperature are known to contain antifreeze proteins. Through advances in marine genomics, such genes can be easily transferred to target fish species which requires cold tolerance. However, first breakthrough in the area of marine genomics was the application of transgenic technology in the case of Atlantic Salmon to attain the market size. A growth hormone (GH) driven by antifreeze promotor gene (AFP) is transferred from Chinook Salmon to Atlantic Salmon to enhance the economic gain in terms of size and length in mere 18 months, which otherwise takes around 30 months (Du et al., 1992; Von Schalburg et al., 2008). In addition, the antifreeze promotor gene inclusion in the gene construct imparts cold tolerance which enhances the flavor of transgenic Salmon (Du et al., 1992; Butler and Fletcher, 2009). Similarly, transgenic *Tilapia* containing pig growth hormone exhibits three times faster growth rate (Rahman et al., 1998). Several other applications of transgenic fish have emerged which includes the use of transgenic fish in sensing pollution at a given site, where a particular reporter gene is cloned into fish that act as pollution indicators. The introduction of heat-shock promotors, hazardous metals response genetic elements fused to green fluorescent protein, luciferase, or any other fluorescent markers in fish facilitates in vivo quantification assays (Carvan et al., 2000) (Fig. 28.2).

Marine transcriptomics

At molecular level, marine transcriptomics have recently emerged far better than genomics in terms of research limitations and data authentication. The advances in the microarray and next-generation sequencing techniques (NGS) opened up new possibilities of studies being carried out in marine animals at molecular level. The application NGS technology has eventually facilitated in the barnacles-based antifouling research. Understanding larval settlement of barnacles and bryozoans, which are major culprits of biofouling, is necessary for formulating its preventive measures (Chandramouli et al., 2015; Wang et al., 2015). Further, the information obtained at genomic level always needs to be validated at transcript level by different available molecular technologies like RNAi studies. Additionally, most of the current knowledge about northern elephant seal, *Mirounga angustirostris*, an important model for studying acute stress tolerance and fasting adaption, comes from de novo transcriptomic analysis of contigs and transcripts data of large families (Khudyakov et al., 2015).

Marine proteomics

The term proteomics refers to the interface between genotype and its cognate phenotype and is therefore, an

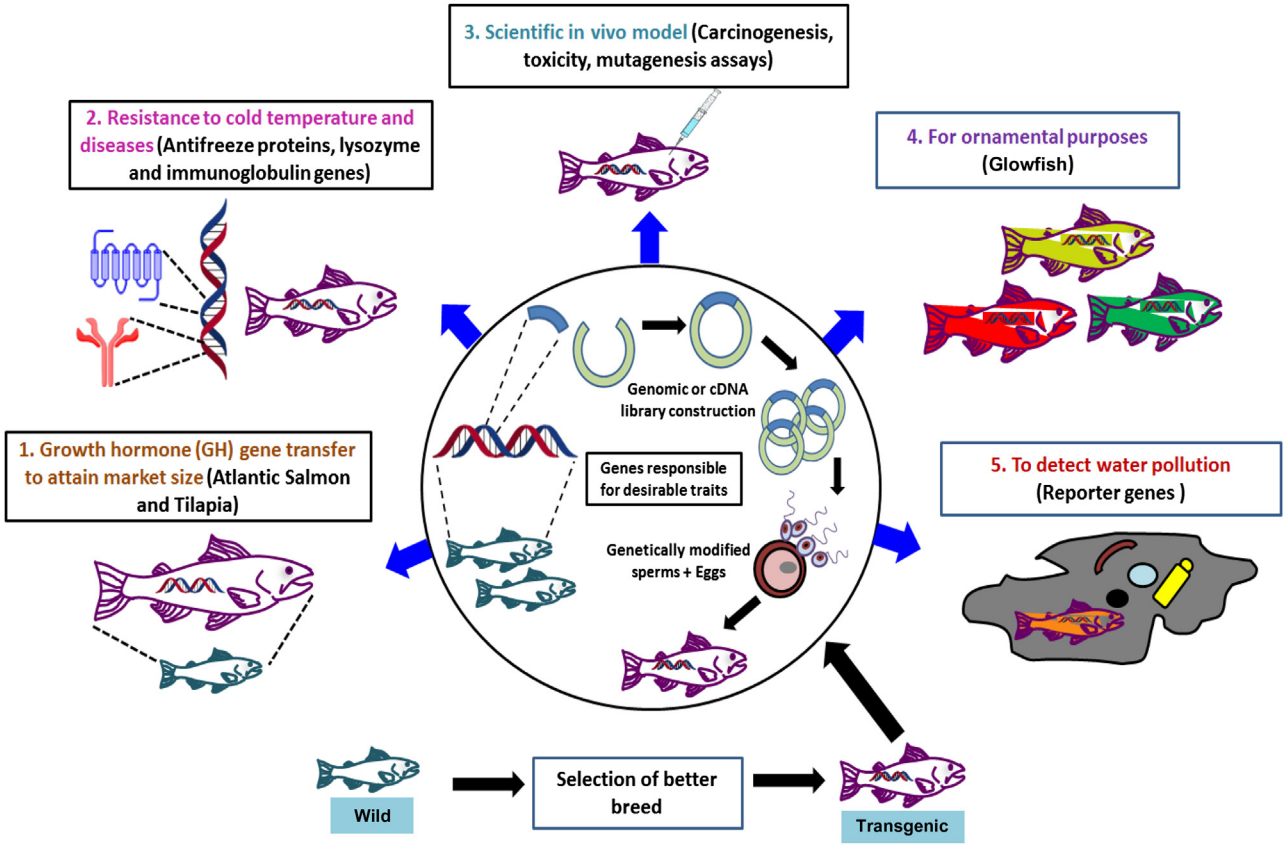

FIGURE 28.2 Transgenic marine fish and its widespread applications.

interesting frontier in better understanding of marine animals. Advances in high-end technologies like 2D electrophoresis, chromatography, ELISA, immunoblotting, and protein expression imaging have allowed generating reference maps that eventually aid in quantification, detection of target, or biologically important protein. With the discovery of fluorescent markers, proteins from animals like green fluorescent protein and red fluorescent protein have made clinical studies easier. The use of clinical protein markers derived from marine animals has been extensively used in species identification, development, and disease diagnostics.

Marine metagenomics

The discovery of the advanced techniques led to the development of genomics research of marine communities. After the successful implication of metagenomics in the year 2001, it has became technically possible by the availability of bacterial artificial chromosomes (BACs), which enables the cloning and sequencing of long stretches of environmental DNA (Liu et al., 2009). Metagenomics works like a shotgun which takes all the genes of a community and putting all these genes in large clone libraries to make them available for use in biotechnological applications. Current metagenomic studies target all domains of life and a broad range of environments. Meta-transcriptomics and meta-proteomics have been successfully applied in addition to marine genomics to provide exciting insights into the functioning of microbial communities. However, these approaches lack broader applications owing to their complexity and are of limited value for biotechnological exploitation. A recently sequencing technology has been discovered which enables 10–100 times faster automated sequencing to nucleic acids. These new sequencing technologies provide a read length of 50–450 nucleotides which can generate 20–200 Mb of raw sequence data per run. Hence, the application of more and more genomic and metagenomic analyses and deep sequencing will generate large data sets from marine environments (Chan, 2009). Bioinformatics resources and tools have been developed in an attempt to maximize the capacity to analyze these vast data sets. This so-called e-infrastructure has to support advanced data acquisition, data storage, data management, data integration, data mining, data visualization and other computing and information processing services over the Internet. Therefore, the provision of dedicated web-based resources and

e-infrastructures are essential for advanced research in marine ecology and biotechnology. Another emerged field called systems biology which aims at system-level understanding of biological systems. In systems biology, organisms are studied as integrated and interacting network of genes and these interactions determine the functions of an organism which largely depends on the mathematical tools to understand gene function relationships (Brown and Botstein, 1999).

Knowledge of metabolic pathways and their link with genomics and other aspects of 'omics' associated with marine organisms are the important basis for the production of unique compounds. In order to increase the productivity of the marine organisms, the metabolic pathways need to be inserted into a new host organism which can grow much easily. Genetic and regulatory pathways should be optimized by metabolic engineering to increase the production of certain compounds by the cells which have been discovered for the prokaryotes, and still, it needs to be developed for the eukaryote systems. If the right targets for metabolic engineering are properly chosen, better processes can be developed. The application of these engineered cells improves the prospects for the commercial production of bioactive compounds for food and pharmaceutical industries which reduces the cost of production and makes it more sustainable. Engineered organisms are expected to become more commonly used in the future but the biosafety and consumer acceptance aspects will need to be taken into account.

Marine animals and nanotechnology

Marine animals in synthesis of nanomaterials

Nanotechnology has a tremendous potential to revolutionize agriculture and allied fields including aquaculture and fisheries. It can provide new tools for aquaculture, fish biotechnology, fish genetics, fish reproduction, and aquatic health management etc. Nanotechnology tools like nanomaterials, nanosensors, DNA nanovaccines, gene delivery, and smart drug delivery have the potential to solve many puzzles related to animal health, production, reproduction, prevention, and treatment of diseases. It is sensible to presume that in the upcoming years, nanotechnology research will reform the science and technology. Nanotechnology applications in the fish-processing industry can be utilized to detect bacteria in packaging, color quality, and safety by increasing the barrier properties. Marine organisms are known to naturally produce remarkable nanostructures like fish bones, shells, and pearls. Additionally, the surface of dolphins, diatoms, whales, and sponges have remarkably arranged architecture that corresponds to nanoridges and other nanostructures which attributes for the efficacy of their biological extracts in the green synthesis of nanoparticles (Asmathunisha and Kathiresan, 2013). Recent advancement in the production of silver nanoparticles with the help of cod liver oil has given an emerging scope in the green nanotechnology. The amine groups and carboxylate ions present in liver oil expedite an in situ generation of organically caped silver nanoparticle by acting as a surfactant and reducing agent. The organic capped nanoparticle is an environment friendly approach and is used for several clinical applications (Khanna and Nair, 2009). A lot of work has been carried out to utilize the organic extracts from marine sponges like *Haliclona* and *Acanthella elongata* that potentially act as reductants in the biosynthesis of silver and gold nanoparticles, respectively (Inbakandan et al., 2010; Hamed et al., 2015). Besides, silver nanoparticles have also been synthesized from organic extracts of marine oyster, *Saccostrea cucullata* (Umayaparvathi et al., 2013) (Fig. 28.3). Additionally, haemolymph of marine crabs, *Carcinus maenas* and *Ocypode quadrata*, serves as excellent organic components for the synthesis of silver nanoparticles and possesses antimicrobial activity against a myriad of pathogenic bacteria (Packia Lekshmi et al., 2015).

Application of nanotechnology in aquaculture and fisheries

The areas related to aquaculture and fisheries where nanotechnology can be applied are as follows:

1. *DNA nano-vaccines*: Use of nanoparticle carriers like chitosan and poly-lactide-co-glycolide acid (PLGA) of vaccine antigens together with mild inflammatory inducers may give a high level of protection to fishes and shellfishes not only against bacterial diseases, but also from certain viral diseases with vaccine-induced side effects. Further, the mass vaccination of fish can be done using nanocapsules containing nanoparticles. These will be resistant to digestion and degradation. These nanocapsules contain short-strand DNA which when applied to water-containing fishes are absorbed into fish cells. The ultrasound mechanism is used to break the capsules which in turn release the DNA, thus eliciting an immune response to fish due to the vaccination. Similarly, oral administration of these vaccines and site-specific release of the active agent for vaccination will reduce the cost and effort of disease management,

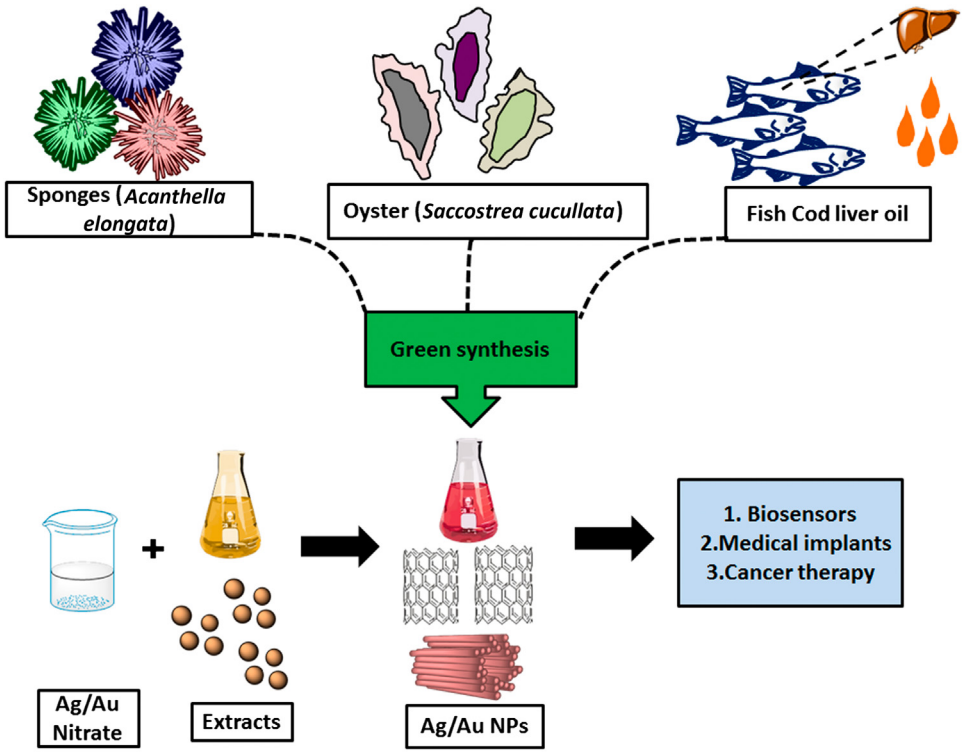

FIGURE 28.3 Marine animals as source of nanomaterial synthesis.

application of drug, and vaccine delivery for sustainable aquaculture (Guddinga et al., 1999).

2. *Gene delivery*: The development of new carrier systems for gene delivery represents an enabling technology for treating many genetic disorders. However, a critical barrier to successful gene therapy remains the formulation of an efficient and safe delivery vehicle. Non-viral delivery systems have been increasingly proposed as alternatives to viral vectors owing to their safety, stability, and ability to be produced in large quantities. Some approaches employ DNA complexes containing lipid, protein, peptide, or polymeric carriers, as well as ligands capable of targeting DNA complexes to cell-surface receptors on the target cells and ligands for directing the intracellular traffic of DNA to the nucleus. Promising results were reported in the formation of complexes between chitosan and DNA. Although chitosan increases the transformation efficiency, the addition of appropriate ligands to the DNA−chitosan complex seems to achieve a more efficient gene delivery via receptor-mediated endocytosis (Kumar et al., 2008). These results suggest that chitosan has comparable efficacy without the associated toxicity of other synthetic vectors and therefore, can be an effective gene-delivery vehicle in vivo.

Pharmaceuticals and therapeutics from marine animals

Most of our medicine comes from natural resources and scientists are still exploring the organisms of marine resources for potentially valuable medical products. Historical records show that human beings were aware of the venomous nature of some sea creatures for, at least, the last 4000 years. More than 2000 years ago, the extracts of marine organisms had been used as medicine.

Marine natural products of animal origin

1. *Jellyfish*: Several marine species turn out to harmful substances that are used for defense/offence purposes. Among which cnidarians (jellyfish) are notably necessary (Mariottini and Pane, 2010). In 1961, Osamu Shimomura of Princeton University extracted green fluorescent protein (GFP) and another bioluminescent protein, called aequorin, from the large and abundant hydromedusa *Aequorea victoria*, while studying photoproteins that cause bioluminescence by this species of jellyfish. Three decades later, Douglas Prasher, a post-doctoral scientist at Woods Hole Oceanographic Institution, sequenced and cloned the gene for GFP. Martin

Chalfie of Columbia University soon figured out how to use GFP as a fluorescent marker of genes inserted into other cells or organisms. Roger Tsien of University of California, San Diego, later chemically manipulated GFP in order to get other colors of fluorescence to use them as markers. In 2008, Shimomura, Chalfie, and Tsien won the Nobel Prize in Chemistry for their work with GFP. Type II collagen derived from Jellyfish is well known to have a variety of applications including immunostimulation, treatment of rheumatoid arthritis, and cosmetics (Hsieh, 2005; Morishige et al., 2011; Leone et al., 2015).

Green flourescent protein is a beta barrel structure, consisting of eleven β-sheets and six α helices containing the covalently bonded chromophore 4-(p-hydroxybenzylidene) imidazolidin-5-one (HBI) in the center. The presence of hydrogen bonding network and electron-stacking interactions with the side chains influence the color, intensity, and photostability of GFP. The fluorescent chromophore is stable to a variety of adverse conditions like heat, extreme pH, and chemical denaturants. GFP comprises of 238 amino acids having excitation peak at 395 nm and emission peak at 508 nm. In cell and molecular biology, GFP gene is reportedly used for reporter of gene expression. In this context, GFP gene can be introduced into the target organism and can be maintained in the genome through breeding, injection with viral vector or transformation. It has been widely applied in many bacteria, yeast, fungus, fish, plants as well as mammalian cells. Apart from gene expression studies, it has wide applications in the field of fluorescent microscopy, viability assay, and a new line of transgenic GFP has been used for gene therapy as well as regenerative medicine.

All jellyfish sting their prey using nematocysts, also called cnidocysts, stinging structures located in specialized cells called cnidocytes. Contact with a jellyfish tentacle can trigger millions of nematocysts to pierce the skin and inject venom, yet the sting of only some jellyfish species causes an adverse reaction in humans. When a nematocyst is triggered by contact by predator or prey, pressure builds up rapidly inside it up to 2,000 lbs/sq. inch until it bursts open. A lance inside the nematocyst pierces the victim's skin, and poison flows through into the victim. Touching or being touched by a jellyfish can be very uncomfortable, sometimes requiring medical assistance; sting effects range from no effect to extreme pain to death. Because of the wide variation in response to jellyfish stings, it is wisest not to contact any jellyfish with bare skin. Even beached and dying jellyfish can still sting when touched.

Scyphozoan jellyfish stings are often uncomfortable, though not generally deadly, but some species of the class *Cubozoa*, or the Box jellyfish, such as the famous and especially toxic Irukandji, can be deadly. Stings may cause anaphylaxis, which may result in death. Hence, victims should immediately get out of the water. Medical care may include administration of an antivenom. The three goals of first aid for uncomplicated jellyfish stings are preventing injury to rescuers, deactivating the nematocysts, and removing tentacles attached to the patient.

2. *Sponge*: Sponge (Phylum: Porifera) is evolutionarily ancient metazoans that have existed for 700−800 million years. They not only solely populate in the tropical oceans but conjointly occur in temperate waters and even in freshwater. Marine sponges have provided a vast resource in the search for bioactive secondary metabolites and potential drug leads (Abbas et al., 2011). These secondary metabolites in sponges play a vital role within their survival in the marine ecosystem and have gained attention in biomedical potential, pharmaceutical relevance, and various biotechnological applications (Thakur and Müller, 2004). Pharmaceutical interest in sponges was aroused within the early 1950s by the invention of unknown nucleosides: spongothymidine and spongouridine within the marine sponge *Cryptotethia crypta* (Alvarez et al., 2000). Apparently, out of several marine natural products that are currently under clinical trials as new drug candidates 20 are derived from invertebrates (Thomas et al., 2010; Malve, 2016). Among them, Porifera remains the foremost necessary phylum, because it provides a large variety of natural products, particularly novel pharmacologically active compounds. Numerous marine sponges are giving raise to many natural products that are currently in clinical trial. L, L-Diketopiperazine called cyclo-(L-Pro-L-Phe) was isolated from South China ocean sponge *Stelletta tenuis* and showed antimicrobial activity (Li, 2009). Coriolin B isolated from Indo-Pacific sponge *Jaspis aff. Johnstoni* exhibited sturdy inhibition of human breast and CNS cell lines (Kim et al., 2011). Sponge *Chondrosia reniformis* produced prugosenes which are used as templates for new anti-infectives (Na et al., 2010).
3. *Cuttle fish and Squid*: Cuttlefish (*Sepia officinalis*) relies for defense on the ejection of a dark ink (commonly known as sepia) that consists of a suspension of melanin granules throughout a viscous colorless medium. At the top of the maturation process, ink gland cells of the digestive tract inside the mantle cavity degenerate and shed

their contents into the ink sac, acting as a reservoir of the exhausted material. In each production and ejection of the ink seems to be regulated by the glutamate/nitric oxide/cGMP signaling pathway that is localized within the ink gland (Palumbo et al., 2000). Besides large amounts of melanin, the ink conjointly contains proteins, glycosaminoglycans, lipids, etc. Cuttlefish ink is an ancient Chinese medicine listed in the *Compendium of Materia Medica* compiled by Shizhen Li, a renouned doctor at the time of the Ming Dynasty, and initially employed to treat heart pain. However, modern clinical medication has proven that it is a good hemostatic medicine that also provides significant curative effects in gynecology, surgery, and so on (Zhong et al., 2009).

Besides, squid ink from *Loligo* spp. also possesses a wide range of biological roles. It has leukocyte-number elevating, anti-retrovirus (Rajaganapathi et al., 2000), and antibacterial properties. As a result of the exhaustive development seen in recent years of medicines from marine sources, the search for effective cell-protective medicines from the ocean has become a crucial activity, and therefore, the detailed biochemical study of cuttlefish ink and squid ink is taking a great intervenes to supply chemotherapeutic drug.

4. *Sea snake*: There are many varieties of venomous snakes in the whole world. Among them, sea snakes are unique in that they spend most of their life in the sea. Generally most sea snakes are venomous containing a mixture of protein, diverse in functions and many highly toxic compounds (Tu, 1977). Closely related to the land kraits and cobras, sea snakes have the same type of venom, only much more potent (Komori et al., 2009). This venom contains neurotoxins which act on the nerve cells of the bitten victim, paralyzing the complete respiratory system and ultimately causing death. Sea snakes are the most successful marine reptiles inhabiting the warm tropical waters of the world. There are 70 species of sea snakes belonging to five subfamilies inhabiting the world's oceans and estuaries. In Indian context, sea snakes are classified into three main families (Hydrophiidae, Colubridae, and Acrochordidae) and having three subfamilies (Hydrophiinae, Laticaudinae, and Homalopsinae) and several genera under each family. All sea snakes are poisonous and very harmful to the victims. Most of the sea snakes produce neurotoxins and very toxic to the prey on which the sea snakes are dependent (Komori et al., 2009).

   All snake venoms are purely protein in nature and have the different unique amino acid sequences and active sites, so they are acting as good inhibitor of the several biochemical pathways of the human body (Nagamizu et al., 2009). Snake venoms comprise a natural library of valuable bioactive substances for haemostasis and thrombosis. The snake venom cofactors are useful for clinical evaluation or subdiagnosis of bleeding disorders as well as for basic investigation into the molecular mechanisms of platelet plug formation induced by von Willebrand factor (VWF) and platelets. Snake venoms contain a variety of bioactive substances that influence hemostasis, thrombosis, and coagulation of mammalian blood (Andrews et al., 2004). These proteins have been not only used as specific reagents for the basic study of thrombosis and haemostasis, but are also expected to have clinical applications, for example, as antithrombotic or diagnostic reagents (Marsh, 2002; Marsh and Williams, 2005; Clemetson and Clemetson, 2008).
5. *Conus*: Cone snails (*Conus*) produce a distinctive repertoire of venom peptides that are used both as a defense mechanism and also to facilitate the immobilization and digestion of prey (Olivera and Cruz, 2001). These peptides known as *conus* act on homologous mammalian ion channels due to the degree of structural conservation exhibited by the voltage- and ligand-gated ion channels across higher eukaryotes. Moreover, mammalian ion channels exhibit diverse tissue expression patterns. This difference in tissue expression patterns was demonstrated with conotoxins that target the nicotinic acetylcholine receptor (nAChR) subtypes present at the invertebrate neuromuscular junctions, while not present in vertebrate neuromuscular junctions, are expressed in tissues relevant to pain. Thus, peptides that target these ion channels may potentially be analgesic therapeutic agents in vertebrates (Olivera et al., 1987).

Conus peptides, such as the μ-conotoxins and ω-conotoxins, are currently being used as standard research tools in neuroscience. The μ-conotoxins are used for the immobilization of skeletal muscles without affecting axonal or synaptic events because of their ability to block the muscle $Na+$ channel (Catterall et al., 2005). The ω-conotoxins are used as standard pharmacological reagents in voltage-gated calcium ($Ca2+$) channel-related research and are used to block neurotransmitter release (Ichida et al., 2000; Olivera et al., 1994).

Many *Conus* spp. have been studied extensively about their peptides and activity. Peptides of *Conus* showed varied activity, and trials have also been conducted for potential treatment for severe chronic pain in patients and for chronic neuropathic (Lubbers et al., 2005). Overall, the conotoxins or conopeptides are mainly responsible for the active blocking or

TABLE 28.4 Amino acid sequences of different conotoxins found in *Conus* targeted to voltage-gated ion channels, Na^+ channels, Ca^{2+} channels, K^+ channels, and ligand-gated ion channels (reviewed in the study by Essack et al., 2012; Kumar et al., 2015).

Peptide	Gene family	Target	Amino acid sequence	*Conus* spp.
Lt5d	T superfamily	Na^+ channel	DCCPAKLLCCNP	*C. litteratus*
Lt6c	O1 superfamily	Na^+ channel	WPCKVAGSPCGLVSECC GTCNVLRNRCV	*C. litteratus*
TIIIA	M superfamily	rNav1.2 rNav1.4	RHGCCKGOKGCSSRECR PQHCC	*C. tulipa*
Cal12a	O2 superfamily	Na^+ channel	DVCDSLVGGHCIHNGC WCDQEAPHGNCCDTDG CTAAWWCPGTKWD	*C. californicus*
Cal12b	O2 superfamily	Na^+ channel	DVCDSLVGGHCIHNGC WCDQDAPHGNCCDTDG CTAAWWCPGTKWD	*C. Californicus*
BuIIIA	M superfamily	Nav1.4	VTDRCCKGKRECGRWC RDHSRCC	*C. bullatus*
BuIIIB	M superfamily	Nav1.4	VGERCCKNGKRGCGRW CRDHSRCC	*C. bullatus*
BuIIIC	M superfamily	Nav1.4	IVDRCCNKGNGKRGCSR WCRDHSRCC	*C. bullatus*
SIIIA	M superfamily	rNav1.2 rNav1.4	ZNCCNGGCSSKWCRDH ARCC	*C. striatus*
SIIIB	M superfamily	rNav1.2 rNav1.4	ZNCCNGGCSSKWCKGH ARCC	*C. striatus*
FVIA	O1 superfamily	(Ca^{2+} channels) N-type	CKGTGKSCSRIAYN CCTGSCRSGKC	*C. fulmen*
Sr11a	I2 superfamily	-	NQQCCWRSCCRGECEAPCRFGP	*C. spurius*
RIIIj	M superfamily	-	LPPCCTPPKKHCPAP ACKYKPCCKS	*C. radiates*
Am2766	O1 superfamily	Na^+ channel	CKQAGESCDIFSQNC- CVGTCAFICIE-NH2	*C. amadis*
Vi1359	T superfamily	Pyroglutamic acid residue	Z*CCITIPECCRI-NH2	*C. virgo*
Mo1659	-	K^+ channel	FHGGSWYRFPWGY-NH2	*C. monile*
κ-PVIIA	-	K^+ shaker channel	CRIONQKCFQHLDDCCS RKCNRFNKCV	*C. purpurascens*
Ac 6.1	-	Voltage gated Na^+ channel	DECFSPGTFCGIKPGLCC SAWCYSFFCLTLTF	*C. achatinus*
Lo959	O2 superfamily	Ca^{2+} channel	GCPDWDPWC- NH2	*C. loroisii*

inhibitions and regulation of different cell transportation channels like voltage-gated ion channels, Na^+ channels, Ca^{2+} channels, K^+ channels, and ligand-gated ion channels (Essack et al., 2012) (Table 28.4).

Commercial bioproducts from marine organisms

In early 1950s, Ross Nigrelli of the Osborn Laboratories of the New York Aquarium extracted a toxin from cuvierian organs of the Bahamian sea cucumber, *Actynopyga agassizi*. He named this toxin as "holothurin," which showed some antitumor activity in mice. From this humble beginning, the number of potential compounds isolated from marine realm has virtually soared and this number now exceeds 10,000 with hundreds of new compounds still being discovered every year. With the combined efforts of marine natural product chemists and pharmacologists, a number of promising identified molecules are already in market, clinical trials, or pre-clinical trials (Table 28.5).

TABLE 28.5 Examples of marine by-products, which are currently in market or in clinical phases (Thakur and Thakur, 2006; Mayer et al., 2010; Malve, 2016).

Product	Source	Application area	Status
Ara-A	Marine sponge	Antiviral	Market
Ara-C	Marine sponge	Anticancer	Market
Okadaic acid	Dinoflagellate	Molecular probe	Market
Manoalide	Marine sponge	Molecular probe	Market
Vent™ DNA polymerase	Deep-sea hydrothermal vent bacteria	PCR enzyme	Market
Aequorin	Bioluminiscent jelly fish, *Aequro victoria*	Bioluminiscent calcium indicator	Market
Green fluorescent protein (GFP)	Bioluminiscent jelly fish, *Aequro victoria*	Reporter gene	Market
Phycoerythrin	Red algae	Conjugated antibodies used in ELISAs and flow cytometry	Market
Cephalosporins	*Cephalosporium* sp. Marine fungi	Antibiotic	Market
Yondelis™	Sea squirt	Anticancer	Market
Zinconotide	Cone snail	Chronic pain	Market
Trabectedin	Sea squirt	Anticancer	Market
Squalamine lactate	Shark	Cancer	Market
Keyhole limpet hemocyanin (KLH)	Gastropod, *Megathura crenulata*	Anticancer	Market
Eribulin mesylate	Marine sponge	Antimitotic	Clinical phase III
Soblidotin	Sea slug, derivative of dolastatin	Tubulin inhibitory activity	Clinical phase III
Tetrodotoxin	Pufferfish	Antitumor	Clinical phase III
DMXBA (GTS-21)	Marine worms	Cognitive functions	Clinical phase II
Plitidepsin	Tunicate (*Aplidium albicans*)	Apoptosis inducer	Clinical phase II
Pseudopterosins	Sea plume		Clinical phase II
Dolastatin	Sea slug	Anticancer	Clinical phase II
Bryostatin-1	Bryozoan	Anticancer	Clinical phase II
Steroid	Sponge	Inflammation, asthma	Clinical phase II
Leconotide	*Conus catus*	Analgesic	Clinical phase I
Hemiasterlin (E7974)	Sponge	Antimitotic	Clinical phase I

Antiviral compound Ara-A (active against *Herpes* virus) (Sagar et al., 2010) and antitumor compound Ara-C (effective in acute lymphoid leukemia) (Grant, 1998) were obtained from the sponge, and these compounds are now in clinical use. Arabinosyl cytosine (Ara-C) is currently sold by the Pharmacia and Upjohn Company under the brand name Cytosar-UP. Apart from these, some of the products such as blood-clotting compounds from cone snail, anti-inflammatory ointment from sea sponge, anticancer substance and disinfectants from shark, gene therapy vehicle and adhesive from shellfish's chitosan are under development.

Enzyme inhibitors have received increasing attention as useful tools in the study of enzyme structures and reaction mechanisms. They also find applications in pharmacology and agriculture. Recently, marine organisms are increasingly recognized as a fruitful source for potential enzymes inhibitors. For example, a bryozoan, *Bugula neritina*, has been the source of a family of protein kinase C (PKC) inhibitors called bryostatins, which are currently in clinical trials for cancer (Trindade-Silva et al., 2010).

In the field of marine biotechnology, living fossil, the horse-shoe crab is important as its amoebocytes

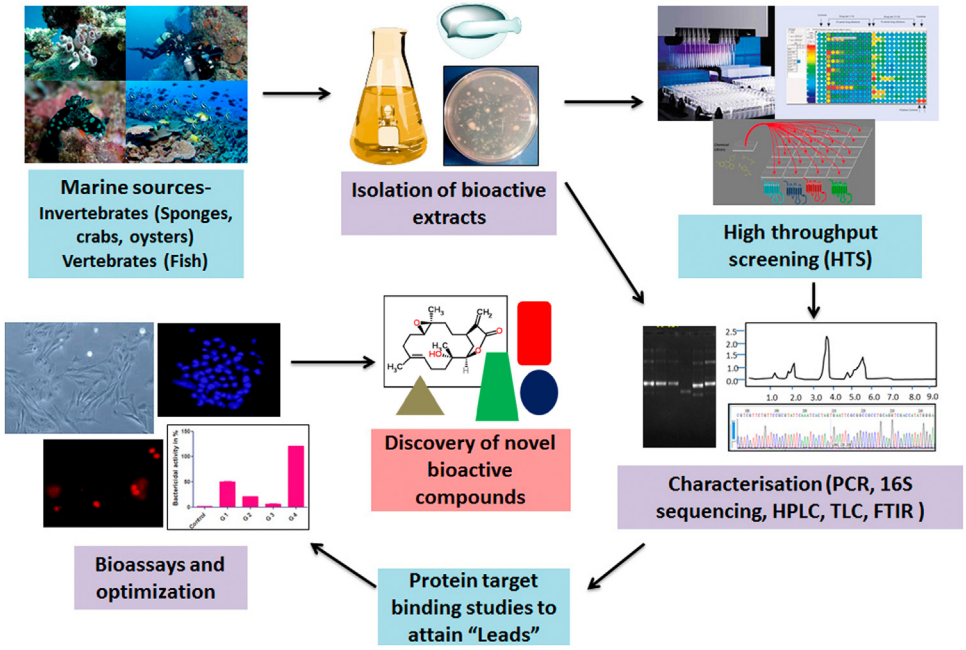

FIGURE 28.4 Workflow in the screening of novel marine bioactive compounds from marine sources.

react with bacterial endotoxins and thus detect early infections in humans as well as traces of lipopolysaccharide or pyrogen in biotechnological products (Ding and Ho, 2010). Many invertebrates, because of their simple cellular structures, provided rich source of new information and serve as desirable non-mammalian models for research. A major area of emphasis is on genetic control of normal development and of tumor formation. In addition, many models threw light on the mechanism of nerve cells in marine invertebrates, which have direct implications in human and other mammalian systems. Sea urchins provided new information to the scientists on fertilization, a fundamental biological process. Thus, marine model systems could provide new insight into basic biological principles that will benefit further developments in pharmaceutical industries (Fig. 28.4).

Green fluorescent protein from jelly fish and its application

Green fluorescence protein (GFP) was first discovered in 1960s and 1970s along with the luminescent protein aequorin from *Aequorea victoria* by Osamu Shimomura (Shimomura et al., 1962). GFP fluorescence occurs when aequorin interacts with Ca^{2+} ions which induces a blue glow, and some of these luminescent energy is transferred to the GFP, thus experiencing the overall color to green. The utilities of these GFP came into lime light when Douglas Prasher cloned and sequenced the wild-type GFP gene (Prasher et al., 1992). GFP is a protein composed of 238 amino acid residues which exhibits bright green fluorescence in blue and ultraviolet light. Although many marine organisms produce similar green fluorescence proteins, but it refers to the protein isolated from the jellyfish *Aequorea victoria*. It is of utmost importance as in cell and molecular biology it is frequently used as a reporter for gene expression by introducing the green fluorescence gene in many bacteria, yeast and other fungi, fish, plants, fly and mammalian cells to be used as biosensors (Soboleski et al., 2005).

In order to increase the potential usage, different researchers have engineered different mutants of GFPs. A single point mutation improving the spectral characteristics of the GFP resulted in increased fluorescence, photostability, a shift of major extinction pick which matches the spectral characteristics of the commonly available FITC filter sets, thus increasing the practical utility of those GFPs. Many other mutants have also been constructed for different color developments in particular blue fluorescent protein (EBFP), cyan fluorescent protein (ECFP), yellow fluorescent protein derivatives (YFP), and BFP derivatives. Genetically encoded fluorescence resonance energy transfer (FRET) reporters are sensitive to cell signaling molecules such as calcium or glutamate, protein phosphorylation state, protein complementation, receptor dimerization, and other processes, thus providing highly specific optical readouts of cell activity in real

time (Chudakov et al., 2010). GFP contains a typical β barrel structure consisting of one β-sheet with α-helix containing the covalently bonded chromophore 4-(p-hydroxybenzylidene) imidazolidin-5-one (HBI), which runs through the center. In the absence of properly folded GFP scaffold, HBI is nonfluorescent which exists mainly in unionized phenol form in wild-type GFP, which is achieved by the post-translational modification called as maturation. Thus, hydrogen-bonding network and electron-stacking interactions with these side chains influence the color, intensity, and photostability of GFP and its numerous derivatives. Protection of the chromophore fluorescence from quenching by water is achieved by the tightly packed nature of the barrel which excludes solvent molecules (Fei and Hughes, 2001).

There is vast use of GFPs in biology and other biologically related disciplines. The other fluorescent proteins are toxic in nature when used in living systems but these naturally fluorescent molecules like GFPs are nontoxic when illuminated in living cells. GFP have been widely used in labeling the spermatozoa of various organisms for identification purposes as in the case of *Drosophila melanogaster* where the expression of GFP can be used as a marker for particular characteristics. GFPs can also be used in various structures enabling morphological distinctions. In other cases, GFP genes can be spliced into the genome of the organisms in the region of DNA that codes for target proteins which is controlled by the same regulatory sequences. Additionally, several spectral variants of GFPs can be used in combination to analyze brain circuitry and as sensors to study neuron membrane potential. GFPs can be used as a useful assay for the detection of viable cells in cryobiology (Fig. 28.5).

Red fluorescent protein from corals and its application

Recent developments in the field of GFP, its spectral variants, and clinical applications have coevolved the discovery of red-fluorescent proteins (RFPs) from anthozoa, *Discosoma striata* (Matz et al., 1999; Miyawaki et al., 2012). The RFP also known as DsRed are known to emit red, far-red, and orange fluorescence. TagRFP, mKOκ, mOrange, and mRuby are all various variants of RFPs used in the labeling DNA, RNA, and protein for live-cell imaging due to their excellent photostability and maturation efficiency (Wu et al., 2011). Additionally, RFPs have emerged as far better fluorescent proteins to be used in studying the localization of intracellular proteins in transgenic embryos like *Xenopus* due to their comparative reduced cytotoxicity levels even at higher concentration (Takagi et al., 2013). The use of mCherry and tdTomato are extensively used as reporter proteins in gene constructs, to monitor DNA vaccine transfection efficiency (Kinnear et al., 2015). Fusion of monomeric RFPs to other fluorescent protein pairs like ECFP-EYFP, ECFP-mRFP, and EYFP-mRFP are widely used in multiparametric analysis during monitoring FRET interactions and in flow cytometry (Piatkevich and Verkhusha, 2011) (Fig. 28.5).

Ethical issues

Marine biotechnology is currently facing a huge variety of ethical and legal issues. The most embarrassing situation is the demarcation of oceanic territories of a country. Here comes the first hurdle in studying and

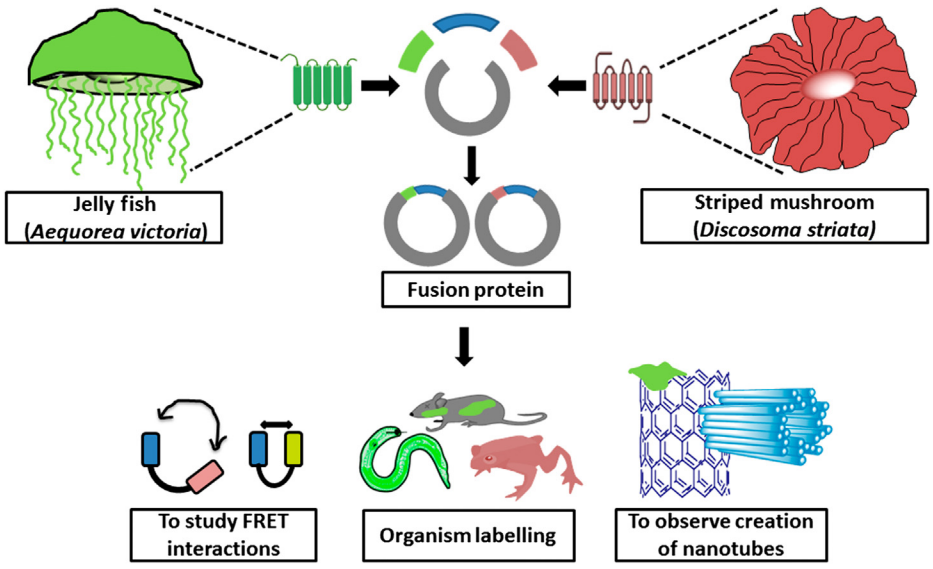

FIGURE 28.5 Green and red fluorescent protein from marine animals: an important biological revolution.

using organism and resources found in other country's territory. If one country is having a rich resource and better selection of marine life, it does not reflect that any country can come and infringe on its territory for their own research. However, still struggle is going on among scientists to come to terms with other countries for the geographical boundaries. Another major ethical issue lies with the maintenance of a stable ecosystem and species. As scientists try to modify living organisms, it may possess threat to damage the surrounding wildlife and habitat. In this regard, marine biotechnologists should be aware of what they are doing to make sure to keep the environment healthy and stable. Another major problem regarding the use of marine resources is its sustainability. Overexploitation of the resources to meet the need of growing human population possesses a major threat. Recently, a total of 80% of the world's fisheries have been overexploited, depleted or in a state of collapse; 29% of fish and sea food species have been collapsed and rest of them are projected to collapse by 2048. People take advantage of the limitation of geographical boundary of marine environment to intrude and overexploit the resources without any hesitation using sophisticated instruments and equipment. Strict legislation and its proper implementation may solve the issue for a sustainable use of marine resources. Hence, many precautionary principles may be followed to explore the potential of marine environment like, movement of the research, that is either from an environmentally sensitive area or to the use of less invasive techniques such as computer modeling.

Translational significance

The success story of marine biotechnology for field application is promising but yet not up to the optimum level. Hence, the primary focus of marine biotechnological research should be more products-oriented and is to solve the problems of local and global issues. So far, the translational research has achieved many mile stones like discovery of GFP proteins, revolution in marine aquaculture for food as well as ornamental field, microbial enhanced oil recovery, treatment for leukemia and cancer. However, many problems are still there like maintaining productivity and sustainability of the ocean, mobilization of knowledge, bioeconomy, promise of marine biotechnology for the benefit of people, treatment of many incurable diseases, treatment of pollutants, and much more. Hence, the research should be targeted to direct transfer of the outcomes to the field conditions for a better society. Collaborative research in spite of the geographical boundaries and overcoming the ethical issues should be the ultimatum for achieving the promised target of marine biotechnology.

Future directions

Blue biotechnology is the newcomer of the group of red, green, and white biotechnology, standing primarily for marine biotechnology. The rich biodiversity of marine biota and their unique physiological adaptations to the harsh marine environment has coupled with new developments in biotechnology. It has opened up new and exciting vista for the exploration of life-saving drugs, novel pharmaceuticals, industrial products, and processes. Applications of scientific and engineering principles to the processing of materials by marine biological agents to provide goods and services are called marine biotechnology. It deals with exploring the oceans to develop novel pharmaceutical drugs, chemical products, enzymes, and other industrial products and processes. However, the field of marine biotechnology is still in its early stage and it faces huge challenges from technical, regulatory, political, and environmental point of view.

Most of our medicines come from natural resources, and more than 2000 years ago, the extracts of marine organisms were used as medicines. The numbers of potential compounds isolated from the marine environments have exceeded 10,000, and still new compounds are discovered every year. A number of promising identified molecules are already in the market after successful clinical trials, by the combined efforts of marine natural product chemists and pharmacologists, and these precious natural products have been obtained from marine microorganisms as well as invertebrates such as sponges, mollusks, bryozoans, tunicates, and fish. The success story of marine biotechnological applications include the commercialization of antibiotic cephalosporin from marine fungus, cytostatic cytarabine from sponge, anthelmintic insecticide kanic acid from red alga, analgesic zincototide from mollusk, and antiviral compounds Ara-A and antitumor compounds Ara-C obtained from sponges. Apart from these blood clotting compounds from cone snail, anti-inflammatory ointments from sea sponge, anticancer substance and disinfectants from shark, gene therapy vehicle and adhesive from shell fish's chitosan are under clinical trials. Bioactive compounds—based antibody conjugates from fish, crustaceans, and gastropods are under development Additionally, the use of marine organic extracts in biosynthesis of nanoparticles have opened up new realms in the frontiers of drug delivery and disease diagnostics.

Mariculture is the most mature and highly successful example of progress in the field of marine biotechnology. Biotechnological applications to improve aquaculture are focused on species diversification, optimum food and feeding, health and disease management with minimum environmental impacts. Use of recombinant technologies is in progress to develop genetically

modified organisms with useful features such as fast growth, resistance to pathogens, temperature, and salinity tolerance. Molecular biology approaches have also resulted in the invention of new feed stocks and vaccines for aquaculture to increase productivity. The field of mariculture needs to be developed continuously to meet the global demand of aquaculture and fish production.

World wide web resources

Sustainable use of marine bioresources needs thorough knowledge, understanding, proper identification, assessment and conservation of local marine inhabitants. Marine animals house abundant unexploited wealth of biomolecules which needs to be explored for bioprospecting, discovery of novel bioactive molecules, and conservation. For further details, web resources given below may be explored:

http://www.marinebiotech.org/
http://www.marinebiotech.eu/
http://www.bioresourcebiotech.com/
http://agsci.oregonstate.edu/brr/
http://www.usda.gov/wps/portal/usda/usda-home?navid = AQUACULTURE
http://www.lsuagcenter.com/en/our_offices/research_stations/Aquaculture/
http://www.euromarineconsortium.eu/fp6net-works/marinegenomics
http://www.marinegenomics.org/
http://www.mendeley.com/groups/1063651/fish-marine-nanotechnology/
http://www.northwestern.edu/newscenter/stor-ies/2013/02/from-sticky-marine-mussels-to-nanotech.html
http://www.marinebiotech.eu/wiki/Marine_derived_nutraceutics

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Glossary

Abalone Common name for any group of small or large edible sea snails and marine gastropod molluscs of family Haliotidae.
Blue biotechnology Application of biotechnology for describing marine and aquatic animals
Clams One or more species of commonly consumed bivalves that burrow in sediments
Cnidocysts A characteristic subcellular organelle responsible for the stings delivered by jellyfish
Cubozoa They are the cnidarian invertebrates distinguished by their cube-shaped medusa commonly known as box jellyfish.
Hydrozoa A taxonomic class of individually very small, predatory animals, some solitary, and some colonial, most living in saltwater
Limpet The common name for a different group of sea and freshwater snails, for those that does not appear to be spirally coiled in adult stage
Metagenomics The study of metagenome, the genetic material recovered directly from environmental sample
Nematocysts A capsule within specialized cells of certain coelenterates, such as jellyfish, containing a barbed, threadlike tube that delivers a paralyzing sting when propelled into attackers and prey, also called stinging cell.

Nudibranch A member of Nudibranchia is a group of soft-bodied, marine gastropod mollusks which shed their shell after their larval stage which are noted for their often extraordinary colors and striking forms

Ostia A small opening or orifice as in case of fallopian tube, vagina, maxillary sinus, etc.

Penaeid shrimps Shrimps belonging to the family Penaeidae of marine crustacean containing economic important species like tiger prawn

Scallops A marine bivalve mollusk of the family Pectinidae which are cosmopolitan in nature

Tunicates Members of the tunicata, a subphylum of the phylum Chordata which are marine filter feeders with a saclike morphology

Long-answer questions

1. Write a detailed note on transgenic fish and its applications.
2. Write a note on prospects and scope of marine biotechnology.
3. Describe the use of green fluorescent protein in medical research.
4. Describe the role of marine animals in green synthesis of nanoparticles.
5. Write a note on marine peptides and their pharmacological applications.

Short-answer questions

1. What is the difference between μ-conotoxins and ω-conotoxins?
2. What do you mean by extenders?
3. Briefly describe about the largest phylum of animals.
4. Why is fish a suitable model for studying transgenic technology?
5. How *Aequorea victoria* is related to in vitro study designs?

Answers to short answer questions

1. μ-Conotoxins possess the ability to inhibit the voltage dependent sodium channels in muscle and are used for immobilization of skeletal muscles. However, ω-conotoxins inhibit N-type voltage dependent calcium channels. Due to having analgesic effect ω-conotoxins are used pharmaceutically to modulate pain.
2. An extender is a solution consisting of organic and inorganic chemicals that resemble blood or seminal plasma which is used for the long term in vitro storage of spermatozoa to maintain its viability.
3. Arthropods, three out of four animals on earth constitute the largest phylum of animals. Marine arthropods include crustaceans like shrimps, crabs, lobsters and many more familiar animals which are mostly decapod in nature.
4. There are many aspects that render fish more suitable than mouse model for transgenic technology that includes the higher fecundity of fish which increases the availability of eggs for microinjection. The other advantages include the external fertilization and incubation of fish eggs and relative large size of fish eggs facilitating handling.
5. *Aequorea victoria*, a jelly fish, is known to produce the fluorescent protein, that is, GFP which is having larger applications in the field of research. It is used as a fluorescent dye to measure the expression level of a gene, confirmation of transformation experiments, use in fluorescence microscopy, and its use in gene therapy as well as regenerative medicine.

Yes/no-type questions

1. Introducing juveniles into the natural environment is called Sea Ranching: (a) Yes, (b) No
2. Androgenesis is a form of sexual reproduction in which females produce eggs without a nucleus and the embryo develops from the male gamete: (a) Yes, (b) No
3. The moulting process in crustaceans is regulated through growth hormone of shrimps: (a) Yes, (b) No
4. Hypophysation in fish refer to the induced breeding by pituitary glands: (a) Yes, (b) No
5. Milk fish migrate from sea to mangrove swamps to survive and spend its most of juvenile phase. It is called anadromous migration: (a) Yes, (b) No
6. Mussel culture farming is carried out in the form of river farming: (a) Yes, (b) No
7. Laminara seaweeds are cultivated in 1-ft depth at low tide: (a) Yes, (b) No
8. White spot syndrome virus commonly found in Shrimps is a double-stranded RNA virus: (a) Yes, (b) No
9. The deepest trench is found in Western Pacific: (a) Yes, (b) No
10. Deep open Ocean is the most productive areas of the ocean. (a) Yes, (b) No

Answers to yes/no-type questions

1. Yes—Sea ranching is the method of introducing the cultured organisms in the sea.

2. Yes—A zygote is formed between a male and a female gamete, but the female genome is eliminated
3. No—Moulting process in crustaceans is regulated through ecdysteroid hormones from the Y-organs
4. Yes—Induced breeding by hypophysis, that is, pituitary is called hopyphysation.
5. Yes—Migration from sea water to brackishwater/freshwater is called anadromous migration.
6. No—Mussel farming is carried out in open sea farming and estuarine farming
7. No—Laminaria seaweed is cultured at approximately one meter's depth at low tide.
8. No—White spot syndrome virus is double-stranded (ds) DNA virus.
9. Yes—Challenger trench is the deepest trench with the depth of 11, 020 m
10. No—Coastal upwelling regions are the most productive areas.

CHAPTER

29

Nanotechnology and detection of microbial pathogens

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Summary

The optical characteristics and functionalization of gold nanoparticles (GNPs) with DNA can be exploited to generate conjugated DNA probes for detection of pathogens. GNP probes with low cost instrumentation or a "spot and read" system can be a viable alternative for onsite detection and monitoring of pathogens.

What you can expect to know

The consumption of unsafe water and food in developing countries is one of the major causes of infectious disease outbreaks. The existing methods for the detection of pathogens prevalence in water and food samples are expensive, time consuming, and highly diverse. A majority of these pathogens often escape detection by conventional methods. The detection of target pathogens requires the development of innovative, simple, rapid, sensitive, and highly specific methods to overcome existing drawbacks for the management of infectious disease outbreaks. The recent advancements in nanotechnology have led to the development of nanoparticle-based facile assays for specific detection of the bioanalytes of clinical interest. Gold nanoparticles (GNPs) with unique optical properties and high surface area are being extensively used for facile detection of bioanalytes of interest in the samples. The outstanding physicochemical properties of GNPs have proved advantageous over conventional detection methods for diagnostic purposes. The colloidal solution of GNPs exhibits intense red and blue/purple colors depending on the size, shape, and degree of aggregation of nanoparticles. This chapter encompasses the application of nanotechnology in the field of pathogen detection and provides an insight on how nanotechnology can be exploited to overcome the problems related to the existing methods of detection.

Introduction

Rapid population growth and industrialization have led to the deterioration in the microbiological quality of water, adversely affecting human health and sustainable development. Water plays a significant role in the transmission of human diseases. Typhoid, infectious hepatitis, cholera, traveler's diarrhea, amoebic and bacillary dysenteries, and other gastrointestinal diseases are waterborne diseases. The occasional outbreaks of waterborne diseases point out toward the need for strict monitoring and management of the "water quality" from public and private water supplies. "Water quality" is a technical term that is based on the characteristics of water in relation to guideline values of what is best for human consumption and for all usual domestic needs, including personal hygiene. Microbial, biological, chemical, and physical aspects

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are important components of water quality. When we refer to microbial aspect, microorganisms that are known to be pathogenic should be absent in drinking water, also addressed as potable water. Potable water is the water that has been treated, cleaned, or filtered and meets established drinking water standards as set by regulatory authorities like World Health Organization, Bureau of Indian Standards, American Public Health Association, and United States Environmental Protection Agency. This water is expected to be realistically free from harmful bacteria and contaminants and considered "safe" for drinking or cooking and baking purposes. Municipal water, that has been UV irradiated, filtered, distilled, or purified, falls in the category of potable water. Hence, water treatment regimen including disinfection and the execution of bacteriological surveillance programs has resulted in the decreased occurrence of water-related illness.

The management of the frequency of waterborne disease outbreaks has become a challenging task. Globally, the source of almost two third of the drinking water consumed is surface water, which may be easily contaminated by sewage discharges, animal defecation, and municipal and industrial wastes. Fecal wastes from domestic animals, wildlife, and humans added to the soil as manure to varying extent are incorporated into the soil. These fecal wastes can also enter into the water stream directly, or through poorly processed sewage effluents, by percolation of water pipelines, malfunctioning septic tanks, and seepage from sanitary landfills. Different pathogenic viruses, bacteria, and parasites may be found in the feces of domestic, wild animals, and humans, along with the nonpathogenic bacteria and parasites that exist in large numbers in the feces of animals as well as soil and water. Hence, it is very important to identify the etiological agent for appropriate treatment, interventions, and control.

However, the identification and monitoring of specific pathogen in very low concentration or low doses, in the presence of large number of background microflora, is a daunting task. The presence of "indicator organisms," generally nonpathogenic microorganisms, points toward the presence of enteric pathogens in sample. Indicator organisms play an important role in predicting the probability of occurrence of pathogens that are quite low in number. There are few criteria for a microorganism to be declared as an "indicator." It must be present when pathogens are present in water, should be absent in uncontaminated water, is present in higher numbers than pathogens in contaminated water, and has better survival in water than pathogens with considerable ease in analysis. Therefore, the need to identify, classify, and delineate the permissible limits of "indicators" of microbial quality of water in different sectors of water have been described in this chapter.

Indicators of microbial water quality

In water quality assessment, the fecal indicator bacteria (FIB) are used to measure the sanitary quality of water for recreational, industrial, agricultural, and water supply purposes. The FIBs are natural inhabitants of the gastrointestinal tract of humans and other warm-blooded animals. Generally harmless, these are released into the environment with feces, and on exposure to a variety of the environmental factors (Ashbolt et al., 2001). In general, it is believed that the fecal indicator adapted to live in the gastrointestinal tract cannot grow in natural environments. However, survival of FIB in water is influenced by the environmental factors like sunlight, temperature, competition for the nutrients with bacteria inhabiting naturally in the water, predation by protozoa and other small organisms, and toxic industrial wastes. Studies have shown that FIB survives from a few hours up to several days in water, but may survive for days or months in sediments, where they may be protected from sunlight and predators. The survival time of FIB in water is a function of many environmental influences, and there is no common factor that applies collectively to all water bodies or even at different seasons in a year for a single water body. It is assumed that the mortality of pathogens and FIB is equal. Therefore, the presence of relatively high numbers of FIB in the environment indicates the likelihood of the presence of other pathogens as well.

The environmental indicators of water quality are described in Fig. 29.1. Coliforms and related pathogens are broadly categorized in total coliforms, fecal coliforms (FCs), or thermotolerent coliforms and other indicator organisms (Fig. 29.2). The study shows that 60%–90% of TCs are FCs and 90% of FCs are

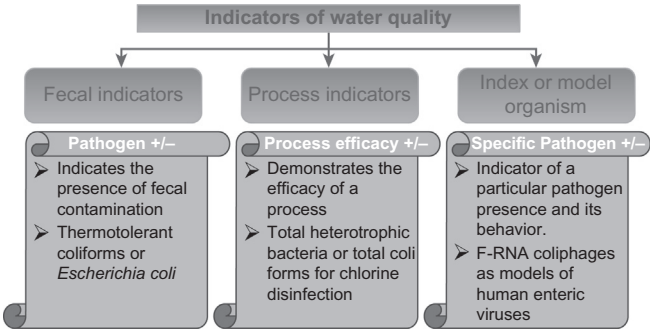


FIGURE 29.1 Types of water indicators.

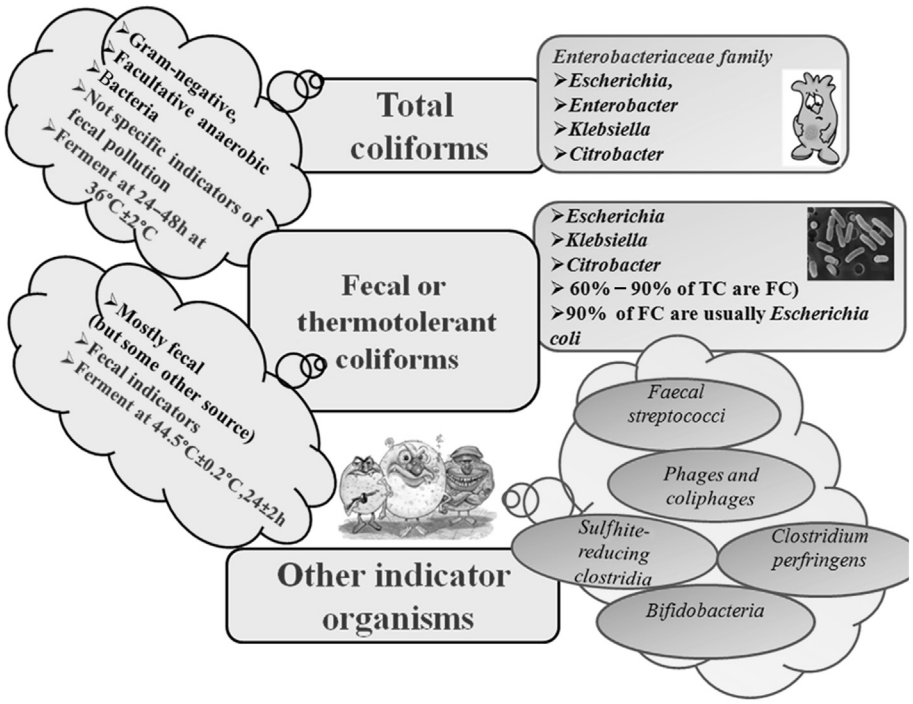


FIGURE 29.2 Different types of Indicator organisms.

TABLE 29.1 Summary of water quality criteria for microbiological indicators.

Water use	*Escherichia coli* (CFU)	*Enterococci* (CFU)	*Fecal coliforms* (CFU)
Raw drinking water - no treatment	0/100 ml	0/100 ml	0/100 ml
Raw drinking water - disinfection only	≤10/100 ml 90th percentile	≤3/100 ml 90th percentile	≤10/100 ml 90th percentile
Raw drinking water - partial treatment	≤100/100 ml 90th percentile	≤25/100 ml90th percentile	≤100/100 ml 90th percentile
Raw drinking water- complete treatment	None applicable	None applicable	None applicable
Livestock - free range animals	None applicable	None applicable	None applicable
Livestock - general livestock use	200/100 ml maximum	50/100 ml maximum	200/100 ml maximum
Livestock - closely confined (no treatment)	0/100 ml maximum	0/100 ml maximum	0/100 ml maximum
Livestock - closely confined (disinfection only)	≤10/100 ml 90th percentile	≤10/100 ml 90th percentile	≤10/100 ml 90th percentile
Livestock - closely confined (partial treatment)	≤100/100 ml 90th percentile	≤100/100 ml 90th percentile	≤100/100 ml 90th percentile
Livestock - closely confined (complete treatment)	None applicable	None applicable	None applicable

CFU, colony forming unit.
Modified from Water Quality Criteria for Microbiological Indicators (Warrington et al., 2001).

Escherichia coli. The permissible limits of "indicators" of water quality are summarized in Table 29.1.

Need for detection of waterborne and foodborne pathogens

We have discussed about "indicators" to access pathogenic microorganisms but why detection of pathogens is urgently needed? Certain most frequently encountered waterborne and foodborne diseases, that is, traveler's diarrhea, typhoid, and cholera, are caused by consumption of foodstuffs including meat products and contaminated water. In the developing world, particularly South East Asia, communities use untreated water for drinking, food preparation, and other domestic purposes particularly in the urban suburbs and rural environment. It has been reported that the presence of waterborne and foodborne bacteria are a major cause of the economic burden on the food industry in developing countries (Wang et al., 2010). Therefore, it is necessary to detect these pathogens at early stage to

circumvent disease spread and epidemics. At this juncture, it is essential to know about the major actors of the troupe of pathogenic microbes.

The pathogenic group of bacteria includes pathotypes of *E. coli* such as enterotoxigenic *E. coli* (ETEC) and enterohemorrhagic *E. coli* (EHEC), *Salmonella* spp., *Vibrio cholerae*, *Campylobacte*r spp., including antibiotic-resistant *Campylobacter jejuni*. Among the *E. coli* pathotype family, EHEC (foremost representative *E. coli* 0157:H7) is the major culprits of the waterborne and foodborne diseases in humans. These bacterial strains are characterized by the production of one or more types of cytotoxins causing tissue damage in humans and animals. ETEC is the causative agent of traveler's diarrhea, which is one of the important pathogen in farming industry and found in cattle and weaning piglets. ETEC strains from humans cause mild or severe watery diarrhea by producing a heat-labile enterotoxin (LTI), similar in structure to cholera toxin and heat-stable enterotoxins (ST IA and/or ST Ib). The heat-labile enterotoxins of *E. coli* are oligomeric toxins with two major serogroups LTI and LTII. The LTI is expressed by *E. coli* strains that are pathogenic for both the humans and animals.

Salmonella, another typical pathogen, causes gastroenteritis and typhoid in humans. The typhoid caused by *Salmonella enterica* serotype typhi remains an important public health problem in developing countries. It has been reported that South Asian countries exhibit high burden of typhoid fever. Furthermore, India, Indonesia, Bangladesh, and Pakistan have been identified as high zones for infections caused by *Salmonella* spp. The severity of infections of *Salmonella* is due to their infective dose that can be as low as 15–100 colony-forming units. This high vulnerability to waterborne *Salmonella* infections in Asia and other developing countries is due to lack of potable water quality and dependence of a large population on natural resources for daily water requirement.

V. cholerae and *Vibrio parahaemolyticus* are pathogens that cause diarrhea in humans. *V. parahaemolyticus* is an invasive organism affecting primarily the colon, whereas *V. cholerae* is noninvasive, affecting the small intestine through secretion of an enterotoxin. *V. cholerae* causes, a globally prevalent gastrointestinal disease, cholera, which remains a persistent problem in many countries. These pathogens occur in both marine and freshwater habitats and in associations with aquatic animals.

C. jejuni has been associated with dysentery-like gastroenteritis, as well as with other types of infections, including bacteremic and central nervous system infections in humans.

Based on the potential risk posed by aforementioned pathogens, it is very clear that the detection of these organisms is the key to the prevention of waterborne and foodborne epidemics or diseases in humans and animals. Therefore, it will be useful to understand the advantages and limitations of existing state-of-the-art detection methods used to detect indicator organisms and identify pathogenic variants of such microbes.

Conventional methods to detect fecal indicator organism and other pathogenic bacteria

Conventional techniques like culture-based methods have been recommended and used routinely for decades for the identification and detection of pathogens. In culture-based methods, microbes grow on specific culture medium at a specific temperature for a particular time period. Their characterization is based on the morphology of bacterial colonies and confirmation by biochemical tests. The most prevalent conventional methods including culture-based methods for pathogen detection are as follows:

Most probable number method

The most probable number (MPN) technique is an important technique in estimating microbial populations in soils, waters, food matrix, and agricultural products. Many soils are heterogeneous; therefore, exact cell numbers of an individual organism are impossible to determine. The MPN technique is used to estimate microbial population where heterotrophic counts are difficult. This technique does not rely on quantitative assessment of individual cells; instead it relies on specific qualitative attributes of the microorganism being counted. The MPN technique estimates microbial population sizes in a liquid substrate, and this method is very tedious and takes 24–48 hours.

Membrane filtration method

The membrane filter (MF) technique is used to test relatively large volumes of sample and yields numerical results more rapidly than the MPN method. The MF technique is extremely useful in monitoring drinking water and a variety of natural water. On the basis of MF technique, the coliform group may comprise all aerobic and many facultative anaerobic, gram-negative, nonspore-forming rod-shaped bacteria that develop a red colony with a metallic sheen within 24 hours at 35°C on an endow-type medium containing lactose. Some members of the TC group may produce a dark red or nucleated colony without a metallic sheen and classified as typical coliform colonies after

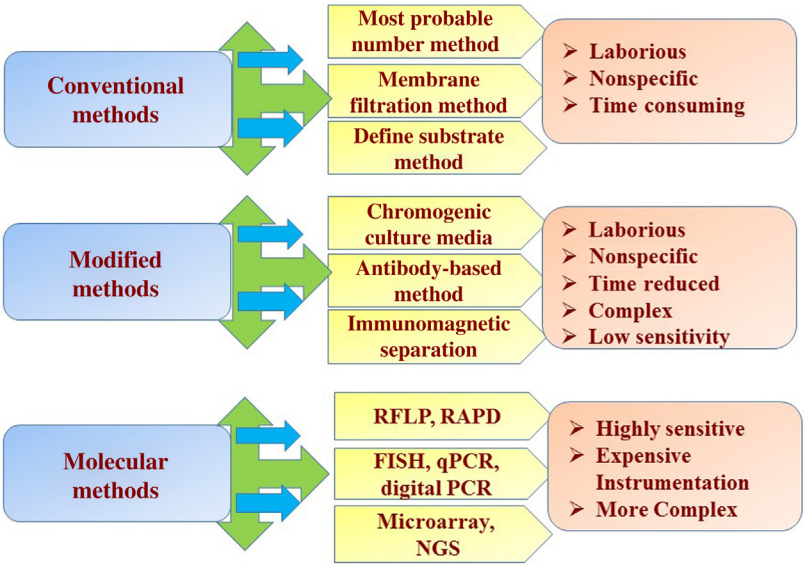


FIGURE 29.3 Different methods of pathogen detection.

verification. Pure cultures of coliform bacteria produce a negative cytochrome oxidase and positive beta-galactosidase reaction. Generally, all red, pink, blue, white, or colorless colonies lacking sheen are considered noncoliforms by this technique. However, the MF technique has limitations, particularly when testing water with high turbidity or the presence of noncoliform bacteria. Hence, for such waters or when the MF technique has not been used previously, it is desirable to conduct parallel tests with the multiple-tube fermentation technique to demonstrate applicability.

Defined substrate methods

Media without harsh selective agents but specific enzyme substrates provide significant improvements in recoveries and identification of target bacteria such as coliforms and *E. coli*. Furthermore, the enzyme-based methods appear to pick up traditionally nonculturable coliforms. TCs are members of genera or species within the family Enterobacteriaceae, capable of growth at 37°C, which possess β-galactosidase.

This defined substrate approach has been advocated by International Standards Organization for miniaturized MPN-based methods for coliforms/*E. coli* and enterococci (ISO/FDIS, 1999). Certain methods in vogue are presented in Fig. 29.3.

On the basis of the defined substrate methodology, Idexx Laboratories (Westbrook, ME) developed products for the microbial assessment of water, Colilert and Enterolert system. Colilert has been used for the enumeration of TCs and *E. coli* simultaneously, and the Enterolert system has been used for the enumeration of *Enterococcus*. This method exploits the target organism's ability to metabolize fluorogenic or chromogenic substrate and convert it to a detectable end product. The coliforms follow the mechanism:

$$\text{o-nitrophenol-}\beta\text{-}D\text{-galactopyranoside} \xrightarrow{\beta\text{-}D\text{-galactosidase}} O\text{-nitrophenol (yellow product)}$$

In *E. coli*, ß-D glucuronidase enzyme converts 4-methylum belliferyl-ß-D-glucuronide in to fluorescent, 4-meth ylumbelliferone end product:

$$\text{4-methylum belliferyl-}\beta\text{-}D\text{-glucuronide} \xrightarrow{\beta\text{-}D\text{-galactosidase}} \text{4-methylumbelliferone (that fluoresces under long wavelength:365 nm UV light)}$$

Enterolert is used to detect enterococci by β-glucosidase that catalyzes the production of 4-methylumbelliferone at 41°C.

Rapid detection using chromogenic substrates

Chromogenic compounds, added to the conventional or newly devised media, were used for the isolation of the indicator bacteria. These chromogenic substances are modified either by enzymes (which are typical for the respective bacteria) or by specific bacterial metabolites. After modification, the chromogenic substance changes its color or fluorescence, thus enabling easy detection of colonies displaying the metabolic activity. In this way, these substances are used to avoid the need for isolation of pure cultures and confirmatory tests. The time required for the determination of different indicator bacteria can be reduced to be between 14 and 18 hours. When necessary, the surface antigens can be selected and used as the pathogen recognition element for confirmation.

Immunological methods

Antibodies-based detection

Antibodies are immunoglobulins secreted by B-cells and are recruited by the immune system to identify and neutralize foreign objects such as bacteria and viruses. Antibodies possess highly specific binding and recognition domains that can be targeted to specific surface structures of a pathogen. Immunological methods using antibodies are widely used to detect pathogens in clinical, agricultural, and environmental samples. As always with immunological techniques, the specificity of the reagents and optimization of their use is a critical issue. Although total coliforms are a broad group and likely to be unsuitable immunological targets in environmental waters, *E. coli* could be identified from other coliforms.

It is evident that aforesaid methods and approaches are either laborious and time consuming or nonspecific with a probability of false positives. Another major disadvantage of the aforementioned methods is the failure to detect noncultivable microbial communities that are viable but nonculturable (VBNC). The VBNC state of a bacterium is its ability to cause the disease and yet fails to respond to enumeration through classical culture procedures. VBNC state of bacterial enteropathogens poses a potential threat to human and animal health because of failure to culture such organisms in current resuscitation protocols that leads to incorrect estimation.

Immunomagnetic separation and other rapid culture-based methods

Immunomagnetic separation offers an alternative approach to rapid identification of culturable and nonculturable microorganisms. The principles and application of the method are simple, but rely on suitable antibody specificity under the experimental conditions. Purified antigens are typically biotinylated and bound to streptavidin-coated paramagnetic particles (e.g., Dynal beads). The raw sample is gently mixed with the immunomagnetic beads and then a magnet is used to hold the target organisms against the wall of the recovery vial, and nonbound material is poured off. If required, the process can be repeated, and the beads may be removed by simple vertexing. Target organisms can then be cultured or identified by direct means. The IMS approach may be applied to recovery of indicator bacteria from water, but it is possibly more suited to replace labor-intensive methods for specific pathogens. *E. coli* O157 recovered from water samples were detected using this technique in some studies. Furthermore, *E. coli* O157 detection following IMS can be improved by electrochemiluminescence detection. However, the IMS/culture methods are also accompanied with disadvantages such as the ability of nonspecific binding, the need for physicochemical conditions such as pH and temperature, sensitivity to chemicals in the samples, the high cost of monoclonal antibody production, and limited shelf life.

Molecular methods based on genetic signature of target pathogen

The nucleic acid sequences are unique to all living organisms. These genetic signature sequences are the potential targets to differentiate an organism from others and diagnose various disease-causing agents. In the postgenomic era, a large number of microorganisms have been sequenced. In early, 2013, ~18,000 prokaryotic genomes have been sequenced (NCBI Genome database, http://www.ncbi.nlm.nih.gov/genome/browse/). This sequence database has made it possible to analyze microbial pathogens at the molecular level.

The application of molecular methods has to be considered in the framework of quality management for potable water. The new methods will influence epidemiology and outbreak investigations more than the routine testing of processed drinking water. Certain molecular approaches like restriction fragment length polymorphism, random amplification of polymorphic DNA, and florescence in situ hybridization (FISH) were extensively used for pathogen detection but each with some limitations. In FISH-based detection method, gene probes with a fluorescent marker were used, typically targeting the 16S ribosomal RNA (16S rRNA). Concentrated and fixed cells are permeabilized and mixed with the probe. The stringency of the homology between the gene probe and the target sequence are influenced by incubation temperature and the addition of chemicals. A single fluorescent molecule within a cell does not allow detection, target sequences with multiple copies in a cell have to be selected (e.g., there are 10^2–10^4 copies of 16S rRNA in active cells). Low-nutrient environments may result in cells entering a nonreplicative VBNC state for many pathogens. Such a state may give a false result that makes culture-based methods unreliable and can be overcome using molecular approaches.

Polymerase chain reaction technique and quantitative polymerase chain reaction

The powerful molecular technique, polymerase chain reaction (PCR), allows amplification of target DNA to generate multiple copies that can be detected.

PCR has been validated by the International Organization for Standardization (ISO) is now used for testing of foodborne pathogens (Malorny et al., 2008). One problem with PCR is that the assay volume is in the order of some microliters and requires that the sample to be concentrated to microliter range. The water sample has to be concentrated and purified using adequate methods as natural water samples often contain inhibitory substances such as humic acids and iron, which concentrate with the nucleic acids. Hence, it is critical to have positive and negative controls with each environmental sample PCR to check for inhibition and specificity. It may also be critical to find out whether the signal obtained from the PCR is due to naked nucleic acids or living and dead microorganisms. The sensitivity of the PCR is often not sufficient, and post-PCR processing and analysis is needed. In addition, PCR requires technical equipment and laboratory setup, which is not suitable for onsite diagnostics. Quantitative PCR (qPCR) is also called real-time PCR; a fluorescence-based detection format is more sensitive than conventional PCR. Samples can be analyzed in real time with higher specificity and sensitivity. No post-PCR processing is required. The technique has been applied to locate nonpoint sources of pathogen contamination and environmental risk assessment (Singh et al., 2010).

qPCR is the most preferred method for the quantitative enumeration of pathogenic microbes in complex environmental matrices. The advantage associated with qPCR depends on its sensitivity, specificity, reproducibility, and wide quantification range. Although qPCR emerged as a suitable and powerful method for the quantification of microbial communities, but still certain issues need serious consideration and might affect analysis of results, especially when dealing with complex sample matrices. Firstly, specificity of primers targeting a specific group and florescent probes followed by nucleic acid extraction efficiency need to be addressed. The quality of template nucleic acid and amplification of DNA from nonviable cells are also important factors that affect qPCR analysis.

A study has shown the application of qPCR for the detection of several bacterial pathogens in a river of Southern Ontario, Canada, which is the major source of drinking water (Banihashemi et al., 2015). In this study, qPCR was coupled to propidium monoazide dye for the quantitative enumeration of selected pathogens such as *Salmonella enterica*, *Campylobacter*, *E. coli* O157:H7, and *Arcobacter butzleri* and suggested that qPCR coupled with PMA dye was able to detect viable pathogens in surface water. qPCR has been used for detection of ETEC and *Salmonella* in environmental water and sediments (Singh et al., 2010, 2013).

Nucleic acid microarrays

Nucleic acid microarrays are based on the principle of hybridization of nucleic acid with their complimentary sequences. The fluorescent or radioactive group labeled oligonucleotide probes specific to gene are attached to a glass slide surface treated chemically. To facilitate hybridization, the extracted DNA/RNA from the samples is incubated with glass slide loaded with oligonucleotides probes. The fluorescence intensity is directly proportional to concentration of the sequence.

The high-density microarrays allow spotting of thousands of probes on the microarray surface, and this allows the detection of multiple reactions simultaneously. The qPCR can be coupled to microarrays to further increase the limit of detection for the targeted DNA. The technique is high throughput and can be used for characterization of microbial community. The method is not fully implemented to complex matrices, such as wastewater and sediments due to the presence of inhibitors that can hinder DNA isolation along with amplification of the target gene or both. Furthermore, low sensitivity for complex environmental matrices, processing of samples, and wastewater complexities limit the application of this technique in wastewater. Several studies have used nucleic acid array for specific and rapid detection of waterborne pathogens in environment.

Recently, Gomes et al. (2015) developed a DNA microarray utilizing 16 probes for targeting 12 different groups, including total and FCs, Enterococci, *E. coli*, *Clostridium perfringens*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. The study concluded that the developed DNA microarray could prove to be a very useful tool for microbiological surveillance of water.

Next-generation sequencing

The enormous potential and continuously reducing cost has revolutionized the applications of next-generation sequencing (NGS) technology in different diagnostic fields. This advanced version of automated Sanger sequencing has capability to sequence 1 kb for 96 individual samples at a time. The existing NGS methods exploit detection system with diverse chemistry; however, the two main steps are common in all NGS types. The first step is library fragmentation/amplicon library preparation followed by the incorporated nucleotides detection. NGS methods can further be classified into two main groups. The first group is PCR-based technology, and other group is non PCR-based termed as single-molecule sequencing technology (amplification is not required before sequencing). The general steps include isolation of DNA from target organism and fragmentation into small pieces. After

this, portions of these fragments are sequenced, usually with the help of sequencing systems. The sequenced fragments were gathered into large pieces to observe overlaps in the sequence each possesses. The gaps between the larger pieces can be filled to determine the complete genome. The NGS methods have now been implemented to detect more than 1000 microbes and multicellular species and have further increased the understanding of the biology and evolution of life.

NGS technologies have potential to generate several hundred thousand to tens of millions of sequencing reads simultaneously. In recent years, the mass sequencing of samples from complex environmental matrices has been an area of great interest for ecological diversity-related research. The application of NGS methods has facilitated the analysis of various environmental samples from different ecosystems. In spite of potential advantages, NGS technologies have faced several challenges. One of the challenges is to improve the sequencing output, in context to read length and accuracy; second, the cost and the labor involved and the third being inclusion of PCR bias as amplification step is essential prior to sequencing.

Huang et al. (2015) utilized 454 pyrosequencing and Illumina high-throughput sequencing for investigating bacterial virulence determinants in drinking water treatment and distribution system. The sequencing results revealed that high bacterial diversity persists in drinking water system, which was found to be decreased after chlorine disinfection. *P. aeruginosa* was found to be in abundance, and *P. aeruginosa* along with *Leptospira interrogans* were detected even after chlorination in the tap water. The study suggested that the combined application of 454 pyrosequencing and Illumina sequencing will be highly beneficial for the characterization of environmental pathogenesis in the drinking water distribution system.

High-throughput sequencing and metagenomic approaches were applied to investigate the variation in antibiotic resistance gene's pattern and bacterial community in a drinking water treatment and distribution system (Jia et al., 2015). The observations revealed the presence of 151 antibiotic resistance genes representing 15 antimicrobial groups in the drinking water; however, after chlorination, the diversity was found to be decreased. The study suggested that the residual chlorine acted as a key factor responsible for the shift in bacterial community and alteration in resistome.

Digital polymerase chain reaction

The third-generation PCR technology has been introduced, termed as digital PCR (dPCR), which further added to the quantification of pathogens based on the genetic analysis. In dPCR, developed on the workflow of qPCR, prior to PCR amplification, the sample is subjected to portioning into hundreds of millions of individual reaction chambers, so that each contains one or no copies of the sequence of interest. Two types of dPCR, based on two different principles, have been used. One is based on chip and another is based on droplets (Baker, 2012). Droplet digital PCR (ddPCR) has been developed as a new platform for DNA quantification. There are certain advantages of ddPCR over qPCR: (1) ddPCR excludes the need to prepare standard curve for quantification, (2) provide high-throughput quantification and based on endpoint PCR, and (3) highly resistant to PCR inhibitors.

The application of droplet digital PCR in detection of zoonotic pathogens in poultry processing water samples was reported by Rothrock et al. (2013). The results showed that ddPCR outperformed qPCR and the culture-based method used for poultry processing zoonotic pathogen quantification.

The problem with digital PCR is the sample size. ddPCR was found to be expensive than qPCR in terms of overall cost of consumables and labor when estimated for 96 samples.

Loop-mediated isothermal amplification

Loop-mediated isothermal amplification (LAMP) is a simple gene amplification method in which reaction can be performed at a constant temperature. The method was first developed by Notomi et al. (2000). The LAMP method has potential to amplify gene copies in less than 1 hour and does not require sophisticated instrumentation. The whole LAMP reaction set up requires an enzyme DNA polymerase with strand displacement activity and a set of four specially designed primers, inner and outer primers. The sequence of both DNA ends are derived from the inner primers, and these primers facilitate the formation of a stem-loop DNA structure followed by self-primed DNA synthesis at the 3′ terminus of the stem-loop DNA. One of the inner primer hybridizes to the loop and initiates strand displacement DNA synthesis yielding the actual stem-loop DNA along with new stem-loop DNA with a stem twice in length. The final products of lamp reactions are stem-loop DNA of the target of interest. A number of methods could be used to detect the amplified LAMP product. The amplicon can be visualized by gel electrophoresis (ladder pattern) or naked eye (color change), by measuring change in turbidity (turbidometer).

The fluorescence labeling is a commonly used and well-established method, but has the disadvantages like a lower stability of fluorescent dyes and the requirement for expensive readout systems. The fluorescence-based detection systems have many significant drawbacks, including susceptibility to photobleaching,

complexity, sensitivity to contamination, cost, and reliance on relatively expensive equipment to probe their presence in an assay.

Although diverse the existing methods have certain disadvantages that limit their use in point-of-care settings and field situations due to requirements sophisticated instrumentation or trained personnel. Faster, simpler, and more reliable detection methods would largely support to help protect consumers. Direct detection methods that provide quick, accurate, simple, and cost-effective devices to be used onsite are highly desired. Advances in human and animal science are placing increasingly stringent demands on diagnostic and clinical tests to enhance sensitivity, specificity, and thresholds.

There is an extensive need for the selection of a unique system, which will allow the pathogen recognition element to be accepted for an ideal detection format. It is becoming evident that new pathogen detection methodologies enabled by "nanotechnology"-based approaches have the potential to provide better options.

Nanotechnology and promises

The term nano derived from the Greek word meaning *dwarf is* usually combined with a noun to form words such as nanometer, nanorobot, and nanotechnology. In the last two decades, nanoscience and nanotechnology have seen a plethora of new developments in almost every field of science and technology, especially in biology and medicine. Nanotechnology has set high expectation in biological and medical sciences to solve key questions concerning biosystems that transpire at the nanoscale. It deals with creation of functional materials, devices, and systems, in the nanometer scale length of 1−100 nm. The ability to manipulate and engineer materials at the nanoscale (atomic, molecular, and macromolecular scale) enables us to tune the physical and chemical properties of desired materials according to specific applications.

The surface functionalization of nanomaterials by biomolecules has led to the development of new interdisciplinary research areas like biomedical nanotechnology, nanomedicine, diagnostic devices, theranostics, contrast agents, nanobiosensors, and targeted drug delivery vehicles. Functionalities can be added to nanomaterials by interfacing them with biological molecules or structures (Fig. 29.4A) that make nanomaterials useful in various technological areas as well as both in vivo and in vitro applications. Nanotechnology has played a significant role in the development of affinity sensors, for example, antibody−antigen interaction−based biosensors and ultrasensitive DNA hybridization detection. At nanoscale, some materials have been shown to exhibit extraordinary optical properties relative to their bulk counterparts. The light-scattering power of nanoparticles is in the order of magnitude greater than fluorescent labels. Moreover, the optical signals generated from these nanoparticles are not prone to photobleaching compared to their counterpart organic dyes. Semiconductor quantum dots are one of the pioneering examples that distinctly show size-dependent emission of different colors with high quantum yield when

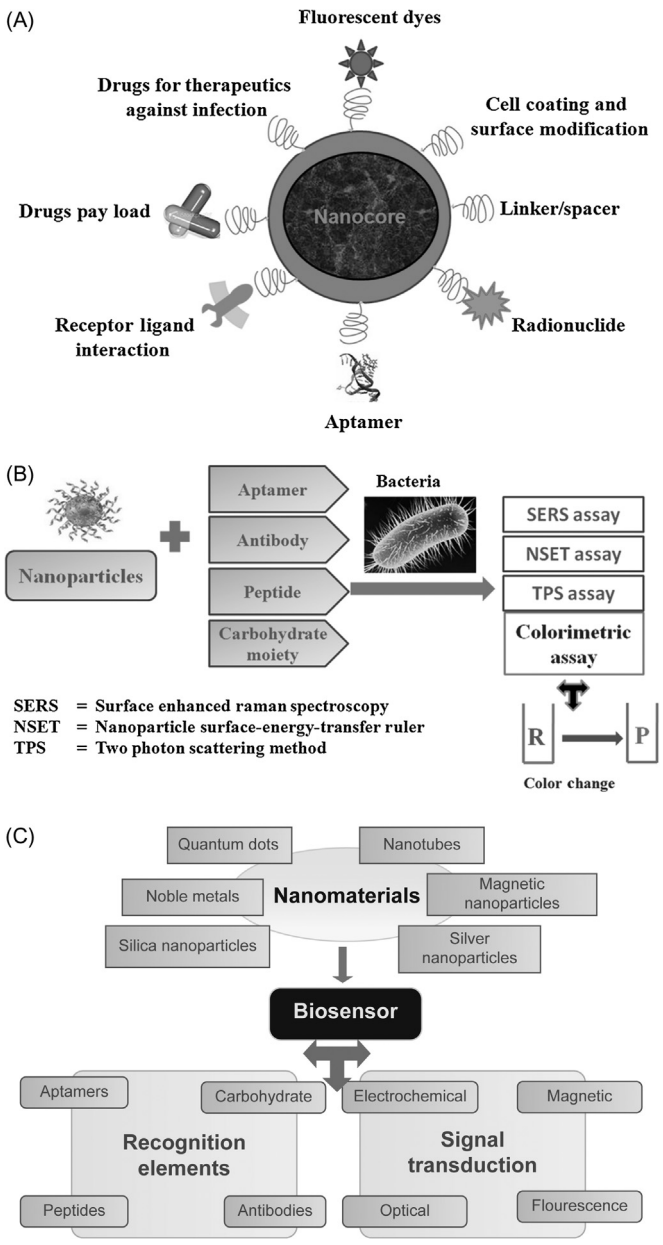


FIGURE 29.4. (A) Functionalization of nanoparticles. (B) Nanomaterial applications in pathogen detection. (C) Nanomaterial components involved in pathogen detection. *Source: Modified from Vikesland et al., 2010.*

excited with single wavelength. The ability to simultaneously tag multiple biomolecules with these quantum dots has given an opportunity to develop new optical diagnostic tools and ability to observe the complex cellular changes and associated events. On the other hand, noble metal nanoparticles have attracted much interest because of their unique physicochemical properties, including large optical field enhancements resulting in the strong scattering and absorption of light in visible region due to the presence of surface plasmon resonances (SPRs). The biochemical assay based on light-scattering signals from metal nanoparticles has been widely used in the determination of affinity interaction among DNA, proteins, and drugs that leads to develop "easy-to-use" optical sensing devices.

Metallic nanoparticles

The colloidal metal nanoparticles, namely, gold and silver nanoparticles have attracted tremendous research interest. Metallic nanoparticles in colloidal solution appear cultured, for example, colloidal GNPs generally appear ruby red, purple, blue, and orange depending on their shape, size, and synthesis conditions. Similarly, titanium (Ti) and platinum (Pt) nanoparticles give blue and dark brown colors. The different colors of metal nanoparticles arise from SPR and their confinement.

Gold nanoparticles–based colorimetric assays

Among metals, gold nanoparticles exhibit excellent biocompatibility, electronic, optical and catalytic properties. GNPs have found a distinguished place in biodiagnostics due to their size-dependent optical properties, variety of surface coatings, and biocompatibility (Mirkin et al., 1996; Storhoff et al., 2004; Sato et al., 2007; Pandey et al., 2008). Spherical GNPs exhibit SPR-related optical absorption at 520 nm, which strongly depend on particle size and morphology.

The rich surface chemistry of GNPs allows surface modification with various biofunctional groups, such as nucleic acids, sugars, proteins via the strong affinity of gold surface with thiol ligands creating multifunctionality to tailor the needs of biomedical applications including imaging, diagnostics, and therapy. The conjugation of nanoparticles with biomolecules, for example, proteins, DNA, can be done either by direct covalent linkage or by noncovalent interactions. Biomolecules are often covalently linked to ligands on the nanoparticles surface via traditional coupling strategies such as carbodiimide-mediated amidation and esterification.

The high surface-to-volume ratio of GNPS makes surface electrons sensitive to minor changes in the dielectric (refractive index) constant of the medium. Therefore, changes to the surface chemistry and environment of these particles (surface modification, aggregation, medium refractive index) lead to colorimetric changes of the dispersions that becomes the basis for detection of any analyte of interest. This has further facilitated the application in bio-detection via numerous detection methods (Boyer et al., 2002). The various synthesis methods of GNPs have been discussed in the methodology section.

Silver nanoparticles colorimetric assays

Silver nanoparticles like GNPs exhibit peculiar properties of localized SPR. As described for GNPs, the colloidal solutions of AgNPs have high extinction coefficients and color change property in the visible region of the spectrum depending on the intermolecular distance. These novel inherent optical properties make AgNPs another viable choice for signal enhancement in a colorimetric assay. AgNPs have been used less frequently than AuNPs in the development of colorimetric assays for pathogens based on aggregation and spectral shift. There are many reports utilizing AgNPs colorimetric assays (based on color change from yellow to brown corresponding to dispersed and aggregated AgNPs) for many organic and inorganic molecules. The role of AgNPs has been explored frequently as an antimicrobial agent rather than colorimetric-based sensing agent. AgNPs more often have been used in combination with AuNPs for the development of colorimetric assays for the detection of waterborne microorganism.

A simple colorimetric assay for determining the *Enterobacter cloacae* P99 β-lactamase activity on the basis of silver and GNPs aggregation was reported (Liu et al., 2010). *E. cloacae* P99 β -lactamase attacks on the β-lactam ring in the cephalosporin substrate and the active sites–modified linker were exposed. This results in aggregation of nanoparticles (silver and/or gold) based on the electrostatic interactions and metal-thiols conjugation between the flexible linker and citrates on the surfaces of silver and GNPs.

Quantum dots

Quantum dots (QDs) are the semiconductor nanocrystals (dimensions in the range of 2–6 nm) and of great interest due to their unique electrooptical properties that lie between the molecular and the bulk semiconductor regime. QDs exhibiting inherent fluorescence are frequently used in various biomedical applications. QDs can enhance detection sensitivity of existing florescent-based methods for pathogen identification. The multicolor properties and photostability of QDs compared to conventional fluorescent dyes for the detection of pathogens makes it a better choice for

biosensing applications. The surface functionalization of QDs with different types of recognition elements targeting viruses, bacteria, and protozoa have been frequently reported (Gilmartin and O'Kennedy, 2012). QDs have found applications in immunoassays for the detection of waterborne and foodborne pathogens such as *E. coli*, *Salmonella*, and *Shigella*.

Silica/other nanomaterials

Silica nanoparticles have found applications in material research due to its unique optical properties. It is composed of honeycomb-like porous structure with plenty of empty channels. Silica nanoparticle's unique properties like tunable pore sizes, high surface-to-volume ratio, and tunable particle diameter made it a preferred choice to be used in the development of pathogen detection methodologies.

Magnetic Fe_3O_4 nanoparticles coated with silica have been used for the isolation of genomic DNA of hepatitis virus B and of Epstein–Barr virus for the qPCR-based quantification of viruses (Quy et al., 2013).

History

The detection of microbial pathogens remains a challenging task despite great strides made in the past three decades. The most frequently used culture-based methods have undergone diverse modifications including specific substrate for enzymes present in target organisms. However, the issues of specificity and sensitivity directed toward low doses of organism, VBNC state, and long incubation periods still elude the culture technique. The immunological methods using antibodies to detect pathogens in different domains of environment as well as in clinical settings followed culture techniques. Immunomagnetic separation evolved to improve detection but faced specificity and limited shelf life problems. The understanding of nucleic acid structure and function led to the era of gene sequencing, DNA hybridization, and consequent genetic signature–based detection of the organisms. The milestone in molecular biology, PCR technique by Kary Mullis in 1983 led to a burst of PCR in pathogen detection between 1992 and 1999. This was followed by advancements in PCR, the real-time PCR or qPCR based on fluorescence-based chemistries. This technique is widely used for quantitative enumeration of pathogens in different domains from 1999 till today. Fluorescence-based detection is complex issues of photobleaching, contamination, cost, and dependence on expensive instrumentation limits application in "onsite detection." These methodologies delineate the need for simple assay and less-expensive detection system. Nanoscience provides new horizons in bionanotechnology for detection of pathogens based on genetic signature. In the last decade, nano-based approaches exploiting the unique properties of nanoparticles have shown potential for the development of novel pathogen detection systems.

Detection principle

The efficient use of nanomaterials in biological systems relies on the knowledge of the *nano-bio interface*. GNPs have found widespread applications in life sciences and serve as excellent standards to understand more general features of the nano-bio interface because of its many advantages over other inorganic materials. The bulk material is chemically inert. Gold's background concentration in biological systems is low, which makes it relatively easy to measure it at the part-per-billion level or lower in water. The unique optical and electronic properties of GNPs enable to conjugate biological molecules like RNA and DNA and serve as scaffolds for nanostructures. GNPs interaction with light is governed by their size, environment, and physical dimensions. The fluctuating electric fields of a light ray promulgating near a colloidal nanoparticle interact with the free electrons causing intense oscillation of electron charge that is in resonance with the frequency of visible light. These resonant oscillations are known as surface plasmons. SPR, for monodisperse GNPs ($\sim$30 nm), causes an absorption of light in the blue-green portion of the spectrum ($\sim$450 nm), while red light ($\sim$700 nm) is reflected, yielding a rich red color. The wavelength of SPR-related absorption shifts to longer wavelengths, with an increase in particle size. Red light is then absorbed, and blue light is reflected, resulting in solutions with a pale blue or purple color. The change in optical properties as a result of shape size and aggregation pave the way for development of colorimetric and sensitive detection system for specific bioanalytes. As an example, the DNA hybridization event using GNP is recognized by change in color that appears as a result of DNA hybridization, which brings GNPS in close proximity.

Several proof-of-concept studies demonstrate the use of GNPs in biomedical applications like in chemical sensing, biological imaging, drug delivery, and cancer treatment. A number of factors, namely, size and shape of the nanoparticle, refractive index of the surrounding media, and interparticle distance, are taken into account for use in colorimetric detection of DNA. Mirkin et al. (1996) reported the colorimetric detection of DNA targets based on the cross-linking mechanism use of GNP probes. The two different batches of probes are designed to target the DNA. Thus, on the

addition of target DNA, a polymeric network of GNP probes is generated due to aggregation, turning the solution from red to blue (Mirkin et al., 1996). This aggregation mechanism is mainly applied to detect small sized targets. The GNP aggregation induced by interparticle cross-linking is relatively a slower process. The relatively slow aggregation is due to the nature of the interparticle cross-linking aggregation mechanism. In general, the aggregation is driven by random collisions between nanoparticles with relatively slow Brownian motion (Sato et al., 2003).

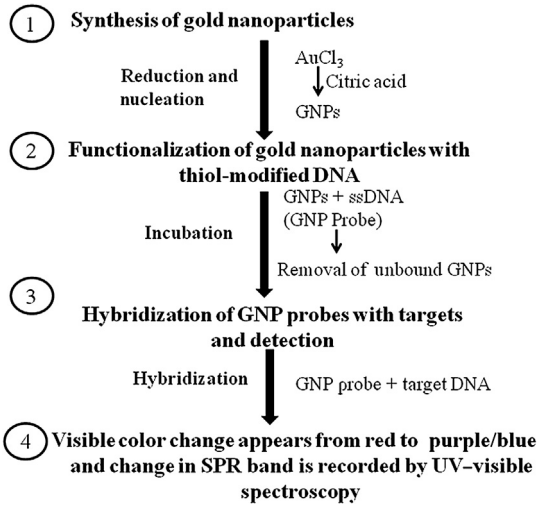
Similarly, the DNA detection based on a noncross-linking mechanism has been well documented. Sato and coworkers described noncross-linking mechanism of GNP aggregation for DNA detection. The single-stranded DNA can be immobilized on GNPs above the physiological temperature. The GNP probes aggregate together at a considerably high salt concentration when the target DNA is perfectly complementary to the probe (Sato et al., 2007). In noncross-linking aggregation systems, the van der Waals force of attraction dominates. Many parameters, including surface charge properties (e.g., charge density, the amount of associated counter ions) and entropy factors, are also involved in aggregation.

Compared to interparticle cross-linking aggregation systems, the noncross-linking aggregation mechanism has some attractive features. Aggregation induced by the noncross-linking process is very rapid leading to the development of faster assays. The interparticle attractive forces (van der Waals forces) dominate over the interparticle repulsive forces, which results in rapid aggregation (Sato et al., 2003). The use of GNP probes for the colorimetric detection of DNA targets represents an inexpensive and simple workable alternative to fluorescence or radioactivity-based assays.

Methodology

Synthesis of gold nanoparticles

GNPs can be synthesized using various synthesis routes. In typical synthesis, tetrachloroauric acid ($HAuCl_4$) is mixed with a reducing agent, which leads to the reduction of Au ions to form nanoparticles. In the most reliable and popular method by Brust et al. (1998), citric acid first reduces gold ions from $HAuCl_4$ and triggers the nucleation to form nanoparticles followed by its adsorption to the surface, which provides colloidal stability to nanoparticles due to its negative charges (). This method produces monodisperse spherical GNPs with a diameter of 10–20 nm (step 1, Flow Chart 29.1).



FLOW CHART 29.1 Step-wise representation of GNP-based detection of microorganism.

Computation of single-stranded DNA sequences for functionalization of gold nanoparticles

The GNPs are biofunctionalized with the thiol-modified ssDNA or oligonucleotides to generate GNP probe for target DNA detection. The bioinformatics tools are used to compute ssDNA sequences or probes complementary to the target gene sequence of pathogen of interest. The first step toward this is the retrieval of nucleotide sequences of targeted genes of selected organisms from GenBank (www.ncbi.nlm.nih.gov). Multiple sequence alignment of retrieved conserved region (www.ebi.ac.uk/clustalW) followed by computation of oligonucleotide probes using web-based or dedicated softwares such as Primer 3, Lasergene, or Beacon Designer. An analysis for cross homology and secondary structure is carried out by BLAST and Mfold servers, (www.ncbi.nlm.nih.gov/Blast, www.bioinfo.rpi.edu/applications/mfold). The computed ssDNA sequences are then synthesized in a DNA synthesizer for functionalization of GNPs.

Functionalization of gold nanoparticles with thiol-modified DNA

The computed oligonucleotides are synthesized with modification, thiol group (-SH) at their 5′ or 3′ end. These modified oligonucleotides are then attached to the GNPs through chemisorption of the thiol group onto the surface of the GNPs for generation of GNP probes. To functionalize with DNA, GNPs (selected size $\sim 20 \pm 0.2$ nm diameter) are incubated with different batches (number varies with the target size) of thiol-modified ssDNAs separately for 16–20 hours

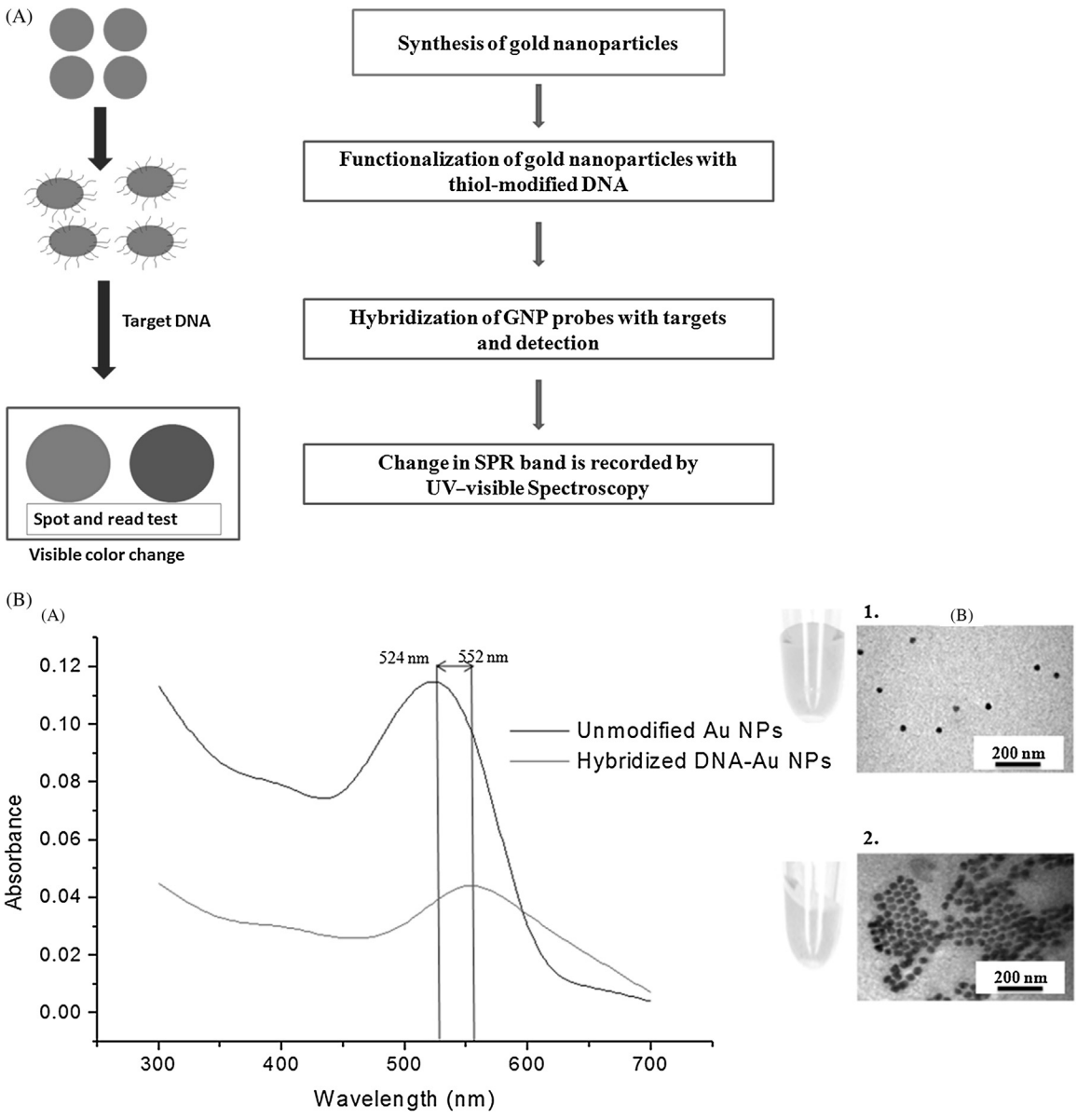


FIGURE 29.5 (A) UV–Vis spectra of unmodified GNPs (*black*) and hybridized DNA–GNPs (*red*); (B) transmission electron micrograph of the (1) monodispersed GNPs (20 ± 0.2 nm) and (2) hybridized polymer network corresponding to the change in solution color from *red*/*pink* to *purple*.

with oligonucleotide concentration of 2 μM. The unbound ssDNA is then removed by centrifugation at 16,000 $\times g$ for 15 minutes, and the pellet of DNA-conjugated GNPs is washed in 0.1 M Tris-acetate and NaCl (pH 8.2) buffer. The ssDNA-grafted GNP is then stored in 0.3 M Tris-acetate NaCl (pH 8.2) at room temperature. The absorption spectra of the biofunctionalized GNP are recorded by UV–visible spectroscopy for confirmation of DNA immobilization (steps 2–4, Flow Chart 29.1).

The basic steps of DNA detection using GNPs probes are depicted in Fig. 29.5A.

Examples of application of gold nanoparticles and few examples of silver nanoparticle and quantum dots for detection of bacteria

EHEC serotype O157:H7 is one of the most deadly pathogens. EHEC produces *stx1* and *stx2* type of enterotoxins and symptoms, such as abdominal pain and watery diarrhea. Many patients develop life-threatening disease, such as hemorrhagic colitis and hemolytic-uremic syndrome, the deadly consequences of these cytotoxins. The natural reservoirs of EHEC are domestic and wild ruminant animals, which shed

TABLE 29.2 Single-stranded thiol-modified oligonucleotide probes and their complementary sequences.

Probe/synthetic target	Nucleotide sequence (5′-3′)	Length (bp)
sx2F	HS-$(C)_6$-GGAGTTCAGTGGTAATACAATG	22
sx2R	HS-$(C)_6$-GCGTCATCGTATACACAGG	19

Modified from Jyoti A., Pandey P., Singh S.P., Jain S.K., Shanker R. (2010). Colorimetric detection of nucleic acid signature of shiga toxin producing Escherichia coli using gold nanoparticles. J. Nanosci. Nanotechnol. 10, 4154–4158.

the bacteria along with their feces into the environment. The products of animal origin, such as meat and milk, are at risk of contamination with EHEC originating from animals. Consumption of food containing EHEC was identified as a major route of human infections with these pathogens in different countries. The hamburger, vegetables, and fruit juices have been frequently contaminated with pathogenic *E. coli*, which have also been sources of infection. Apparently, cattle, the natural reservoir for pathogenic strains, have often been implicated in *E. coli* infections. Following are the examples for the detection of EHEC using GNPs.

Colorimetric detection of DNA of shiga toxin producing *E. coli* using bioconjugated gold nanoparticles

EHEC causes bloody diarrhea in humans through the production of shiga-like toxin. The toxin is encoded by *stx2* gene in *E. coli*. The existing methods for detection of EHEC are culturing of the bacteria on fluorogenic-substrate media that are time consuming. Molecular methods including PCR and real-time PCR assays are also used, which requires expensive instrumentation. In recent studies, the optical properties of GNPs have been exploited for the detection of nucleic acid of *E. coli*. The PCR product of *stx2* gene representing EHEC signature has been targeted using the GNP probes. GNPs of 20 ± 0.2 nm were synthesized by the citrate reduction method and characterized by UV–visible spectroscopy and transmission electron microscopy (TEM). Two different batches of thiolated ssDNA (19 and 22 bp) complementary to target are grafted onto the GNPs (Table 29.2). The hybridization of GNP probes with target DNA led to change in color from red to purple that is visible by naked eye (Jyoti et al., 2010). The hybridization-induced aggregation was also observed by TEM.

Colorimetric detection of *enterotoxigenic E. coli* gene using gold nanoparticle probes

In the present example, the colorimetric detection of heat-labile toxin gene, *LT1* (1257 bp), of ETEC has been shown. Multiple probes were used to target the gene to increase specificity toward target as well as the aggregation of GNPs. A total of eight GNP probes were used to target different locations on the target DNA sequence. The oligonucleotides were computed based on the conserved signature gene of ETEC. The GNPs were functionalized with the thiol-modified ssDNAs to facilitate hybridization with the target. After hybridization, a change in SPR-related band is observed in UV–visible spectroscopy. This leads to a visible colorimetric change of reaction assay mixture from red ($\lambda_{max} = 524$ nm) to purple ($\lambda_{max} = 552$ nm), which is clearly visible to the naked eye. The aggregation and reduction in the interparticle distances of GNPs are evaluated by TEM. TEM confirms the hybridization, aggregation, and reduction in the interparticle distances of the GNP probes in the presence of target DNA (Fig. 29.3). In addition, the assay shows its specificity by differentiating the DNA of EHEC from ETEC, a closely related pathotype of *E. coli*.

The aggregation and the spectral shift in the plasmon band leading to change in color observed with target DNA indicates the possibility of a simple and rapid colorimetric "spot and read" test in contrast to amplification-based fluorescence detection methods (Fig. 29.5B).

Clinical significance of nanoparticle-based detection

In recent years, nano-based approaches have been exploited for detection of other pathogens of clinical significance like *Mycobacterium* and other pathogenic species that affect humans and animals as causative agents of tuberculosis, leprosy, and paratuberculosis. Direct detection of unamplified DNA from pathogenic mycobacteria using DNA-derivatized GNPs was reported by Liandris et al. (2009). Different nanodiagnostics systems have been developed for the molecular diagnostics of tuberculosis like nanoparticle-based systems, such as gold, silver, silica, and QDs, have been the most widely used for TB diagnostics due to their unique physicochemical properties. For detection of multiple bacterial genomic DNA detection, gold nano rods probes were used with high sensitivity and excellent specificity by reading the decrease in its sensitive longitudinal absorption band (Wang et al., 2012). The development of nano-enabled assays and onsite detection systems for EHEC and ETEC can perhaps help in reduction of morbidity and mortality in children in rural ecozones. In summary, high sensitivity and selectivity, easy to use, and rapid

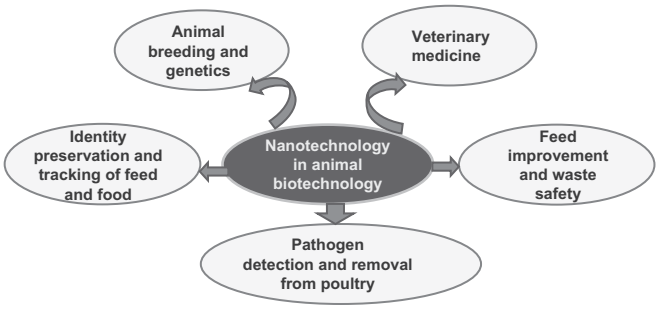


FIGURE 29.6 Applications of nanotechnology in animal biotechnology.

detection systems are the immediate need, and nanoparticle-based detection shows a great promise in the development of such nanodevices.

Ethical issues

Containment of hazards in laboratory experiments is the key to safety. Microbiologists have gained valuable experience over decades in handling extremely dangerous natural organisms, such as smallpox virus and cholera bacteria. According to *Biosafety in Microbiological and Biomedical Laboratories* (Centers for Disease Control and Prevention National Institutes of Health, 2009), safe handling and containment of infectious microorganisms and hazardous biological materials are the mandatory principles of working with microbes. The basics of containment include the safety equipment, microbiological practices, and safeguards facility that protect laboratory workers, the sterile environment, and the public from exposure to infectious microorganisms that are handled and stored in the laboratory. This inclusion of risk assessment in our routine practice can prevent laboratory-associated infections. Individual workers handling the pathogenic microorganisms must understand the containment conditions under which infectious agents can be safely maneuvered and secured. The correct information and the use of appropriate techniques and equipment will enable the microbiological and biomedical community to prevent personal, laboratory, and environmental exposure to potentially infectious agents or biohazards. Furthermore, our limited understanding on the adverse impact of engineered nanomaterials or nanoparticles in biological systems desires safe handling by following basic laboratory safety norms and disposal as per the guidelines available internationally (Dhawan et al., 2011).

Translational significance

Nanomaterials-based DNA detection and biosensors for sensing pathogen are attracting much attention due to their comparatively high sensitivity and noncomplexity (Fig. 29.4B). The replacement of fluorescently labeled DNA probes with a class of metallic nanoparticle-conjugated probes appears promising because it can minimize or eliminate the necessity of using expensive and complex instrumentation. Nanosized, multipurpose sensors are being developed to detect almost everything from physiological parameters to toxic compounds. Nanosensors can detect very small amounts of a chemical contaminant, virus, or bacteria in food systems. Carbon nanotubes are being investigated as biosensors to detect glucose, ethanol, hydrogen peroxide, immunoglobulins, and as an electrochemical DNA hybridization biosensor (Fig. 29.4C). Nanotechnology has a tremendous potential to revolutionize agriculture and livestock sector. It can provide new tools for molecular and cellular biology, biotechnology, veterinary physiology, animal genetics, and reproduction, which greatly increase the sensitivity of detection of biological materials with quantities usually in nanoliter to picoliter range (Fig. 29.6).

Nanoparticles are being used to remove *Campylobacter* and *E. coli* from poultry products (Manuja et al., 2012). *Listeria monocytogenes*, another foodborne pathogen, was detected in spiked milk samples by magnetic nanoparticle-based immune-magnetic separation combined with real-time PCR. GNPs have been used to detect contamination of melamine in raw milk samples by the naked eye, and no sophisticated instruments are required. The method is also promising for detection of melamine contamination in other foods, such as eggs and animal feeds. A fluorescent biobarcoded DNA assay has been developed for the rapid detection of the *Salmonella enteritidis* based on two nanoparticles (Zhang et al., 2009). A hand-held chip detects animal *cytochrome b* genes in food or feed products. Nanosensors can detect very small amounts of a chemical contaminant, virus, or bacteria in food systems.

Futuristic approach

Nanotechnology has the potential to revolutionize biomedical science of the 21st century through a paradigm shift in diagnostics, drug discovery and delivery, vaccine development, and tissue engineering. In the domain of veterinary science, nanotechnology has improved animal reproduction, hygiene and health, and animal nutrition. Food science and technology have seen new nanoscience-based developments in food storage especially in animal food like red meat and fish by increasing their shelf life and fortified nutritional value.

In microbiology, the nanomaterials are paving way as new bactericidal promising to fight even multidrug resistance. Nanotechnology tools could enable the

understanding of how bacteria work, while providing new opportunities to probe the dynamic and physical aspects of molecules, molecular assemblies, and intact microbial cells, whether in isolation or under in vivo conditions. Furthermore, developments in nanoscience are leading to the new, sensitive and faster methods for pathogen detection in the form of nanosensors. The detection of relatively large-sized dsDNA using stable DNA-functionalized GNPs is quite simple colorimetric approach without the use of complex instrumentation. However, the developed colorimetric approaches still need research advancement in terms of sensitivity and onsite instrumentation before commercialization.

World Wide Web resources

1. NCBI Genome database: http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi
2. Multiple sequence alignment: www.ebi.ac.uk/clustalW
3. BLAST (basic local alignment search tool): www.ncbi.nlm.nih.gov/Blast
4. Mfold: www.bioinfo.rpi.edu/applications/mfold/
5. Wikipedia: http://en.wikipedia.org/wiki/Surface_plasmon_resonance

The NCBI houses genome sequencing data in GenBank and an index of biomedical research articles in PubMed Central and PubMed, as well as other information relevant to biotechnology.

ClustalW is a program for global multiple sequence alignment. The program clustal W constructs pairwise sequences alignment. This heuristic method does a pairwise progressive sequence alignment for all the sequence pair that can be constructed from the sequence set. A dendrogram (guide tree) of the sequences is then generated according to the pairwise similarity of the sequence. Finally, a multiple sequence is constructed by aligning sequences in the order, defined by the guide tree.

BLAST, is an algorithm for comparing primary sequence information, such as nucleotides of DNA sequences or the amino acid sequences of different proteins. A BLAST search compares a query sequence with a library or database of sequences and identifies library sequences that resemble the query sequence above a certain threshold. Different types of BLASTs are available according to the query sequences.

In silico is an expression used to mean "performed on computer or via computer simulation." The phrase is coined from the Latin phrases in vivo and in vitro, which are is commonly used in biology and refers to experiments done in living organism and outside of living organism, respectively. The in silico approach reduces time and expense involved in the bench work on testing several PCR primers experimentally to reach a validated protocol.

The mfold web server is one of the oldest web servers in computational molecular biology. The program predicts the secondary structures of RNA and DNA mainly by using thermodynamics methods.

Clinical correlations

The infections caused by bacteria are still a major health challenge throughout the world. The detection of bacterial pathogens is a critical issue in water and food safety, clinical medicine, agriculture, and human health. Despite advancements in the identification and quantification methods for bacterial pathogens, some of the extensively used methods face challenges, for example, requirement of sophisticated instruments, availability of skilled staff, involvement of labor, and cost. The unique properties of nanomaterials have been utilized for the detection of targeted bacteria in more complex matrices like blood. The use of nanotechnology (viz, GNPs and their plasmonic shifts) has revolutionized the detection of bacterial pathogens, virulence factors, and nucleic acids with desirable detection limits. These nanotechnology-oriented diagnostic methods are urgently needed in generating early warning systems and point-of-care applications. The nanotechnology-based diagnostics methods will help to detect pathogens and prevent outbreaks and ultimately assist in safeguarding human health.

Turning point

The detection system based on simple, rapid, highly sensitive, and specific methodology for detection of antimicrobial-resistant microbes is desired in aggressively populating planet. The advances in gene detection techniques and nanoscience indicate the development, in not too distant future, of nanoparticle probe based "spot and read" enabled hand-held robust device for onsite detection of pathogens not only in animal to clinical samples but also in a range of environmental matrices to protect life on earth.

1. This is the first step of synthesis of GNPs by Turkevitch and Brust method in which citric acid reduces gold chloride, that is, $AuCl_3$, and triggers the nucleation of Au ions to form nanoparticles followed by its adsorption to the surface, which provides colloidal stability to nanoparticles due to its negative charges.

2. This is the second step, in which GNPs are functionalized with ssDNA. GNPs are incubated with thiol-modified ssDNAs for 16–20 hours and unbound ssDNA is removed by centrifuging the solution. The pellet of DNA-conjugated GNPs is washed and stored in 0.3 M Tris-acetate NaCl (pH 8.2) at room temperature.
3. The third step is hybridization of formed GNP probes with targets and subsequent detection. After hybridization with the target, the GNP probes come in the close proximity; as a result, the particles aggregate and the solution appears purple/blue.
4. This is the last step in which visible color change appears from red to blue/purple change in the SPR band, which is recorded by UV–visible spectroscopy.

Awards/recognitions

Richard Phillips Feynman was a theoretical physicist and he gave a lecture "There's Plenty of Room at the Bottom" at an American Physical Society meeting at Caltech in 1959. By his lecture, he introduced the world to the nanotechnology. He laid the foundation for the field of nanotechnology and imagined a day when things could be miniaturized.

Acknowledgment

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Glossary

DNA detection It refers to the identification and detection of DNA molecules using molecular techniques.
Gold nanoparticles A suspension of nanometer-sized particles of gold in a fluid having intense red color (less than 100 nm).
Nanotechnology Nanotechnology is the engineering of functional systems at the molecular scale.
Surface plasmon resonance (SPR) SPR can be described as the resonant, collective oscillation of valence electrons in a solid stimulated by incident light.
Thiol-modified DNA The single-stranded DNA having thiol modification (-SH group) at either 5′/3′-terminal.

Abbreviations

BLAST	Basic local alignment search tool
ddPCR	Droplet digital PCR
dPCR	Digital PCR
FC	Fecal coliforms
FIB	Fecal indicator bacteria
FISH	Florescence in situ hybridization
GNPs	Gold nanoparticles
LAMP	Loop-mediated isothermal amplification
MF	Membrane filter
MPN	Most probable number
NGS	Next-generation sequencing
NSET	Nanoparticle surface-energy-transfer ruler
PCR	Polymerase chain reaction
QDs	Quantum dots
qPCR	Quantitative polymerase chain reaction
SERS	Surface enhanced Raman spectroscopy
SPR	Surface plasmon resonances
TEM	Transmission electron microscopy
TPS	Two photon scattering method
VBNC	Viable but nonculturable
WHO	World Health Organization

Long answer questions

1. Define nanotechnology.
2. What is nanobiotechnology?
3. Discuss the following applications of nanotechnology.
   a. Medical
   b. Nanotoxicology
   c. Nanotechnology and environment
4. Discuss in detail about the principle of working of gold nanoparticles for DNA detection.
5. Describe the applications of nanomaterials for pathogen detection?

Short answer questions

1. What is the significance of particle size in the nanodomain?
2. Brief about metal nanoparticles synthesis by the colloidal route.
3. What is the principle behind the color change in hybridization solution?
4. Differentiate the working of cross-linking and noncross-linking pattern of GNP probes in DNA detection.
5. How electron microscopy confirms the detection of target DNA?

Answers to short answer questions

1. Nanotechnology deals with creation of functional materials, devices, and systems, in the nanometer scale length (1–100 nm). The small particles exhibit high surface-to-volume ratio compared to their bulk counterparts and becomes more reactive due to the

presence of large number of atoms on the surface. The quantum confinement effects at the nanoscale imparts exceptional physicochemical properties to nanoparticles that makes them promising for various technological applications. In biology, these small size particles find immense potential in improving our understanding of cell functioning as most of the biological activity happens at that nanoscale.

2. The metal nanoparticles are synthesized by reduction of metal cation using appropriate reducing agent in aqueous and/or nonaqueous solvents. A stabilizer is also used during the synthesis to prevent the aggregation of the nanoparticle. The synthesis could be generalized the following equation:
   $M^{+} + ne^{-} + \text{stabilizer} \rightarrow M^{\circ}$.
3. After DNA hybridization, the nanoparticles come closer to each other and acts like an aggregate. This aggregate of nanoparticles causes shift in the light scattering that leads to change in color.
4. The method based on the GNP cross-linking involves attachment of noncomplementary DNA oligonucleotides capped with thiol groups to the surfaces of two batches of GNPs. A polymer network is formed when DNA, complementary to the two grafted oligonucleotides, was added to the solution. This condensed network brings the conjugated GNPs self-assemblies into aggregates with a concomitant change of color from red to purple. This technique is mostly suitable for the tracking of small synthetic target sequences up to 50 bp. Another GNP aggregation system induced by noncross-linking DNA hybridization involves immobilization of ss DNA on gold nanoparticles above the physiological temperature. The nanoparticles conjugated with oligonucleotide probes aggregate together at considerably high salt concentration when the target DNA is perfectly complementary to the probe. This can help in tracking only the short synthetic oligonucleotides (up to 20–30 bp) targets, which again do not represent the sequences of a pathogenic DNA or PCR product.
5. In transmission electron microscopy (TEM), a beam of electrons is transmitted through an ultra-thin specimen, interacting with the specimen as it passes through. TEMs are capable of imaging at a significantly higher resolution so if there is hybridization of GNP probes with target DNA the GNP probes come in the close proximity, as a result, the particles aggregate. This aggregation can be visualized by TEM imaging.

Yes/no type questions

1. Do nanotechnology deals with creation of functional materials, devices, and systems, in the nanometer scale length (1–100 nm)?
2. Do nanoparticles differ in their properties from their bulk counterparts in terms of high surface-to-volume ratio?
3. Do the metal nanoparticles are synthesized by reduction of metal cation using appropriate reducing agent in aqueous and/or nonaqueous solvents?
4. Do the stabilizer used during the synthesis of nanoparticles increases the aggregation of the nanoparticle?
5. Do the aggregation of nanoparticles cause color change in hybridization solution?
6. Do the colloidal solution of GNPs exhibit intense red and blue/purple colors depending on the size, shape, and degree of aggregation of nanoparticles?
7. Do the spherical gold nanoparticles exhibit SPR-related optical absorption at 520 nm, which strongly depends on particle size and morphology?
8. Do the quantum dots (QDs) are the semiconductor nanocrystals (dimensions in the range of 2–6 nm) and of great interest due to their unique electrooptical properties?
9. Do the silver nanoparticles is composed of honeycomb-like porous structure with plenty of empty channels?
10. Will the combination of molecular methods and nanotechnology lead to the development of efficient detection tools for bacteria?

Answers to yes/no type questions

1. Yes.
2. Yes.
3. Yes.
4. No—A stabilizer is used during the synthesis of nanoparticles to prevent the aggregation of the nanoparticle.
5. Yes.
6. Yes.
7. Yes.
8. Yes.
9. No—Silica nanoparticles are composed of honeycomb-like porous structure with plenty of empty channels.
10. Yes.

CHAPTER

30

Herbal medicine and biotechnology for the benefit of human health

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Summary

Since ancient times, plants are known to have tremendous ethnopharmacological significance and are recognized as valuable resource for healthcare. India is the vast repository of these medicinal plants, and despite their innumerous medicinal uses, most of them remained unexplored using biotechnological tools. These plants naturally synthesize organic compounds called "secondary metabolites" by central metabolism of primary metabolites. These metabolites not only play major roles in adaptation of plants to the environment by interacting with the ecosystem but also are prominently used in pharmaceuticals. This chapter discusses the commercial utilization of plants using alternate biotechnological strategies but without affecting their natural population. Furthermore, it highlights the procedures involved in processing of plants and their derivatives for the production of herbal medicine and in drug discovery.

What you can expect to know

This chapter provides useful information on uses and beneficial effects of herbal medicines over conventional drugs. The methods on processing of plants and their parts for production of herbal medicines are described in details. Besides, alternative strategies of plant biomass production using in vitro tools and techniques and know-how of recent analytical techniques for herbal medicine production are also elaborately mentioned in this chapter.

Introduction

Herbal medicines refer to the use of plant seeds, berries, roots, leaves, bark, or flowers for medicinal purposes (Fig. 30.1). These plants are important for pharmacological research and drug development, not only when their constituents are used directly as therapeutic agents but also as starting materials for the synthesis of drugs or as models for pharmacologically active compounds (Wachtel-Galor and Benzie, 2011). Medicinal plants have been a major source of drugs for thousands of years, and even today they are the basis of systematic traditional medicines in almost all countries of the world. Unani and Ayurveda systems of medicine are two of the classic and oldest examples of this category. Around 80% of the population in developing countries is completely dependent on plants for their primary healthcare (Bannerman, 1983). Even in developed countries, which are enormously advanced in terms of medicinal chemistry, over one-fourth of all prescribed pharmaceuticals originate directly or indirectly from plants (Newman et al., 2000). Furthermore, of 252 drugs considered as indispensable by the World Health Organization (WHO), 11% are mainly derived from flowering plants and 28% of synthetic drugs are obtained from natural precursors (Namdeo, 2007). Few important compounds of plant origin used as drug or drug precursors are listed in Fig. 30.2.

Herbal medicines are secondary metabolites (Kubmarawa et al., 2007) derived from plants. Understandably, these pharmaceuticals are produced solely from massive quantities of whole plant parts,

Animal Biotechnology.
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FIGURE 30.1 Herbal medicines. *Source: Courtesy Google.*

which have certain limitations. One limitation is that excessive harvesting can diminish local plant populations and erode genetic diversity. Second, it causes inconsistency in the production of compounds in terms of quality and quantity. The latter can spell trouble in terms of safety, supply, and economic feasibility of these herbal products on a commercial scale. To overcome these bottlenecks, domestication and use of good agricultural practices are crucial, especially for revival of diminishing plant populations. However, the conventional methods of plant propagation are lengthy and time consuming. The long cultivation periods between planting and harvesting make the entire process cumbersome and uneconomical, which in turn leads to the high cost of drugs. Moreover, wild populations are susceptible to problems of disease, drought, environmental fluctuations, low rate of fruit set, and poor seed yield, germination, and viability. Genetic variability also poses a concern in out-breeding plants, and owing to all these vulnerabilities batch-to-batch consistency of derived metabolites becomes questionable. Clearly, there is an urgent need of alternative and complementary methods for uniform qualitative and quantitative production of herbal medicine. An assured consistency of the metabolite could be achieved if the same plant is grown under controlled conditions of in vitro culture. In this context, tools and techniques of biotechnology, like in vitro plant, cell, tissue, and organ culture, offer solutions by maximizing the number of plantations and thus restoration of natural plant stock in a short time span. It also favors uniform metabolite production all year round, irrespective of seasons and vagaries of climatic conditions.

Traditional medicine

The WHO defines traditional medicine as being the "sum total of knowledge, skills, and practices based on the theories, beliefs and experiences that are indigenous to different cultures, which are used to maintain health, as well as to prevent, diagnose, improve, or treat physical and mental illnesses." Every early civilization used plants as their main source of medicine, and most of the world's population still relies on them. The first recorded literature on medicinal plants can be traced back to early human history, the Atharvaveda (2000 BCE) in India. With time, the original population of an area gained knowledge with which plants could be used for certain diseases or states of illness. In addition, they also gained knowledge of the harmful and poisonous plants. It is evident that the modern drug industry has been developed to a considerable degree as a result of plant-based traditional medicines.

There are a few closely related terms in use today, the meanings of which should be understood clearly. *Traditional medicine* refers to the following components: acupuncture (China), Ayurveda (India), Unani (Arabic countries), traditional birth attendant's medicines, mental healer's medicines, herbal medicines, and various forms of indigenous medicines. *Complementary or alternative medicine* refers to a broad set of healthcare practices that do not form the part of country's own tradition and are not integrated into the dominant health care system. Traditional medicine has maintained its popularity in all regions of the developing world, and its use is rapidly spreading in industrialized countries (Liu, 2011).

Ancient system of medicine

Ayurveda and traditional Chinese medicines are perhaps the most ancient of all medicinal traditions. Ayurveda means the "science of life" and is derived from "Ayur" meaning "life" and "Veda" meaning "knowledge." It takes a holistic view of human beings, their health, and illness. It aims at positive health, which has been defined as a well-balanced metabolism coupled with a healthy state of being. According to Ayurveda, disease can arise from the body and/or mind due to external factors or intrinsic causes. The origin of Ayurveda is lost in prehistoric antiquity, but its characteristic concepts appear to have matured between 2500 and 500 BCE. in ancient India. The earliest references to drugs and diseases can be found in the Rigveda and Atharvaveda.

Ayurvedic drugs have been found to perform very well against chronic ailments. Today, they are also attracting attention for diseases for which there are no or inadequate drugs for treatment in modern

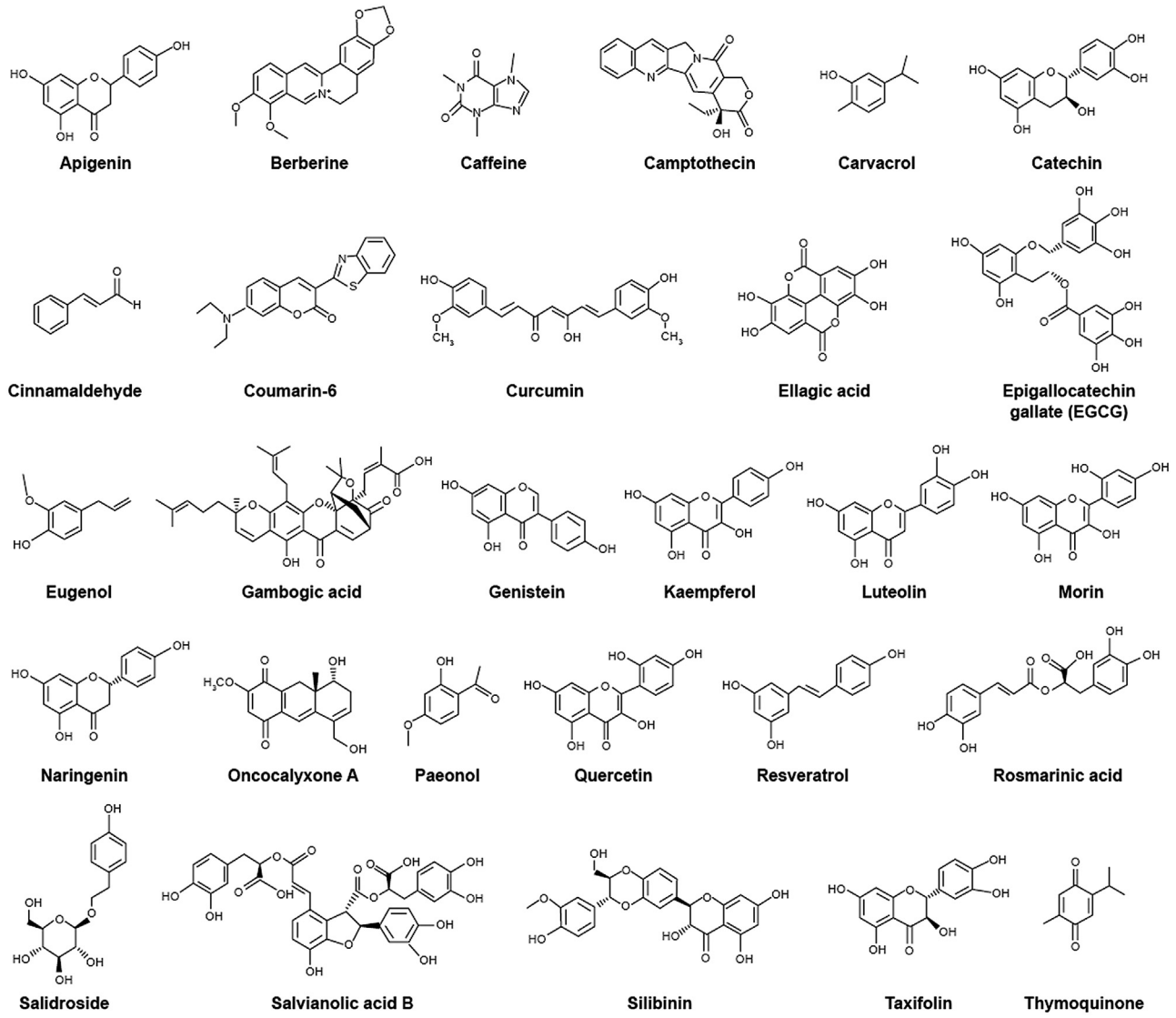

FIGURE 30.2 Chemical structures of some plant origin compounds used as drug or drug precursors. *Source: Adapted from Watkins, R., Wu, L., Zhang, C., Davis, R.M., Xu, B., 2015. Natural product-based nanomedicine: recent advances and issues. Int. J. Nanomedicine 10, 6055–6074.*

medicine, such as metabolic and degenerative disorders. Most of these diseases have multifactorial causation, and there is a growing awareness that in such circumstances, a combination of drugs, acting at a number of targets concurrently, is likely to be more effective than drugs acting at one target. Ayurvedic drugs, which are often multicomponent, have a promising impact on such conditions. Detailed chemical characterization in terms of composition and concentration of each ingredient in the formulation and studies of the biological activity of multicomponent Ayurvedic drugs will bring Ayurveda into the mainstream of scientific investigations. Recently there have been efforts to realize this objective (Fig. 30.3).

Methodology

Investigation of medicinal plants

Medicinal plants have formed the basis of healthcare throughout the world since the earliest days of civilization. They are still widely used and have noteworthy significance in the international trade. Recognition of their clinical, pharmaceutical, and economic value is still growing, although this varies widely between countries.

Each plant species has its own specific set of secondary metabolites. Apart from the family Poaceae, which harbors the world's worst weeds but is low in medicinal plants, many of the top 12 weed families are

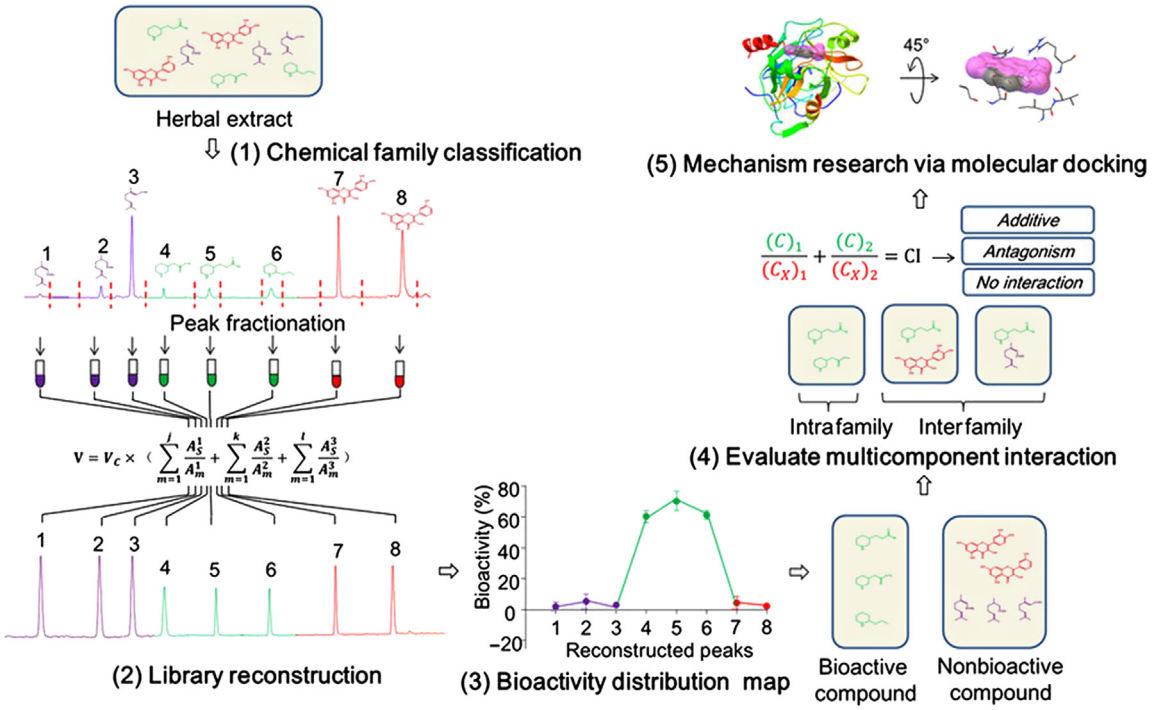

FIGURE 30.3 Diagram of the chemical family-based strategy for uncovering hidden bioactive molecules and multicomponent interactions in herbal medicines. *Source: Adapted from Song, H.P., Wu, S.-Q., Hao, H., Chen, J., Lu, J., Xu, X., et al., 2016. A chemical family-based strategy for uncovering hidden bioactive molecules and multicomponent interactions in herbal medicines. Sci. Rep. 6.*

also the ones that are important for medicines. The ecological and biochemical evidence suggest the preponderance of weeds in medicinal floras. Secondary compounds in plants are involved in the interaction of the plant with its environment and are important for ecological functions, such as allelopathy, insect and animal attractants for pollination, seed dispersal, and for chemical defense against microbes, insects, and herbivory (Bourgaud et al., 2001). These compounds do not participate in the vital metabolic processes of the plant system, but are the ones that exhibit bioactivity and can serve as medicine for humans. The spectrum of chemical structures synthesized by the plant kingdom is broader than that of perhaps any other group of organisms (Rao and Ravishankar, 2002).

In the present scenario, a large proportion of the drugs used in modern medicine are either directly isolated from plants or synthetically modified from a lead compound of natural origin. However, rarely is the drug isolated in the pure, usable form. What is initially obtained is the crude extract, which requires stepwise purification to obtain the finished product. The finished product as herbal medicine most of the time is a mixture of several compounds. When each and every component in the mixture is characterized qualitatively and quantitatively, it is called "characterized extract," which is understandably more desirable than the "uncharacterized extract." Plant extracts are known to consist of many chemicals, and among them, a few compounds could be acting synergistically. Sometimes, isolation of compounds from the extract may cause a decrease in desired activity, which underlines the importance of extract screening (Orhan et al., 2009).

Evidence-based studies on the efficacy and safety of traditional Indian medicines are limited. The essential ingredients in most formulations are not precisely defined. This is one of the most important challenges to scientists attempting to identify a single bioactive compound. Therefore in-depth studies and more stringent conditions should be followed to make a herbal formulation, so that the role of each and every component is known.

Drug discovery is the process by which drugs are discovered or designed. Plants have long been a very important source of drugs, and many plant species have been analyzed to see if they contain substances with therapeutic activity. Many plant drugs of folklore were investigated to determine the active ingredient in the mixture. Several reviews are available in the literature pertaining to approaches for selecting plants as candidates for drug discovery programs.

Today, many new chemotherapeutic agents are obtained synthetically based on "rational" drug design. The study of natural products has many rewards over synthetic drug design. The former leads to materials having new structural features with novel

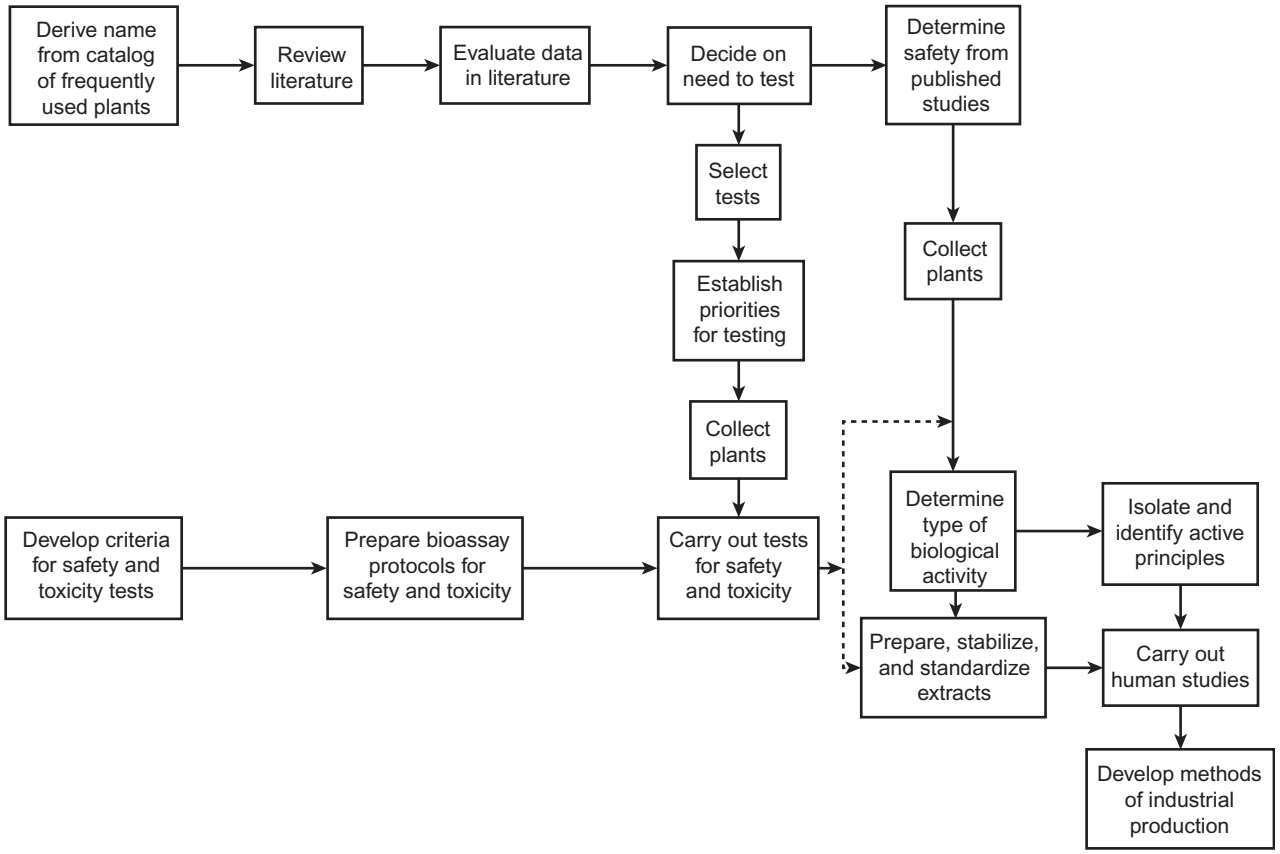

FIGURE 30.4 Flow chart of sequence for the study of plants used in traditional medicine. *Source: Adapted from Fabricant, D.S., Farnsworth, N.R., 2001. The value of plants used in traditional medicine for drug discovery. Environ. Health Perspect. 109, 69–75.*

biological activity. In this context not only do plants continue to serve as possible sources for new drugs but also chemicals derived from the various parts of these plants can also be extremely useful as lead structures for synthetic modification and optimization of bioactivity. The starting materials for about one-half of the medicines come from natural sources. There is no doubt that the future of plants as source of medicinal agents for use in investigation, prevention, and treatment of diseases is very promising.

Drug discovery from natural resources is a very tedious process. It involves identification of plant material, extraction, preliminary phytochemical screening of the crude extract, evaluation of biological activity, isolation of various bioactive compounds, and finally elucidation of structures. If the molecule is appealing, with strong pharmacological properties, then further preclinical studies are conducted on the molecules, such as toxicity, stability, and solubility studies. After undertaking these studies, if it is found that a molecule is substantially more active than the currently used drug, only then processes are developed for its economical and easy isolation from the source so that it can be readily available for therapeutic use.

In context of isolation and screening of chemicals from plants that possess medicinal properties, different approaches can be used. The process of obtaining bioactive substances and their chemical characterization are schematically represented in Fig. 30.4.

Extraction

Extraction involves the separation of medicinally active fractions of plant from inactive or inert components by using selective solvents through extraction procedures. The products so obtained from plants are relatively complex mixtures of metabolites in a liquid, a semisolid, or (after removing the solvent) a dry powder form. This is the critical first step in the investigation of medicinal plants.

The selection of a solvent system mainly depends on the exact nature of the bioactive compounds being targeted because during the extraction process, solvents diffuse into the solid plant material and solubilize compounds of similar polarity. The extraction of hydrophilic compounds uses polar solvents, such as methanol, ethanol, or ethyl acetate. For extraction of more lipophilic compounds, dichloromethane is used.

In a few cases, extraction with hexane is used to eliminate chlorophyll and oil.

As the target compounds may be nonpolar to polar and thermally labile, the suitability of the methods of extraction must be well thought out. Different methods, such as sonication, heating under reflux, soxhlet extraction, and others, are commonly used for plant sample extraction. In addition, plant extracts are also prepared by maceration or percolation of fresh green plants or dried powdered plant material in water and/or organic solvent systems.

Other modern extraction techniques include solid-phase microextraction, supercritical-fluid extraction, phytonics process, pressurized-liquid extraction, microwave-assisted extraction, counter-current extraction, solid-phase extraction, and surfactant-mediated techniques, which possess certain advantages (Handa et al., 2008; Patil and Shettigar, 2010).

Chemical screening

This technique is also known as phytochemical screening. In this method, aqueous and organic extracts are prepared from those plant samples that are the reservoir of secondary metabolites, such as leaves, stems, roots, or bark. The plant extracts are then analyzed for the presence of secondary metabolites like alkaloids, terpenes, and flavonoids. Standard tests are available in the literature for each class of compounds to be analyzed. Following this, a simple separation technique like thin-layer chromatography (TLC) is generally used to analyze the number and type of components present in the mixture. In TLC, the extracts are loaded on a glass coated with silica gel or other adsorbent, which is then kept in a chromatographic chamber containing a suitable running solvent. This technique mainly consists of a mobile phase and a stationary phase, whereby the compounds are separated based on their polarity. Sometimes a developing solvent might also be used after the plate has been taken out of the chromatographic chamber to detect chemicals. This approach has been used in the past and is still being used in developing countries. Since the isolation of pure bioactive components is a long and tedious process, this procedure enables the early recognition of known metabolites in the extracts and is thus economically viable. The tests are simple to perform; however, it is not suitable for the efficient separation of metabolites and has low selectivity and sensitivity of detection, which makes it difficult to detect traces of components in the sample.

Biological assays

Plant extracts have served as an important source of bioactive compounds for many drug discovery programs, and several important drugs have been isolated and identified from plants. In any isolation program in which the end product is a drug or lead compound, some type of bioassay screening or pharmacological evaluation must be necessarily used to guide the isolation process toward the pure bioactive component.

The selection of the biological assay to be adopted usually depends on the target syndrome and on the available information about the plant to be studied. For instance, if a plant has an ethnopharmacological history of use against a particular disease, then one would rationally use a specific bioassay technique that can predict the reputed therapeutic activity to isolate the lead that is responsible for that biological activity.

Bioassays can be categorized into primary and secondary assays. Primary bioassays, such as antimicrobial, antiviral and cytotoxic, anthelmintic activity, hepatotoxicity, antiinflammatory are performed when a large number of samples (plants or extracts) are to be screened for bioactivity. Primary bioassays, most of the time qualitative and not quantitative, provide reproducible results and offers potential tolerance against extract impurities. These assays are usually low cost and provide the results quickly. Secondary bioassays are performed after screening of lead compounds and are usually low capacity, slow, and costly assays (Mukherjee, 2019). It involves more exhaustive and comprehensive investigation of lead compounds on a number of model systems to select compounds for clinical trials. Model systems can be lower organisms, isolated subcellular systems, isolated intact cells of human or animal, and isolated organs of humans.

The major limitations of bioassay techniques are the use of biological organisms, particularly mice and rats, which are most often have to be sacrificed. Moreover, the phytochemical extracts are highly heterogeneous due to the presence of a mixture of different bioactive components. A desired biological response may not be due to a single bioactive compound, but due to a mixture of several bioactive compounds. Finally, isolation, screening, and quantification of a specific bioactive compound are difficult.

Identification, quantification, and characterization of bioactive compounds

Due to the fact that plant extracts usually contain various types of compounds with different polarities, their separation still remains a big challenge for the process of identification, quantification, and characterization of bioactive compounds. Apart from this, there are always chances of wide variations with respect to their chemical content in crude drugs/raw materials of plant origin due to varied reasons, such as climatic conditions, geographical distribution, source and

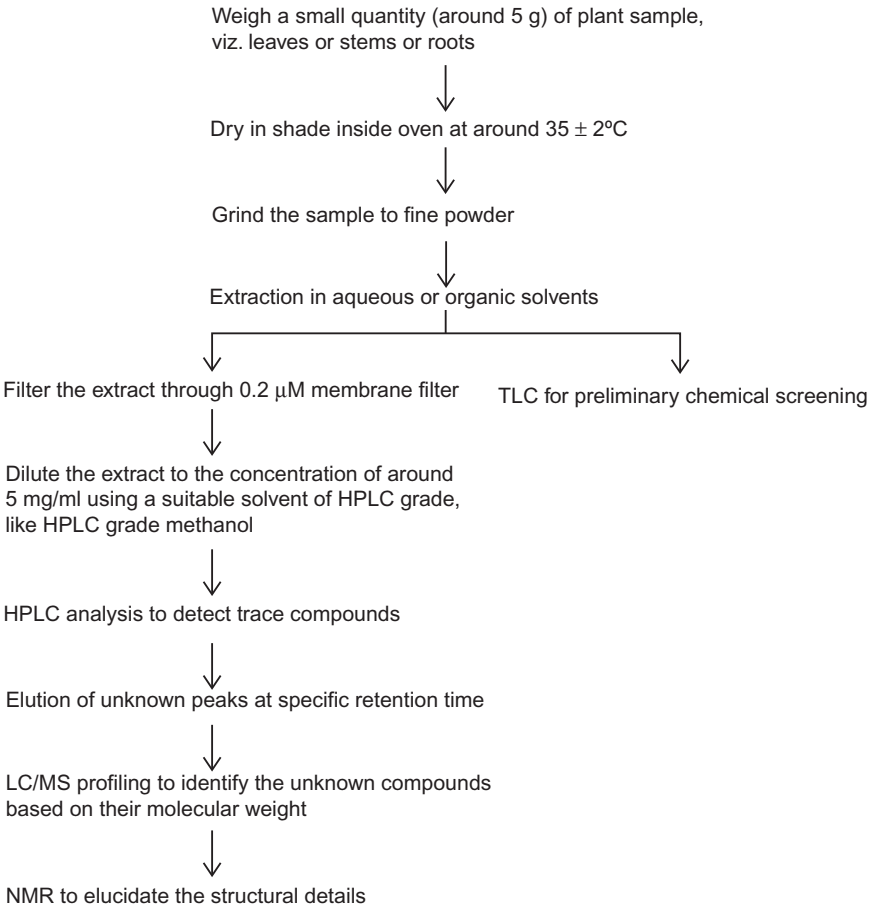

FIGURE 30.5 Schematic representation showing the process of chemical screening, isolation, and characterization of bioactive substances from plants.

season of collection, and lack of scientific methods of postharvest processing, storage, and preservation. Therefore identification, quantification, and characterization of bioactive compounds are essential prerequisites for herbal drug development (Fig. 30.5).

Various spectrophotometric, chromatographic, and chromatospectrophotometric methods are in use to obtain precise results and to identify, quantify, and characterize the phytoconstituents present in the plants. Chromatographic techniques, such as TLC, high-performance thin-layer chromatography (HPTLC), and high-performance liquid chromatography (HPLC), are used for the identification and quantification of known compounds. With the advancement in technology, superior hyphenated chromatographic and spectrophotometric techniques like Liquid chromatography coupled to mass spectrometry (LC-MS), liquid chromatography coupled with high field nuclear magnetic resonance (LC-NMR), liquid chromatography coupled with high field nuclear magnetic resonance and mass spectrometry (LC-NMR-MS), liquid-chromatography-Fourier transform infrared spectroscopy (LC-FTIR), and gas chromatography/mass spectrometry (GC-MS) are in practice. These hyphenated techniques are used with biological screening methods to circumvent reisolation of known compounds as well as for structural elucidation of the promising novel compounds (Philipson, 2007; Patel et al., 2010).

TLC is a rapid and simple analytical tool extensively used in herbal medicine analysis due to the fact that it requires minimum sample clean-up. Moreover, it gives qualitative and semiquantitative information of the separated compounds (Doughari, 2012). HPTLC, an extension of TLC, is one of the most versatile, reliable, rapid, and cost-efficient analytical tool in the quantitative analysis of compounds. This separation technique is based on TLC, but with the increased resolution, reproducibility, and accuracy of the compounds to be separated (Attimarad et al., 2011).

High-pressure liquid chromatography, also called as HPLC, is an important analytical tool for detection, separation, quantification, and qualitative assessment of bioactive compounds. It involves the injection of a small volume of liquid sample into a tube packed with porous particles (stationary phase), and the individual components of the sample are pulled along the packed tube (column) by a solvent (mobile phase). A pump forces the liquid through the column at a specific flow rate and generates high pressure. The column packing separates the components from the sample by various

physical and chemical interactions between the molecules and the packing material. The separated compounds collected at the exit column are detected by several techniques, like ultraviolet, fluorescence detection, diode array detection, etc. Data are generated in the form of chromatograms, where individual components show peaks at specific retention times (RTs) at which the component was eluted. Since HPLC has a high resolution and is very sensitive, this technique is suitable for the detection of trace components whose concentration in the sample is very low (Doughari, 2012).

The processing of a plant crude extract to provide a sample suitable for HPLC analysis, as well as the selection of solvent for sample reconstitution, can have a significant bearing on the overall success of natural product isolation and identification. The source material (e.g., dried powdered plant) will initially need to be treated in such a way as to ensure that the compound of interest is efficiently liberated into solution. This is where an efficient extraction protocol becomes important. An organic solvent may be used for extraction, and then solid material is removed by centrifugation and filtration of the extract. The filtrate is then concentrated and injected into an HPLC instrument for separation. The use of guard columns is necessary in the analysis of crude extract. Many natural product materials contain significant levels of strongly binding components, such as chlorophyll and other endogenous materials, that may in the long term compromise the performance of analytical columns.

LC-MS is a newer technique and is one of the most sensitive methods of molecular analysis. It yields information on the molecular weight and structure of the analytes and is suitable for the analysis of large, polar, ionic, thermally unstable, and nonvolatile compounds. In LC-MS, LC separates the different components of crude extracts, which are analyzed by mass spectrometry (MS) to selectively detect and confirm molecular identity. An MS detector senses a compound eluting from the LC column first by ionizing it using electro spray, thermo spray, and ion spray ionization techniques, and then by measuring its mass or by fragmenting the molecule into smaller pieces through collision-induced dissociation that are unique to the compound. The MS detector can sometimes directly identify the compound since every compound has its own unique mass spectrum and acts as a fingerprint for that particular compound. LC-MS in combination with biological assays have been proved to be very effective for the rapid analysis of herbal drugs (Herderich et al., 1997; Patel et al., 2010).

LC-NMR combines HPLC, and nuclear magnetic resonance (NMR) spectrometer is one of the most effective chromatospectrophotometer techniques for the separation and structural elucidation of unknown compounds and mixtures (Wann, 2005). The recent advancement in NMR devices has improvised LC-NMR sensitivity, like use of high magnetic field magnets and sensitive probes, employment of detection cells with smaller volumes, and automatic measurement software most appropriate for multicomponent analysis. Introduction of pulsed field gradient technique in high-resolution NMR has further improved the NMR techniques for structural elucidations and molecular weight information of usually light and oxygen-sensitive compounds (Wolfender et al., 1998; Doughari, 2012). It is useful for the identification and separation of chiral and isomer compounds. This equipment is usually supplemented with a parallel MS detector (LC-NMR-MS) to provide additional chromatographic traces (total ion current, extracted ion chromatogram, etc.) and complementary structural data (molecular ions and their fragments) (Burton et al., 1997).

LC-FTIR is a hyphenated technique coupling LC with FTIR. FTIR spectroscopic technique is used for the quantitative measurement of the interaction between IR radiation and organic materials. It is generally used when specific detection and identification of bioactive compounds (e.g., complex mixture of isomers) is required. It gives information related to functional groups (e.g., -OH, -COOH) that are present in the molecule (Patel et al., 2010).

GC-MS is based on the partitioning of compounds between a liquid and a gas phase. This technique is widely used for the qualitative and quantitative analyses of a large number of herbal drugs because it has high sensitivity, reproducibility, and speed of resolution. It has proved to be most valuable for the separation of volatile, nonpolar, and semipolar bioactive compounds. In GC-MS, the sample is injected into a long tubular column, the chromatography column, which has a high boiling point stationary phase, such as silicon grease. The basis of the separation is the difference in the partition coefficients of volatilized compounds between the liquid and gas phase as the plant metabolites are carried through the column by the inert carrier gas (e.g., nitrogen, helium, hydrogen, or argon). The time taken by the sample to pass through the length of the column is referred to as its RT. The RT for a given sample is an identifying characteristic. The detector for the GC is the MS detector. As a sample exits the end of the GC column, it is ionized into gaseous ions in the ionization source and then enter into the mass analyzer. The major principle of ionization is electron impact and chemical ionization techniques. Fragmented ions with different mass-to-charge ratio are sequentially sorted, and an electron multiplier finally multiplies the fragmented ions signal to

generate electric signal (Philipson, 2007; Patel et al., 2010; Mukherjee, 2019).

Supercritical fluid chromatography (SFC), a new analytical tool, combines the best feature of gas and liquid chromatography. It is an important chromatography that is beginning to find use in herbal medicine. It is mainly used for the separation and detection of compounds that are nonvolatile and thermolabile and contain no functional groups (Henry and Yonkar, 2006).

Biotechnological approaches for herbal drug production

Intact plants in the field or wild habitats produce high-value bioactive compounds. However, as mentioned in the forgoing sections, the quantity and availability of these economic products from natural resources restrict their maximized uses for the benefit of humankind. As the demand for bioactive compounds has increased in the last few years, exploitation of medicinal plants has also increased. Hence, there is an urgent need to develop alternative methods for large-scale production of metabolites and quality plants. In this respect, biotechnology puts forward an attractive alternative to whole-plant extraction for homogeneous, controlled production, especially, when we take the commercial demand into picture. It also results in more consistent yield and quality of the products, irrespective of the seasons and the regions. Biotechnology offers an opportunity to exploit plant cells, tissues, organs, or entire organisms by growing them in vitro and genetically manipulating them to get desired compounds (Rao and Ravishankar, 2002). Many biotechnological strategies, such as embryogenesis, organogenesis, screening of cell lines, media optimization, and elicitation, can be carried out for the enhanced production of secondary metabolites from medicinal plants. The subsequent sections briefly discuss various in vitro culture techniques that can be used for herbal drug production.

Organ cultures

The selection of an appropriate technique depends on the results. In plants where molecules of interest are localized in specialized cells, dedifferentiated (callus) cultures are not desirable but establishment of organogenic cultures would be advantageous. Under in vitro conditions, redifferentiation is generally associated with an improved synthesis of secondary metabolites (Collin, 2001). This is probably due to the appearance of complex cells and tissues that are metabolically more proficient. In all redifferentiated cell lines, along with the shoot-forming nodules, nonmorphogenic cell masses are also present. Although nonmorphogenic it might have a certain degree of differentiation at the cellular stage and, due to coevolution, imitate the biochemistry of redifferentiated cells (Brown and Charlwood, 1986). The reports on *Artemisia annua* and *Azadirachta indica* stated that artemisinin and azadirachtin production, respectively, were very poor in dedifferentiated callus cultures, and a certain degree of redifferentiation was obligatory for compound production (Singh and Chaturvedi, 2013). Organogenesis was also found to be an essential prerequisite for steroidal saponin production in *Ruscus aculeatus*. Similar observations were made for the biosynthesis of picroside in *Picrorhiza kurroa*, wherein the metabolite did not accumulate in the dedifferentiated callus cultures, but occurred specifically in the redifferentiated cultures. Berkov et al. (2010) also demonstrated that alkaloid synthesis in *Pancratium maritimum* is closely related to tissue differentiation.

Since it was observed that the production of bioactive compounds is generally higher in organized plant tissues, there are attempts to regenerate whole plant organs (i.e., shoots or roots) under in vitro conditions, either directly from explants or indirectly via an intervening callus phase (Fig. 30.6). As expected, such regenerating cultures produce patterns of secondary metabolites that are similar to the field-grown parent plant, with the added advantage of improved production of metabolites. Another advantage of using the

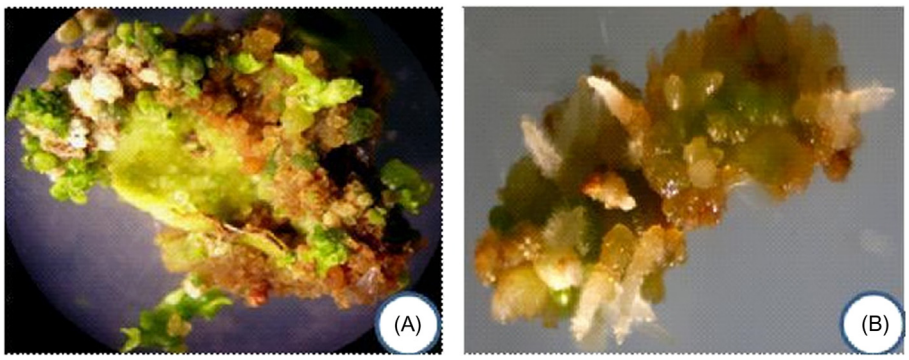

FIGURE 30.6 Neem organogenesis from leaf explants indirectly via callusing. (A) Shoot differentiation. (B) Root differentiation.

organized cultures is that they are relatively more stable in the production of secondary metabolites than cultures of undifferentiated cells, such as cells in callus or suspension cultures (Rao and Ravishankar, 2002).

Callus cultures

Callus cultures are considered as dedifferentiated mass of cells obtained from explants inoculated in vitro on the medium consisting of relatively higher auxin concentrations or a combination of equal concentration of both auxin and cytokinin. In plants, where sought after metabolites are present in leaves, establishing in vitro cultures from leaves and using them for the extraction of compounds would be an ideal alternative. Callus cultures containing the bioactive substances are collected at a specific stage (usually during the stationary phase of their growth cycle, since secondary metabolite production is greater during the stationary phase), dried, extracted, and the extract then taken for identification and quantification of the desired medicinal compound using HPLC, LC-MS, etc. The further scale-up and yield enhancement studies of the compound are performed by raising the callus in suspension, first in a shake-flask culture, and then in a suitably designed bioreactor, to maximize its production.

Suspension cultures

A breakthrough in cell-culture methodology occurred with the successful establishment of cell lines capable of producing high yields of secondary compounds in cell suspension cultures (Zenk, 1978). During the past decades, this approach of metabolite production has attracted much academic and industrial interest. The technique of using plant cell suspension cultures for secondary metabolite production is based on the concept of biosynthetic totipotency of plant cells, which means that each cell in the culture retains the complete genetic information for the production of range of compounds found in the whole plant. Cell suspension cultures are initiated from established callus cultures by inoculating them into liquid media. The cultures are then kept in glass flasks under continual agitation on horizontal or rotating shakers; they can be eventually transferred to a specialized bioreactor. Cells in suspension cultures grow much better than in semisolid media because of better mixing of oxygen and nutrients during shaking conditions.

Productivity of suspension cultures is critical to the practical application of this cell technology for bioactive compound production. To improve the production of secondary metabolites in in vitro cultures, various strategies such as the manipulation of parameters of the environment and medium, selection of high-yielding cell clones, precursor feeding, and elicitation can be opted for.

Case study: *Lantana camara* L

Owing to the tremendous relevant ethnopharmcological significance and demand of the genus, *L. camara L*, an alternate strategy is explored in this case study to maximize the cell biomass utilization and, thereby, restoration of natural plant resources. The plant tissue culture technique offers to generate the cell biomass in a shorter duration, throughout the year, irrespective of the seasons and regions with a provision of tunability of the cells to increase the metabolite production. *L. camara* L. [Sage (in English) or Caturang (in Hindi)] is an aromatic, evergreen shrub belonging to the family Verbenaceae. It is a reservoir of several important bioactive molecules. It has been listed as one of the important medicinal plants in the world. For many years, natural products from *Lantana* have been used in the prevention and cure of many serious diseases, including cancers. The most significant bioactive molecules of this plant are presented in Fig. 30.7.

For establishing tissue cultures, the first prerequisite is the selection of healthy plant material. Thus, for this study, leaves from *Lantana* plants bearing pink-yellow flowers were picked. Leaves were disinfected using 1% (v/v) Tween-20 and 0.1% (w/v) mercuric chloride, followed by three rinses in sterile distilled water after each step. The leaf disk explants were prepared using a cork borer of 5 mm diameter. The basal media used in all the experiments related to callus induction and proliferation consisted of MS (Murashige and Skoog, 1962) medium enriched with 30 g/L sucrose and solidified with 0.8% agar (HiMedia Laboratories, Mumbai, India). The pH of the media was adjusted to 5.8 before autoclaving at 1.06 kg/cm^2 and 121°C for 15 minutes. The media was supplemented with different plant growth regulators (auxins and cytokinins) at defined concentrations. Remaining steps are explicitly described in Fig. 30.8.

Opportunities and challenges

The consumption of herbal medicines and the importance of the herbal medical industry are fast growing and widespread. According to estimates of the World Health Organization, more than 80% of the world's population depends primarily on herbal medicines. The ancient art of herbal medicine is fast developing today and is undergoing something of a

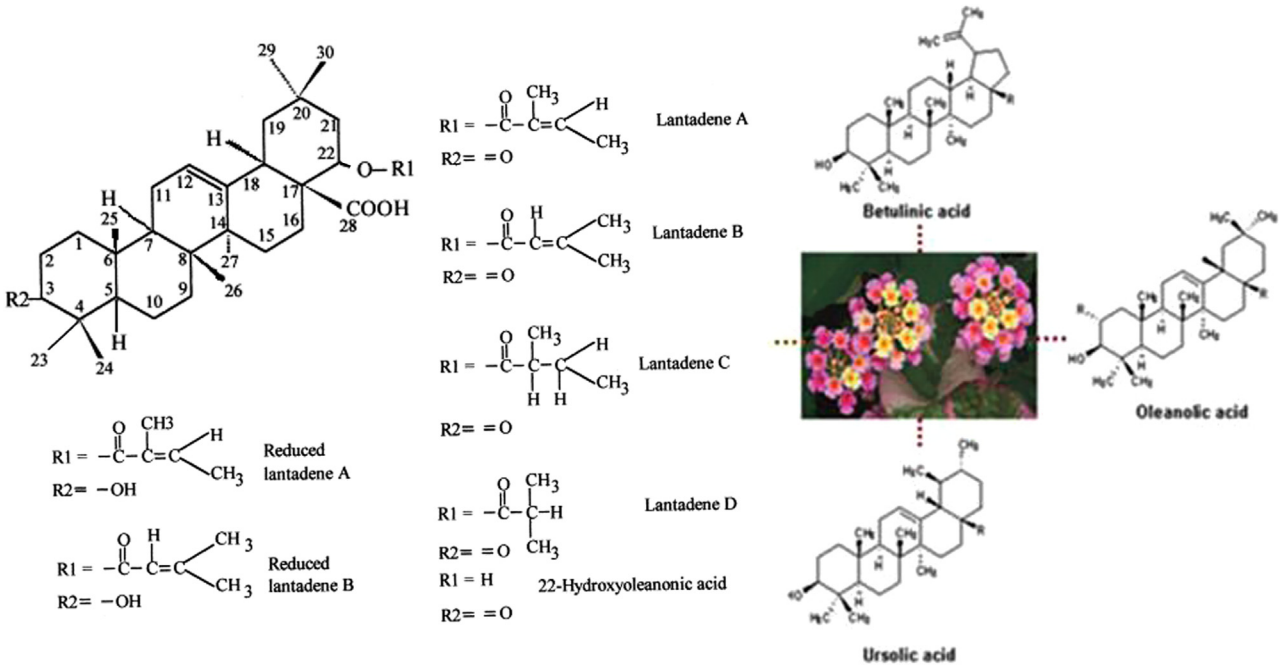

FIGURE 30.7 Bioactive compounds of *Lantana*.

renaissance all over the world, particularly in developed countries. Most of the ingredients used in herbal medicines are taken from wild plants, and the increasing demand for medicinal plants, along with habitat loss and climate change, is putting pressure on many species. Indiscriminate harvesting from the wild has led to loss of genetic diversity, diminishing populations, local extinctions, and habitat destruction. This has raised the ire of plant conservationists.

Large-scale cultivation of medicinal plants offers a viable conservation strategy and also eliminates the problems that are generally faced in herbal extracts, such as misidentification, genetic and phenotypic variability, extract variability and instability, toxic components, and contamination. Optimized yield and uniform high-quality product can also be achieved through cultivation. However, in a rapidly shifting and fashion-prone market, the cultivator has to make the difficult decision of which particular species to grow.

Therefore, the difficulty in predicting which extracts will remain marketable is another serious obstacle in bringing medicinal plants into successful commercial cultivation.

Although a large number of plant species used in herbal medicine are cultivated, a great majority of them are still utilized from the wild population. There are certain difficulties faced by growers in the cultivation of herbal plants because of low germination rates, unavailability of quality planting materials, or specific ecological requirements. Lack of knowledge about the specific requirements for pollination, seed germination, and growth are the main hindrances in the cultivation of herbal plants. Fungal infection or mechanical damage frequently results in low germination rates that can be easily overcome by improved seed treatments and by ensuring optimal storage conditions. Moreover, difficult-to-grow herbal plants can be easily cultivated on a commercial scale by using controlled environments, including hydroponic systems.

Another major challenge faced in the production of herbal medicines is that the main bioactive component, which is the major ingredient in the herbal medicine, is synthesized in a very small quantity in the specific plant. This is obvious, as the bioactive components are mainly produced as secondary metabolites in plant cells that are produced in small quantities. This leads to cutting down of a large number of herbal plants for producing a single drug. However, by the use of modern tissue culture techniques and genetic transformation that can alter the pathways for the biosynthesis of target metabolites, today this wasteful harvesting technique can be easily prevented.

Together with supporting the use of herbal medicines, it is high time for everyone, herbalist and conservationist alike, to reduce the overexploitation of the world's wild plants. In the modern world, the trade in medicinal plants is everincreasing, but largely unmonitored. At the moment, many harvesting practices are unsustainable, which is threatening population of medicinal plants and their habitats and also the

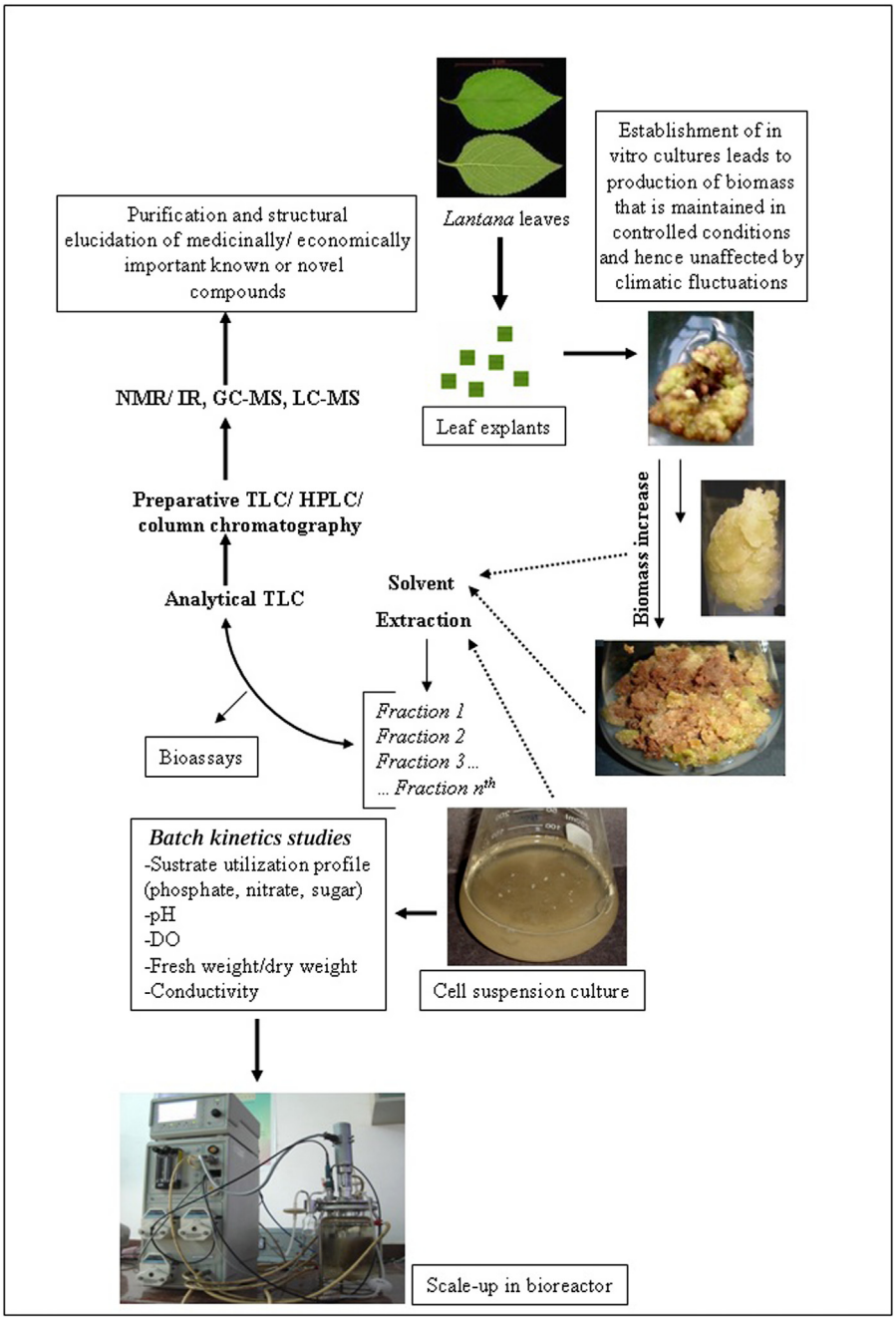

FIGURE 30.8 Isolation of bioactive compounds from *Lantana camara*, a medicinal plant.

livelihoods of those people engaged in their collection. It is time for the conservationists, the government, and each and every one of us to find workable global solutions.

Conclusions and outlook

Medicinal plants are widely used by the people living in both rural and urban areas. Globalization has greatly renewed the interest in herbal medicines, and today most people prefer to take herbal medicines as an alternative therapy. This resurgence in plant remedies has mainly resulted from the following factors:

1. Herbal medicines are found highly effective in curing diseases including chronic symptoms.
2. Most modern drugs have one or more side effects.
3. Development of science and technology.

In addition to these factors, economic benefits also contribute to their everincreasing popularity. Development of modern science and technology, and further studies into traditional plant medicines, conducted with modern theories and techniques have greatly enriched the use of herbal medicines by

absorbing new ideas, and concepts from traditional plant medicine are in use across the world. This has led to the tremendous expansion of herbal medicine industry and employment generation in a last few decades. Realizing these facts, it is understood that in not-too-distant future, traditional plant medicine will emerge as an area of prime importance in healthcare system. However, efforts are to be concentrated on sustainable harvesting of medicinal plants to avoid their overexploitation and extinction. Also, to utilize the available resources of medicinal plants to their fullest, social, cultural, and economic problems, lack of well-planned and integrated strategies, and poor access to scientific information must be dealt with first.

Ethical issues

Although approximately 80% of the people today depend on herbal medicine as a component of their primary healthcare according to the World Health Organization, there is still concern about the safety and efficacy of herbal drugs. Despite the fact that herbal medicine can potentially contribute to the improvement of healthcare, many major challenges must be overcome prior to the successful incorporation of herbal remedies into medicine. Beneficence, nonmalfeasance, patient autonomy, justice, and public accountability are the pillars of bioethical principles, which are religiously followed in conventional medicine. They guide the clinicians such that the patients' interests are best served. As the use of complementary medicine (including herbal medicines) becomes increasingly popular, it is becoming apparent that the same bioethical principles are applicable to these alternate forms of healthcare (Kemper and Cohen, 2004). Beneficence is the principle that says it is a clinician's responsibility to promote a patient's wellbeing; clinicians must take appropriate measures to ensure that some positive outcome will occur. Nonmalfeasance is the responsibility to not hurt others. This ethical principle is almost the same as beneficence, but with important distinctions, as one's duty to prevent harm is not the same as the duty to promote wellbeing (Beauchamp and Childress, 2009). Patient autonomy is a foundation of conventional medicine that is pertinent to the use of herbal medicines too. In most parts of the world, consumer access to herbal medicines is controlled by prescription, thus allowing for extensive use. With self-care as one component of patient autonomy, another key element is that the patient must consists of complete information to make an informed treatment decision (Ernst and Cohen, 2001). Time and again researchers come across cases where a patient has gathered information about herbal medicines from relatives, friends, magazines, and Internet (Gardiner and Riley, 2007; Khader et al., 2008; Low, 2009), all of which are perceived as less reputable than official sources.

Translational significance

Animal models are used in study on human diseases because both animals and humans are similar in genetics, anatomy, and physiological aspects. Also, animal models are often preferable because of their easy and abundant supply and ease of manipulation. Also, for statistical analysis, a sufficient number of specimens must be used for a particular experiment. Therefore, scientists cannot conduct research on just one animal or human, and it is easier for scientists to use sufficiently large numbers of animals instead of humans to get reliable results. Only in cases of advanced clinical trials, humans are used for investigations. Otherwise, animals, like mice, rats, monkeys, dogs, and several fungal, bacterial, and plant species, are used as model organisms for such studies. However, even with the evident similarities between animal models and humans, only about 1% of drugs reach the last phase of clinical trials. As far as herbal medicines are concerned, the chemical constituents present in them are a part of the physiological functions of living plants, and therefore, they have better compatibility with the human body. However, scientific proof of this statement is not sufficient, and this is, therefore, one major area where research can be carried out.

Clinical correlation

Unhealthy lifestyle, rise in environmental toxins due to pollution, and other factors increase the risk of diseases. In addition, continued usage and side effects of allopathic drugs are a cause of concern. In 2013, WHO developed and launched "WHO Traditional Medicine Strategy 2014–2023," which emphasized on integration of traditional and complementary medicine to promote universal healthcare while ensuring the quality, safety, and effectiveness of such medicines (Sen and Chakraborty, 2017).

In spite of familiarity and possible benefits, integration of herbal medicine faces a number of problems and challenges. Among many, incorrect identification of plants, adulteration, and/or incorrect formulation process are the main problems that incorporate inconsistency and reduce the effectiveness of herbal medicines. Besides these, lack of good manufacturing practices, quality control and regulatory measures,

ignorance on side effects are other bottlenecks that prevent integration of herbal medicines in mainstream. Due to similar reasons, clinical trials that are important for examining the efficacy and safety of these medicines on humans are sparse and limited. Even after all these shortfalls, the good news is that research and funding bodies like AYUSH are profoundly supporting studies on herbal and traditional forms of medicine. AYUSH research portal includes information on numerous clinical trials, preclinical researches, and drug researches related to plants used in these systems. AYUSH practitioners have the rights to prescribe herbal formulations to patients and have a wider reach in rural and remote parts of India (Roy, 2015). In a nutshell, although a lot still remains, but a pioneering step has already been taken, to integrate herbal medicines with contemporary forms of treatment.

Turning point

Although there is a long way to go, but the subject will certainly revolutionize the therapeutic industry. The human connection with the herbal drugs from nature is boundless since ancient times. Written documents, preserved plant materials, and original plant medicines are some of the evidences that elucidate the use of plants as medicines by human. Even today, most of the pharmacologists prefer to use only plant resources as medicine due to its less toxic side effects. About 80% of world population depends on herbal medicine to treat various kinds of ailments. However, the significant number of medicinal plants are either endemic or are on the verge of extinction. Therefore, the ultimate challenge is to increase the availability of plant biomass as a raw material and also to increase the production of valuable metabolites at an industrial scale. This can be achieved by adopting biotechnological methods, like plant tissue culture techniques, where cell biomass can be generated constantly throughout the year, irrespective of the seasons, and regions and environmental fluctuations, in large bioreactors. This cell biomass can serve as raw materials for the production of useful drugs. However, the plant cell cultures are not as commercially utilized as microbial cell cultures due to the increased size of plant cells that make it more sensitive to shear stress. The other major limitations are lack of availability of data, mostly the published work are dealt with plant cells at lab scale due to their comparatively large size and rigid cell wall than animal cells. Bioprocess parameters like, engineering considerations, optimization of process parameters, and process strategies can be applied to increase the production of medicinal metabolites from plant cells. Therefore, establishment of in vitro cultures and investigating their potential by using various in vitro assays is a route to conquer many life-threatening diseases.

World Wide Web resources

One of the first steps in the use of herbal medicine is to find out the best source for complete information about herbs and/or derivatives. At present the Web is the most prominent (and perhaps most familiar) tool, but the Internet, like other resources, has its own strengths and weaknesses. The major strength of Internet is that it is an especially valuable research tool when looking for information that is current and frequently updated. It is also quick to access.

As far as weaknesses are considered, the Internet is not the best place to find established viewpoints in their original form since it is often the case that information is changed from its original source. Information on the Internet is often second, third, or even fourth hand. Published books remain the safest place to get established facts and opinions, especially when looking for traditional ideas.

However, following websites do provide comprehensive information on herbal medicines:

http://ethnomedicinetomodern.blogspot.in/

http://www.umm.edu/altmed/articles/herbal-medicine-000351.htm

http://www.nlm.nih.gov/medlineplus/herbalmedicine.html

http://www.herbs.org/herbnews/

http://www.journals.elsevier.com/journal-of-herbal-medicine

http://www.who.int/bulletin/volumes/86/8/07-042820/en/

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Glossary

Bioactivity Specific effect on, or a reaction in, living being upon exposure to a substance.
Biosynthetic totipotency The inherent potentiality of a plant cell to give rise to a whole plant.
Dedifferentiation The phenomenon of mature cell reverting to the meristematic state and forming undifferentiated callus tissue is termed dedifferentiation.
Plant metabolite *Plant metabolites* are the intermediates and products of *metabolism*. It is usually restricted to small molecules of plant.
Morphogenic The development of form and structure during growth.
Redifferentiation The phenomenon of whole plant formation from undifferentiated callus tissue.
Secondary metabolite Secondary metabolites are organic compounds that are not directly involved in the normal growth, development, or reproduction of the plant but often have an ecological role, such as attractant of pollinators and chemical defense against microorganisms. Humans use secondary metabolites as medicines, flavorings, and recreational drugs.
Traditional medicine Traditional medicine refers to the knowledge, skills, and practices based on the theories, beliefs, and experiences, used in the maintenance of health and in the prevention, diagnosis, improvement, or treatment of physical and mental illness.
Natural product Natural product is a chemical compound or substance produced by a living organism. Natural product often has pharmacological or biological activity for use in pharmaceutical drug discovery and drug design. A natural product can be considered as such even if it can be prepared by total synthesis.

Abbreviations

GC/MS Gas chromatography/mass spectrometry
HPLC High-performance liquid chromatography
LC/MS Liquid chromatography/mass spectrometry
MS Mass spectrometry
NMR Nuclear magnetic resonance
RT Retention time
TLC Thin-layer chromatography
WHO World Health Organization

Long answer questions

1. Write an essay on plant secondary metabolites. Discuss the preponderance of weeds in medicinal flora.
2. Elucidate various steps for study of plants in traditional medicine.
3. What is drug discovery? What are different ways for drug discovery from natural products?
4. Write a detailed account of the tools and techniques of plant tissue culture and highlight the importance of each.
5. Enlist and describe in detail important analytical techniques associated with characterization of medicinal metabolites.

Short answer questions

1. Define the term "secondary metabolites."
2. What is ethnobotany?
3. Differentiate between *characterized* and *uncharacterized* plant extracts.
4. Give the names of three solvents that can be used for the extraction of hydrophilic compounds?
5. Which analytical technique can be used for the separation and identification of volatile compounds? Which spectroscopic technique analyzes functional groups of the compounds?

Answers to short answer questions

1. Secondary metabolites are compounds that are not directly involved in primary metabolic processes of an organism. They generally defend the organisms from environmental stresses and predators.
2. Ethnobotany is the study of how people of a particular region relate to the plants of their environment.
3. Characterized extracts are ones where each component, its concentration, and function are known; for uncharacterized extracts, the entire components of the mixture and the role they play are not known.
4. Methanol, ethanol, and acetone.
5. GC-MS, FTIR

Yes/no type questions

1. In TLC, the extracts are loaded on a glass coated with silica gel or other adsorbent.
2. Primary bioassays are performed when less number of plant extracts are to be screened.
3. HPLC is used for the identification and quantification of unknown compounds.
4. LC-MS gives information on the molecular weight and structure of the analytes.
5. Pulsed field gradient technique is introduced in GC-MS to elucidate structure of oxygen-sensitive compounds.
6. LC-NMR is useful for the identification and separation of isomer compounds.
7. LC-MS is used to analyze thermally unstable compounds.
8. Nonvolatile compounds are separated in GC-MS.
9. SFC is mainly used for the separation and detection of thermolabile and nonvolatile compounds.

10. Production of bioactive compounds is generally higher in unorganized tissues.

Answers to yes/no type questions

1. Yes, silica gel is the most widely used adsorbent and remains the dominant stationary phase for TLC.
2. No, primary bioassays are performed when large number of plant extracts are to be screened because it is easy to perform and cost effective.
3. No, HPLC is used for the identification and quantification of known compounds for which standards are available.
4. Yes, LC-MS gives information on the molecular weight and structure of the analytes by detecting ions.
5. No, introduction of pulsed field gradient technique has improved the NMR to elucidate structure of oxygen sensitive compounds.
6. Yes, LC-NMR can separate and identify isomer compounds without reference compounds.
7. Yes, LC-MS is used to analyze thermally unstable compounds because soft ionization techniques are used in LC-MS.
8. No, GC-MS separate and detect volatile and thermally stable compounds as gas phase is use to elute analytes.
9. Yes, SFC can provide high resolution at low temperature and thus thermolabile compounds are separated and detected easily.
10. No, production of bioactive compounds is generally higher in organized tissues due to the appearance of complex cells and tissues that are metabolically more proficient.

CHAPTER

31

Enzyme inhibition assay for metabolic disorders—exploring leads from medicinal plants

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Summary

Herbal medicines serve as the most important source for treatment of many diseases and ailments since ancient times. Quality assessment including authentication, phytochemical evaluation, standardization, validation processes, safety, efficacy, and clinical aspects are the major parameters for the development of herbal products. Starting from different extraction techniques followed by bioassay-guided fractionation and isolation of the plant bioactive molecules with potential therapeutic activity are the key focuses in drug discovery from natural products. The present article highlights some of the major aspects of natural product-based drug development process, specifically focusing on some major enzymes involved in metabolic diseases.

What you can expect to know

Drug discovery from medicinal plants (MPs) has reclaimed the attention of the researchers, scientists, and pharmaceutical industries globally. Herbal products have been used by folklore traditions for the treatment of several diseases. Herbal medicines (HMs) have the diverse source of chemical entities such as alkaloids, glycosides, terpenoids, flavonoids, and lignans. Evaluation of its phytochemistry and therapy is necessary to validate their use through various modern techniques to meet the requirements to be used in modern drug. In this context, in vitro bioassays based on the high-throughput screening (HTS) are pioneering and considered to be unique techniques for the evaluation of MPs and their constituents. It is very specific, sensitive, and robust method for biological evaluation of herbal extract. Among these techniques, microplate-based assay is most commonly used in the form of fully or semiautomatic ways. Thin-layer chromatography (TLC) bioautography is another technique for finding lead compounds in a plant extract or fractions that can be the basis of drug discovery in future. The major highlights of the article are as follows:

- extraction, fractionation, and bioguided isolation of phytoconstituents;
- chemoprofiling and standardization of plant secondary metabolites;
- quality assessment and marker profiling for HMs;
- in vitro bioassays for screening of MPs in metabolic disorders; and
- finding new chemical entities from the medicinal herbs for the treatment of several metabolic disorders like diabetes, obesity, and cardiovascular diseases including atherosclerosis, hypertension, renal disease, glaucoma, etc.

Introduction

MPs are the richest source of drugs for traditional systems of medicine, modern medicines, nutraceuticals,

Animal Biotechnology.
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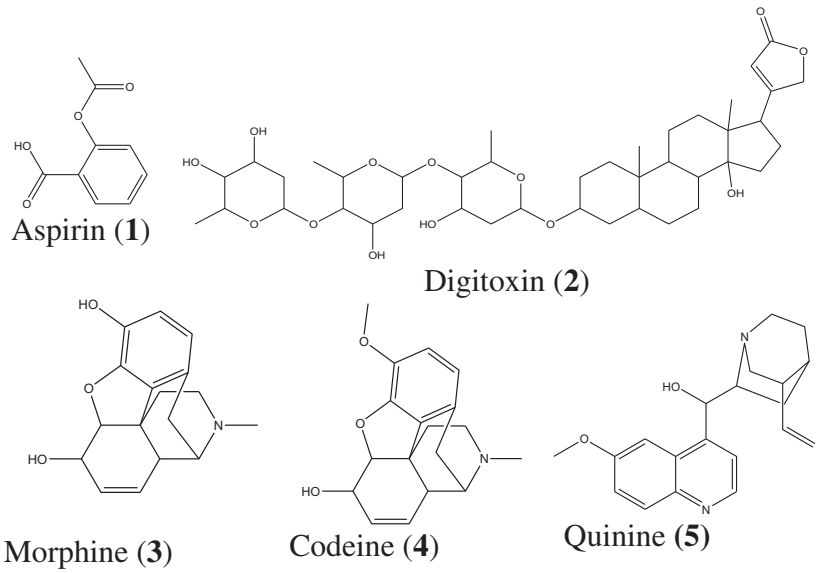
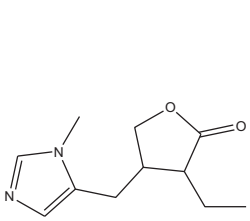
food supplements, folk medicines, pharmaceutical products, and new chemical entities for synthetic drugs. MPs are traded as such in bulk from many developing countries for further value addition in developed countries. MPs are being used by human civilization throughout the world since long ago because of their several therapeutic benefits. HM implies generally to those medical and healthcare systems that are practiced in a traditional manner and not presently considered to be a part of modern Western medicine (Cordell and Colvard, 2012). It is quite popular among people due to their practical benefits, traditional beliefs, economical advantage, easy access, and other reasons, which have a regional, religious, and social basis. HM is time honored and are being used by the people for their own healthcare, so these systems are well rooted with profound clinical basis, where scientific validation is some time the major constrains for their development. In spite of all these setbacks, HM remains with a constant growth in the global market. This discipline has devolved over years by exploiting natural products for numerous diseases for indigenous resources (Houghton and Mukherjee, 2009).

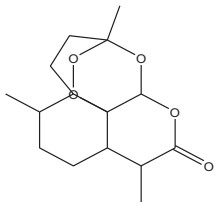
However, HM has to virtually cross miles in providing its therapeutic efficacy scientifically due to its immensity and wide acceptability to a variety of disease and targets according to the principle of synergy. HMs includes extracts, crude drugs, herbal preparations, etc. Herbal drugs are unprocessed part of herb or whole plant. Herbal preparations include powdered materials or extracts, tinctures, and fatty oils, which may be produced by extraction, fractionation, purification, concentration, or other physical and biological processes (Patwardhan et al., 2015). Quality assessment and scientific validation of HMs are very important parameters to be considered in assuring the quality, efficacy, and safety to promote their use in healthcare. Metabolite fingerprinting and marker compound analysis are pioneering in this area for the standardization of HMs. This approaches helps not only in establishing the correct herbal identity but also helps in finding the chemical holiness of the plants (Mukherjee et al., 2016, 2017a).

Natural products (NPs) have been the basis of treatment of human diseases dates back practically to the existence of human civilization. It has been the basis of the modern system of medicine and will exist as one important source of future medicine and drug therapeutics. Several medicines are derived either in the form of NPs or semisynthetic derivatives. Approximately 40% of all medicines are discovered from the NPs, and so it can play a crucial role in pharmaceutical research and drug development. Drugs like aspirin (**1**), digitoxin (**2**), morphine (**3**), codeine (**4**), quinine (**5**), pilocarpine (**6**), artemisinin (**7**), paclitaxel (**8**), vincristine (**9**), and reserpine (**10**) have been predominantly obtained from ethnomedicine based on their chemical, pharmacological, and clinical studies (Lahlou, 2013).

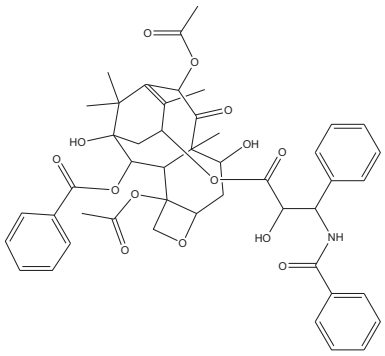




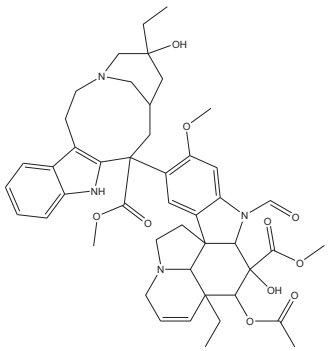
Pilocarpine (**6**)



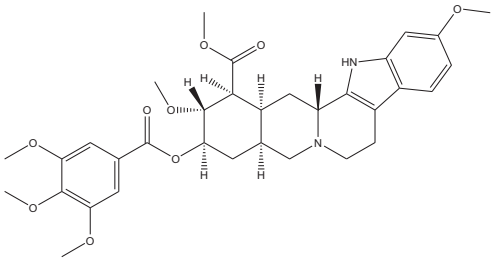
Artemisinin (**7**)



Paclitaxel (**8**)



Vincristine (**9**)



Reserpine (**10**)

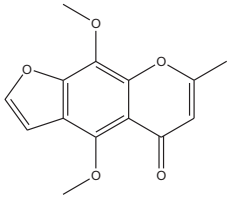
Despite competition from other drug discovery methods, natural products still play a significant role for finding new drugs for several disease such as anticancer, antihypertensive, antiinfectives, immunosuppression, and neurological disorders. Plants have vast chemical and structural diversity than synthetic compounds; therefore, it can be the major resources of bioactive compounds and will continually play as protagonists for discovering new drugs (Cragg and Newman, 2013).

In the past, most of new drugs have been discovered as secondary metabolites and derived molecules from natural resources. Interest in natural products research (NPR) are being continued due to the failure of alternative drug discovery techniques to produce many lead molecules in several therapeutic areas such as immunosuppression, antiinfectives, metabolic disorders, etc. NPs have been exploited as the big source of finding new lead structures, which might be used as a model for the development of new molecules or drugs in the area of pharmaceuticals. It is obvious from the natural product research that the NPs have been and will be an important source of new drug for pharmaceutical industry. Newer techniques have been introduced to improve and accelerate the natural product drug discovery and development to find the drug targets, lead molecules, and structures elucidation (Patwardhan et al., 2015). Drug discovery leading to robust and viable drug candidates is a challenging scientific task. This is the transition from a screening hit to a drug molecule, which requires expertise and experience. There are various important techniques including automated separation techniques, HTS, and combinatorial chemistry, which are used for enhancing drug discovery from NPs. These methods can be used for minimizing the inherent limitations of natural products and offers a unique opportunity to re-establish NPs as a major source for drug discovery (Lahlou, 2013). This chapter attempts to describe the utilization of natural resources as drug candidates, with a focus on the success of these resources through in vitro enzyme inhibition assay.

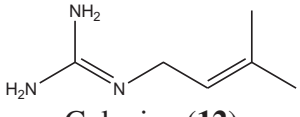
Lead molecules from herbs

MPs are being used in therapy by different cultures worldwide, and it has been extensively exploited and documented. It continues to play an important role in healthcare due to several health benefits. According to the World Health Organization (WHO) report, it is estimated that approximately 80% of the world population predominately relied on plant-based HMs for their primary healthcare. WHO-Traditional Medicine Centers reported that 122 pure compounds have been identified from only 94 plant species, which have been used as drugs, among them 80% are used for therapeutic and other purposes (Cragg and Newman, 2013).

Several drugs have been discovered and developed from the MPs. Some relevant examples of plant derived drugs such as khellin (**11**), from *Ammi visnaga*, which led to the development of sodium chromoglycate as a bronchodilator. Galegine (**12**) was derived from *Galega officinalis*, which was the lead compound for the synthesis of metformin and other biguanidine antidiabetic drugs.

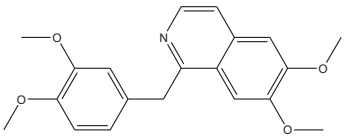


Khellin (**11**)

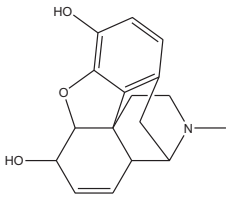


Galegine (**12**)

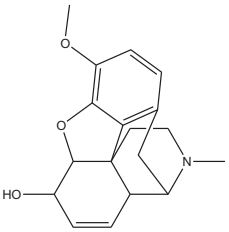
Papaverine (**13**) was isolated from *Papaver somniferum*, which was the basis for the development of verapamil as an antihypertensive drug and also the most powerful painkillers such as morphine (**14**) and codeine (**15**) has been developed. Quinine (**5**) derived from the bark of *Cinchona officinalis* was a precursor for the synthesis of the commonly used antimalarial drugs, chloroquine and mefloquine, and later on it largely replaced quinine (Cragg and Newman, 2013).



Papaverine (**13**)



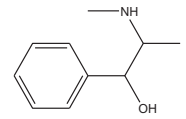
Morphine (**14**)



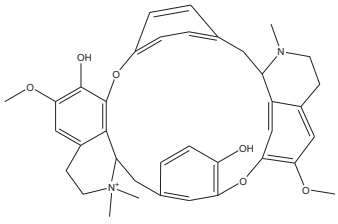
Codeine (**15**)

In this regard, another novel antimalarial drug, artemisinin (**7**), was discovered from the Traditional Chinese Medicine (TCM), *Artemisia annua* (Quinhaosu) with the emergence of resistance to both the above mentioned drugs. Now different artemisinin (**7**) analogues have been developed for the treatment of malaria in many countries. In 2015 Dr. Youyou Tu won the Nobel Prize in medicine for her discovery of artemisinin (**7**), which was isolated from *A. annua* for malaria. It would encourage research in phytopharmaceutical drug development for academia, researchers, and industry (Bhatt, 2016).

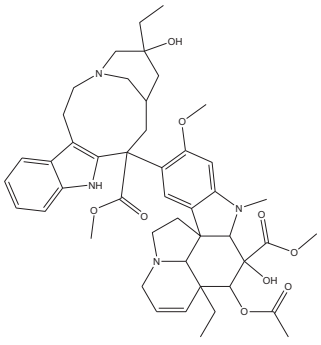
Several other drugs developed from HM were reserpine (**10**), isolated from *Rauwolfia serpentina* (Sarpagandha) as the antihypertensive drug (Kapoor, 1990); ephedrine (**16**) from *Ephedra sinica* (Ma Huang) as a antiasthmatic agents, which was the basis for the synthesis of other antiasthmatic drugs like beta agonists, salbutamol, and salmeterol; tubocurarine (**17**) from *Chondrodendron tomentosum* and Curare species (arrow poison), which was used as the muscle relaxant (Buss and Waigh, 1995). Anticancer drugs such as vincristine (**9**) and vinblastine (**18**) from *Catharanthus roseus* (Gueritte and Fahy, 2005) with the two clinically active agents, etoposide and teniposide (**19**), are semisynthetic derivatives of the natural product podophyllotoxin (**20**) (Lee and Xiao, 2012).



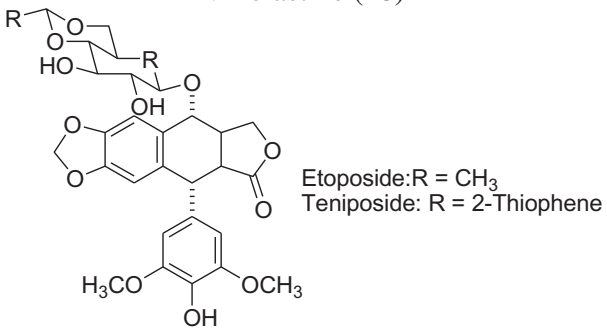
Ephedrine (**16**)



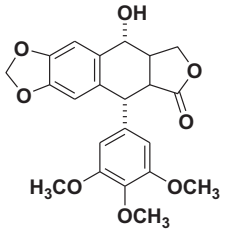
Tubocurarine (**17**)



Vinblastine (**18**)

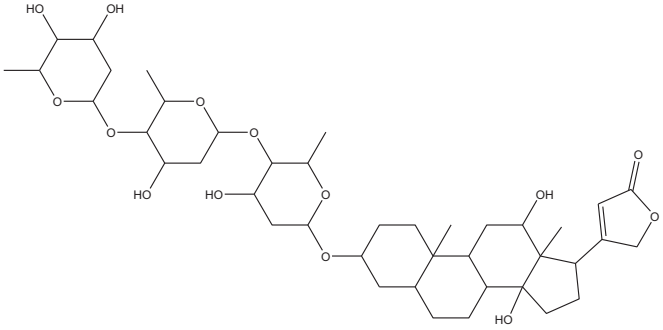


Etoposide & teniposide (**19**)



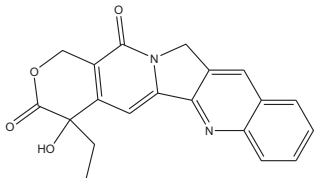
Podophyllotoxin (**20**)

Paclitaxel (**8**) (Taxol) along with several key precursors [baccatins, docetaxel (Taxotere)] has been discovered from *Taxus* species used as the most exciting anticancer drug for ovarian and breast cancer (Kingston, 2012). It has become a blockbuster drug with annual sales of over $1 billion throughout different pharmaceutical companies. Plant-based cancer chemotherapeutic agent, camptothecin (**21**) and its analogues topotecan, irinotecan and belotecan have been originated from the Chinese ornamental tree, *Camptotheca acuminate* (Rahier et al., 2005). Maytansine (**22**), tubulin interactive compounds, isolated from the Ethiopian tree *Maytenus serrata* is used as an anticancer drug (Kirschning et al., 2008). A cardiotonic drug, digoxin (**23**), has been isolated from Foxglove (*Digitalis purpurea*), which is most widely used in cardiovascular diseases.

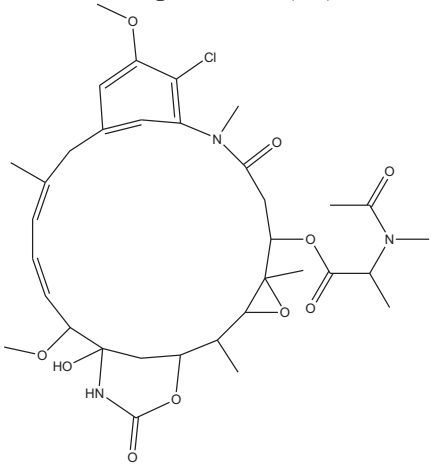


Digoxin (**23**)

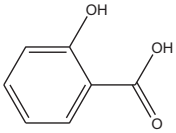
Salicylic acid (**24**) and its semisynthetic derivative drug, aspirin (acetyl salt) (**1**), was isolated from Willow bark (*Salix alba*), which is used as a pain killer and anticoagulant agent (Mahdi, 2010). In 2009 the USFDA approved capsaicin (**25**) (Qutenza) as a drug, which was developed from *Capsicum annum* (Chili peppers) by NeurogesX, Inc., which is used for postherpetic neuralgia, pain etc. Another drug colchicine (**26**) (Colcrys) was discovered from *Colchicum autumnale* (saffron), which was also approved by the USFDA in 2009 to treat acute flares in patients with gout and patients with familial Mediterranean fever (USFDA, 2009).



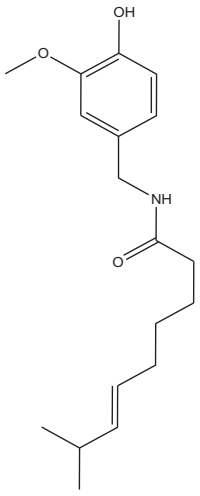
Camptothecin (**21**)



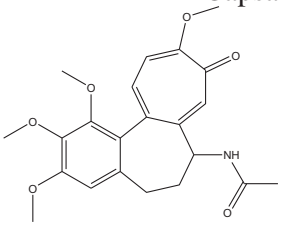
Maytansine (**22**)



Salicylic acid (**24**)

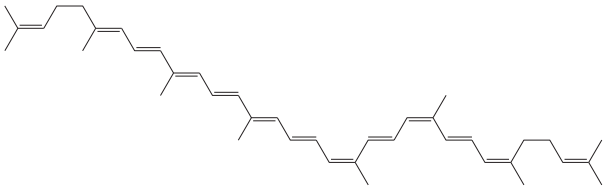


Capsaicin (**25**)

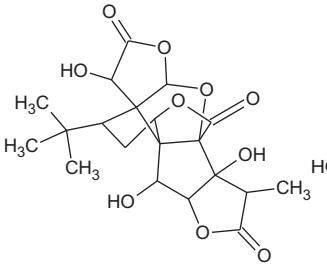


Colchicines (**26**)

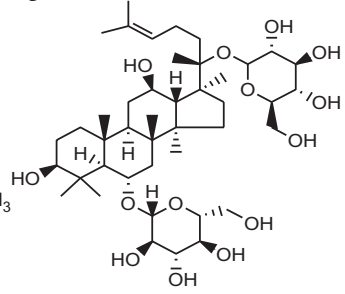
Botanicals may be derived from conventional primary food sources, for example, soy extracts containing isoflavones, tomato extracts rich in lycopene (**27**), or from secondary sources such as herbs and spices, for example, garlic oil, rosemary extracts, green tea extracts. Other botanical products may have no significant history of use as food ingredients but are derived from sources that have been used in herbal medicinal products in various regions of the world and are considered as the dietary supplements and nutraceuticals, for example, ginkgolide (**28**) from *Ginkgo biloba*, ginsenoside (**29**) from *Panax ginseng*, and hypericin (**30**) from *Hypericum perforatum* (St. John's Wort) (Mukherjee et al., 2017b).



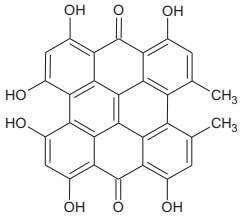
Lycopene (**27**)



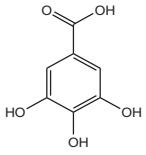
Ginkgolide (**28**)



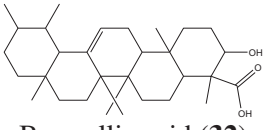
Ginsenoside (**29**)



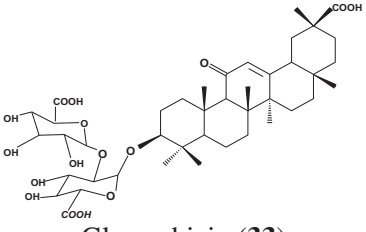
Hypericin (**30**)



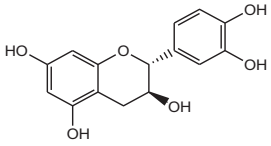
Gallic acid (**31**)



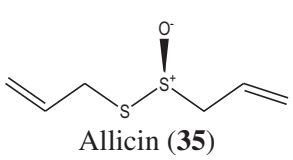
Boswellic acid (**32**)



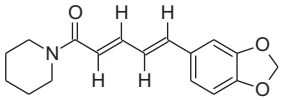
Glycyrrhizin (**33**)



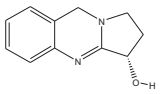
Catechin (**34**)



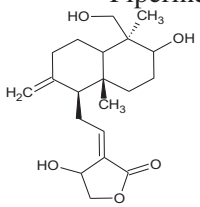
Allicin (**35**)



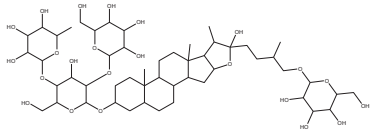
Piperine (**36**)



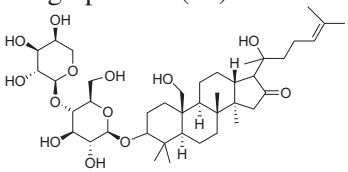
Vasicine (**37**)



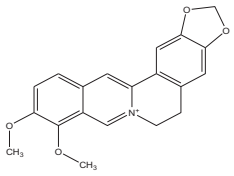
Andrographolide (**38**)



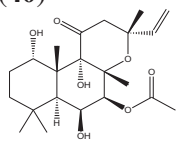
Shatavarin I (**39**)



Bacoside (**40**)

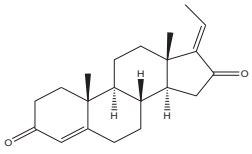


Berberine (**41**)

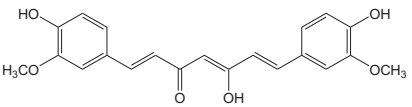


Forskolin (**42**)

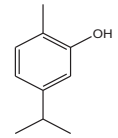
Several leads have been developed as the gallic acid (**31**) from *Terminalia chebula* and *Emblica officinalis*, boswellic acid (**32**) from *Boswellia serrata*, glycyrrhizin (**33**) from *Glycyrrhiza glabra*, catechin (**34**) from *Camellia sinensis*, allicin (**35**) from *Allium sativum*, piperine (**36**) from *Piper* species, vasicine (**37**) from *Adhatoda vasica*, andrographolide (**38**) from *Andrographis paniculata*, shatavarin I (**39**) from *Asparagus racemosus*, bacoside (**40**) from *Bacopa monnieri*, berberine (**41**) from *Berberis aristata*, forskolin (**42**) from *Coleus forskohlii*, guggulsterone (**43**) from *Commiphora mukul*, curcumin (**44**) from *Curcuma longa*, carvacrol (**45**) from *Ocimum sanctum*, withanolide A (**46**) from *Withania somnifera*, tinosporoside A (**47**) from *Tinospora cordifolia*, sennoside (**48**) from *Cassia angustifolia*, etc. (Mukherjee et al., 2015, 2016, 2017a).



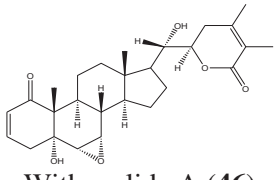
Guggulsterone (**43**)



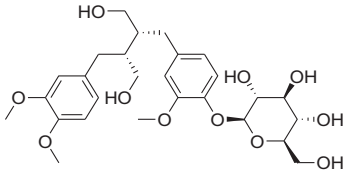
Curcumin (**44**)



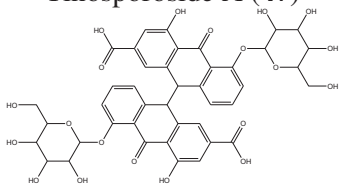
Carvacrol (**45**)



Withanolide A (**46**)



Tinosporoside A (**47**)



Sennoside (**48**)

Drug discovery from natural products particularly MPs has reclaimed the attention of the researchers, scientists, and pharmaceutical industry and is on the threshold of a comeback due to modern scientific inputs that assures better outputs on investment.

Principle

Extraction involves the separation of medicinally active portions (phytoconstituents) of plant tissues from the inactive or inert materials by using selective solvents in standard extraction methods. For aromatic plants, hydrodistillation techniques including water and steam distillation, hydrolytic maceration followed by distillation, expression, and enfleurage (cold fat extraction) may be used. Apart from the traditional solid–liquid extraction methods, such as maceration, infusion, decoction, and boiling under reflux, a wide range of modern techniques have been introduced in the past decades. These include microwave-assisted extraction (MAE), ultrasound-assisted extraction (UAE), and supercritical fluid extraction (SFE) for plants. There are several methods for extraction (Handa et al., 2008; Brusotti et al., 2014) of MPs, which are discussed later.

Methodology

Maceration

In this process the coarsely powdered plant material is placed in a stoppered glass container with the solvent and kept for at least 72 hours at room temperature with everyday agitation until the soluble matter has dissolved. Then mixture is strained, and the marc (solid material) is pressed, and the combined liquids will be filtered.

Infusion

Infusions are prepared by macerating the MPs for a short period of time with cold or boiling water. These are dilute solutions of the readily soluble constituents of crude drugs.

Digestion

This is another form of maceration in which only gentle heat is used in the extraction process. It is used when moderately elevated temperature is not objectionable. In this process solvent efficiency of the menstrum is slightly improved.

Decoction

This process is most suitable for the extraction of water-soluble and heat-stable constituents. In this process the crude drug is boiled in a specified volume of water for a specific time and then cooled and filtered. The ratio of crude drug to water is fixed as either 1:4 or 1:16 at initially and then volume is reduced to one-fourth of its original volume by boiling after the concentrated extract is filtered.

Percolation

This method is mostly frequently used for the extraction of active ingredients in the preparation of tinctures and fluid extracts. A percolator (a narrow, cone-shaped vessel open at both ends) is generally used in this process. The cure plant materials are moistened with an appropriate amount of the stated menstrum and kept for the 4 hours in a well-closed container, and then the mass is packed and the top of the percolator is closed. Furthermore, additional menstrum is added to form a shallow layer above the mass and the mixture is allowed to macerate in the closed percolator for 24 hours. After that outlet of the percolator is opened, and the liquid contained therein is allowed to drip slowly. Then marc is pressed, and the expressed liquid is added to the percolate. Sufficient menstrum is added to produce the required volume, and the mixed liquid is clarified by filtration.

Hot continuous extraction

In this extraction procedure, the finely powdered crude drug is placed in a "thimble," which is made of strong filter paper and placed in a chamber of the Soxhlet apparatus. The extracting solvent in the flask is heated, and its vapors condense in a condenser. The condensed extractant drips into the thimble containing the crude drug and extracts it by contact. When the

level of liquid in a chamber rises to the top of siphon tube, the liquid contents of chamber siphon into the flask. This is the continuous process and is carried out until a drop of solvent from the siphon tube does not leave residue when evaporated. This method required less time, is more economical, and large amounts of drug can be extracted with a very small amount of solvent.

Aqueous alcoholic extraction by fermentation

Some medicinal preparations of Ayurveda (like *asava* and *arista*) adopt the technique of fermentation for extracting the active principles. This extraction process involves soaking the powdered crude plant material for a specified period of time, during which it undergoes fermentation and generates alcohol in situ and facilitates the extraction of the active plant constituents.

Countercurrent extraction

In this method of extraction, wet plant material is pulverized through toothed disc disintegrators to produce fine slurry. In this method, the material to be extracted is moved in one direction within a cylindrical extractor where it comes in contact with extraction solvent. After that starting material moves, the extract becomes more concentrated. This method is more efficient and requires less time and pretense no risk from high temperature. This extraction process has significant advantages including requirement of smaller volume of solvent compared to other methods like maceration, decoction, and percolation.

Ultrasound-assisted extraction (sonication)

The UAE method involves ultrasound waves (20−2000 kHz) for extracting the phytomolecules from crude herbs by damaging the cell wall of the plants through cavitation, which may enhance phytoconstituents permeability in the solvents. This technique is very helpful for simultaneous extraction of plant materials with the maximum yield.

Microwave-assisted extraction

MAE extraction techniques involved combination of microwaves in the conventional solvent extraction system, which can enhance the penetration of solvent into the crude plant materials promoting the dissolution of the phytoconstituents. Similarly, in the UAE, the ultrasonic waves rupture the cell walls of plant material and improve the release of bioactive molecules into the solvent. There are several methods of MAE, which includes high-pressure MAE, nitrogen-protected MAE, vacuum MAE, ultrasonic MAE, solvent-free MAE, and dynamic MAE for higher yields and purity with shorter time as compared with conventional extraction processes

Supercritical fluid extraction

The SFE method is an alternative extraction procedure aiming to reduce the use of organic solvents and improvising the total yield of extract. There are several significant factors that need to be considered during the whole SFE process, such as temperature, pressure, sample volume, analyte collection, modifier (cosolvent) addition, flow and pressure control, and restrictors. In this technique, cylindrical extraction vessels are used most commonly for better performance. Carbon dioxide (CO_2) is used as an extracting fluid in this technique. Solvent polarity is an important factor for the extraction of polar solutes when strong analyte−matrix interactions are present. Organic solvents are frequently added to the CO_2 extracting fluid to alleviate the polarity limitations. In this system, argon gas can be used as the alternative fluid for Co_2 because it is inexpensive and more inert. The component recovery rates generally increase with the increasing pressure or temperature with the highest recovery rates in case of argon obtained at 500 atm and 150°C.

Crude products obtained from herbs through different extractions are relatively impure liquids, semisolids, or powders intended only for oral or external use. These products are produced in the form of different preparations such as decoctions, infusions, fluid extracts, tinctures, pilular (semisolid) extracts, and powdered extracts. Such preparations popularly have been called galenicals, named after a famous Greek physician, Galen. The aims of standardized extraction techniques for crude drugs are to gain the therapeutically active substance and to eliminate the inert material by treatment with a suitable solvent known as *menstruum*. Therefore, the extract may be ready for pharmaceutical application as a medicinal agent in the form of tinctures and liquid extracts. It may be further processed to be incorporated in any dosage form such as tablets or capsules, or it may be fractionated to isolate individual lead compounds, which may be the basis for the development of modem drugs. Hence, standardization of extraction methods offers significantly to the final quality of the medicinal products (Handa et al., 2008).

Bioguided fractionation of extract and isolation of phytoconstituents

Phytochemical researches based on traditional practices are being considered as an effective system for

the drug discovery from the MPs. Herbal products have been used by folklore traditions in the treatment of several diseases, which have a diverse source of chemical entities, but less information about their phytochemical aspects are available. These crude products contain complex mixture of many constituents, such as alkaloids, glycosides, terpenoids, flavonoids, and lignans. Its evaluation is necessary to validate the traditional use and to look for the most active extracts. The plant extracts may be further processed through various modern techniques for fractionation and isolation of bioactive compounds to meet the requirements to be used as a modern drug. These include the combination of extraction/sample preparation tools and analytical techniques for fractionating, isolating, and characterizing bioactive compounds from botanicals as potential lead compounds in the drug discovery process. Furthermore, the crude plant extract was evaluated for their biological tests and selected on the basis of the hypothesized bioactivity. In vitro bioassays are based on the HTS and considered to be ideal, which require very small amounts of compound. However, it may not be suitable to clinical specifications and can be specific, sensitive methods for biological screening of plant extract. The 96-well microplate-based tests are most commonly used methods through fully or semiautomatic system (Mukherjee, 2015).

TLC bioautography is another method and very useful to find out active compounds in a crude extract or fractions. Then plant sample (extract or fraction) is spotted on a TLC plate and allowed to run with an appropriate solvent system that offers a remarkable resolution of the compounds in the form of different bands. These methods involve the screening of a huge number of phytoconstituents against several targets using an automatic system. Typically, several thousands of compounds with very minute quantity can be tested simultaneously (Brusotti et al., 2014). Bioassay-guided fractionation has been the state-of-the art method for identifying bioactive natural products for many years. Detail of the extraction, fractionation of extract, and isolations of the phytoconstituents have been shown in Fig. 31.1.

This technique involves the repetitive preparative-scale fractionation and the assessment of biological activity till the isolation of pure compounds with the selected biological activity. There are several techniques such as column chromatography, flash chromatography, and other hyphenated chromatographic techniques employed for the fractionations and

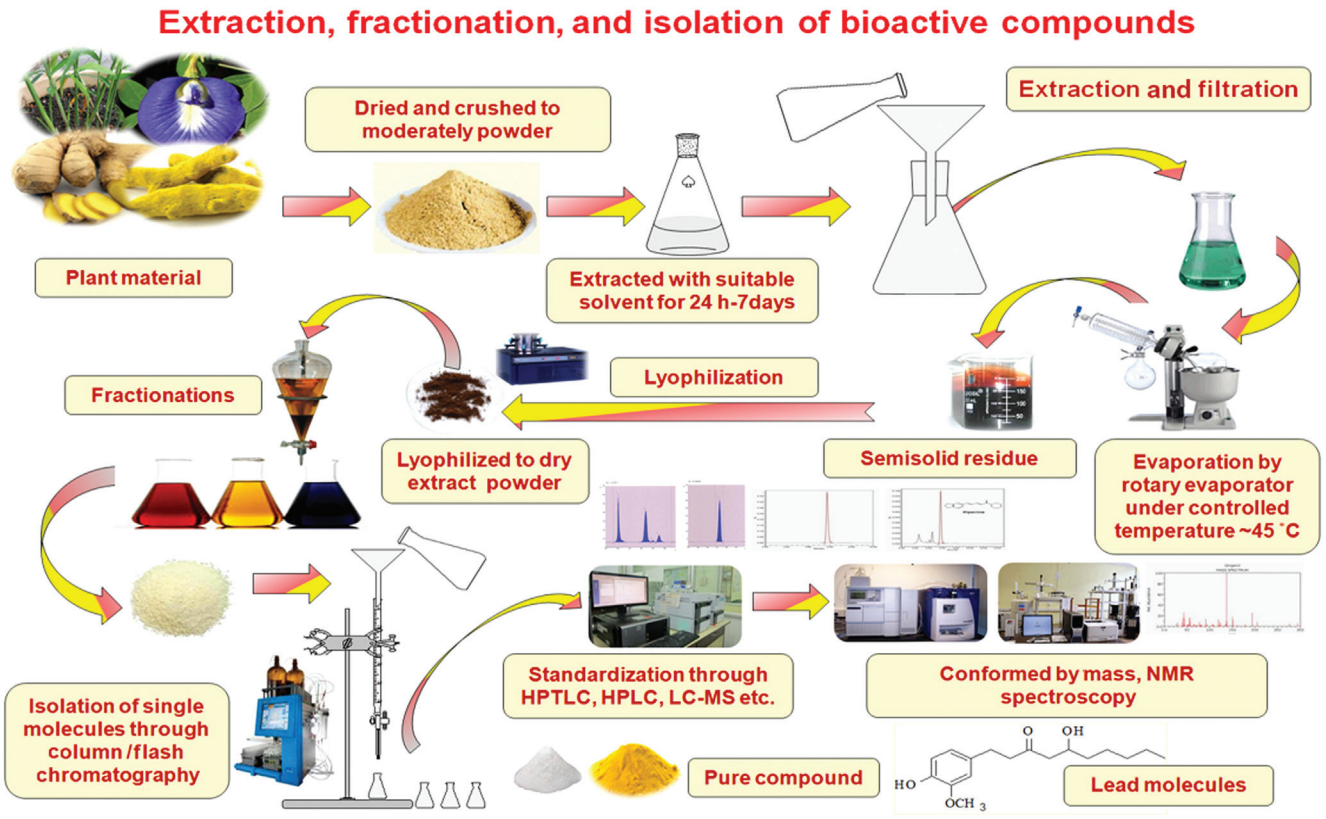


FIGURE 31.1 Extraction, fractionation of crude drugs, and isolation of phytoconstituents.

isolations of the plant secondary metabolites. In addition, the analytical techniques includes mass spectrometry (MS), nuclear magnetic resonance (NMR), high-performance liquid chromatography (HPLC), gas chromatography (GC), capillary electrophoresis (CE), HPLC-NMR, HPLC-MS, GC-MS, and CE-MS are utilized for the characterization and identification of the isolated molecules (Brusotti et al., 2014).

Examples with applications

The QC and standardization of HM and raw materials are the major important factor in justifying their acceptability in modern medicine healthcare system. Herbal drug standardization is the main responsibility of the regulatory authorities to ensure that the consumers get the proper medication, which can guarantee their quality, safety, potency, and efficacy. The QC of crude and herbal drugs is an important issue in justifying their acceptability in modern medicine (Mukherjee, 2015). Thus MP parts should be authentic and free from harmful materials like pesticides, heavy metals, microbial, radioactive contamination, etc. The bioactive extract should be standardized on the basis of active principle or major compound(s) with the help of modern techniques. Phytochemistry has an important and indispensable role in the implementation of each of those steps. Quality control of HM requires knowing what is chemical constituents in the plant, what is happening to those constituents during the processing of the plant, and assuring appropriate analyses when materials are being tested biologically and eventually delivered to the patient in a finished product (Mukherjee et al., 2012).

A serious issue is being expressed all over the world with respect to the contamination and adulteration of herbs. Contaminants may include various pesticides, herbicides, insecticides, heavy metals (arsenic, cadmium, lead, and mercury), microbial species, and radiation. Adulterants may include spurious and allied substances, which are cheaper and similar in appearance, or synthetic drugs (Cordell, 2011). The regulatory requirement for the herbal materials including food supplements and other related products in the food market raises concern about the safety assurance of such products (Kroes and Walker, 2004).

Instrumental techniques for quality evaluation

Due to the complexity of the herbal products, the use of advanced analytical techniques such as MS, NMR, HPLC, GC, CE, HPLC-NMR, HPLC-MS, GC-MS, and CE-MS are useful to perform the quality assessment of HMs. There are several modern techniques used for quality control and standardization of the herbal product confirming their composition from batch to batch uniformity and assuring the safety of the finished product. It is assumed that high doses (in common with conventional medicines) of herbal drugs may cause unwanted side effects. These techniques are typically used in a combined way for the product development at the initial stages of drug discovery. The next important step is stabilization of the bioactive extract with a minimum shelf-life of over a year in the final product (Mukherjee, 2015).

A fingerprinting of all the compounds is usually characteristic for individual plant or plant extract. The analyzed compounds may or may not be the bioactive substances, but quite often characteristic marker substances only. The chemical fingerprints produced by chromatographic techniques like TLC, high-performance thin-layer chromatography, and HPLC of herbal preparation should also be documented. Amalgamation of sensitive and rapid analytical techniques with online spectroscopic methods is known as "hyphenated chromatographic techniques," which all together generates both chemical- and biological activity-related information, which plays a significant role in the study of the effects of phytopharmaceuticals and in the QC of HMs. Nowadays, these techniques particularly, HPLC coupled with simple detectors [UV, evaporative light scattering detector (ELSD), electron capture detector], or detectors for hyphenated systems [i.e., photo (UV)-diode array (PDA/DAD), MS and NMR] have been used for fingerprinting and quantitative analyses of complex herbal extract in terms of rapid online identification of known compounds as a dereplication (Wolfender, 2009).

Liquid chromatography (LC) coupled with ELSD is applied mainly for the detection of plant secondary metabolites with weak chromophores, such as terpenes, in both aglycone and glycosidic forms, saponins and some alkaloids. In this conjecture, ultrahigh-pressure liquid chromatography (UHPLC) has been employed, which increases the speed of analysis and allows higher separation efficiency and resolution, higher sensitivity and much lower solvent consumption. For example, metabolite profiling of Brazilian *Lippia* species, *Radix scrophulariae*, and *Desmodium styracifolium* has been performed through UHPLC-DAD-TOF-MS, HPLC-DAD-ESI-MS, and LC-DAD-MS techniques, respectively. Likewise, GC-MS and LC-MS are usually used for detection and identification of volatile components and bioactive metabolites (Brusotti et al., 2014). Schematic representations of several hyphenated techniques have been represented in Fig. 31.2.

The rapid identification of known compounds from phytopharmaceuticals is an important step for finding

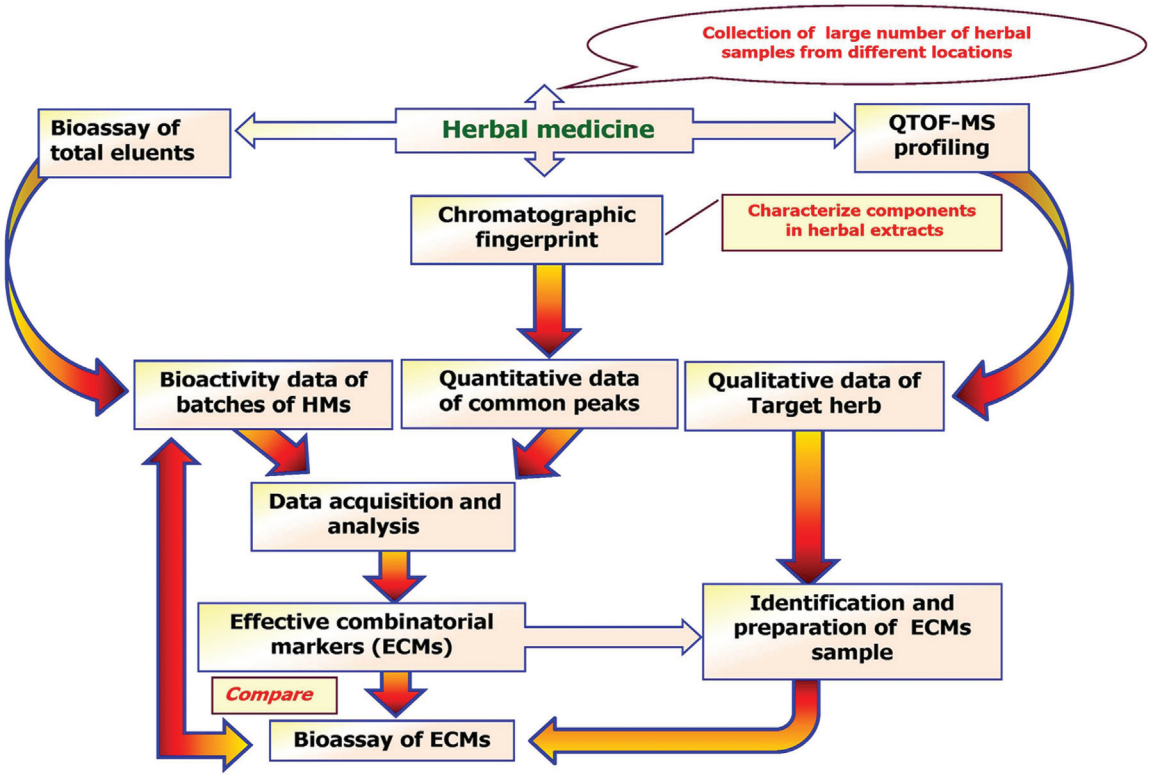


FIGURE 31.2 Techniques for the evaluation of herbal medicines.

lead compounds, which may be the basis for drug discovery from plants. Herbal drugs and its marketed products are widely used for the prevention, diagnosis, treatment, and management of disease for better health of human beings, while quality control and proper regulation are still a big challenge globally. Its wide application and continuous use have created a global health challenge in terms of quality, safety, and efficacy. Scientific validation and standardization of HM is needed for the globalization of traditional medicine. The appropriate use of products of assured quality could also do much to decrease any risks linked with HM (Mukherjee et al., 2016, 2017a).

Regulation and legislation on HMs have been endorsed in very less countries; most countries do not have proper regulation of natural products, and the quality of herbal products sold is generally not guaranteed. Proper research methodology and documentation are still needed although much more research is done on natural products most of them are inadequate, very few trials produce satisfactory results that are included in the healthcare system. Good manufacturing practices should be applied to assure the high quality of herbal drugs and products. In order for a drug regulatory agency to meet the high demand of the public, there is a need for well-designed, randomized, double-blind, and placebo-controlled clinical trials to establish the safety and efficacy of herbal drugs along with modern drugs (Mukherjee, 2015).

In vitro enzyme inhibition assays for screening of medicinal plants in metabolic disorders

Metabolic disorders

Metabolic disorder disrupts normal metabolic process of converting food to energy on a cellular level. The onset of symptoms usually occurs when the body metabolism comes under stress. Most of the metabolic disorders perturb numerous metabolic pathways where thousands of enzymes are involved interdependently. Metabolic disease affects the ability of the cell to perform critical biochemical reactions that comprise various processes, namely, transport of proteins (amino acids), carbohydrates (sugars and starches), or lipids (fatty acids) (Meisinger et al., 2006). It also increases the risk of cardiovascular disease, type 2 diabetes mellitus, obesity, hypertension, etc. The major pathophysiology underlying these diseases are insulin resistance, visceral adiposity, atherogenic dyslipidemia, endothelial dysfunction, genetic susceptibility, elevated blood pressure, hypercoagulable state. Metabolic syndrome is a cluster of risk factors for cardiovascular disease that includes

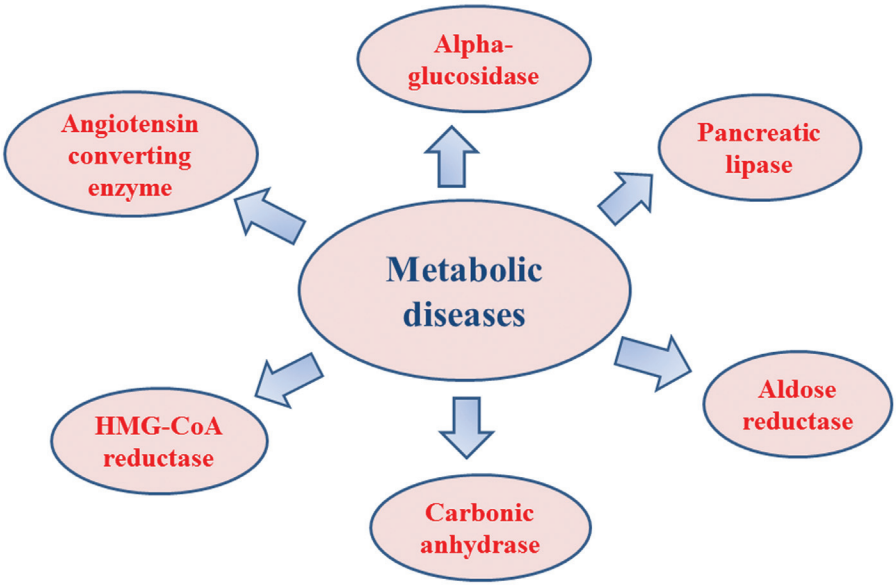


FIGURE 31.3 Enzymes involved in metabolic disorders.

abdominal obesity, dyslipidemia, elevated blood pressure, and impaired glucose tolerance (Halcox and Quyimmi, 2006). There are several enzymes involved in major biochemical pathways in the disease pathogenesis, namely, α-glucosidase, angiotensin-converting enzyme, pancreatic lipase, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, carbonic anhydrase (CA), and aldose reductase, as shown in Fig. 31.3. The interaction between the components of clinical phenotype with its biological phenotype (insulin resistance, dyslipidemia, etc.) contributes to the development of these chronic metabolic disorders.

Enzyme inhibition: a target of drug discovery

Enzymes are considered to be an attractive target for drug development due to their essential catalytic roles in many physiological processes in our body. The enzyme activity may be altered in various disease conditions. In most of the cases, enzyme catalysis can be hindered by small-molecular-weight, drug-like molecules that are known as inhibitors. The in vitro evaluation of enzyme–inhibitor interactions have been found as a key point for primary screening of new drug molecules. The development of enzyme inhibitors as therapeutic agents involves the optimization of multiple pharmacologic properties beyond the affinity and selectivity of the molecule for its target enzyme. Many of these pharmacologic properties have their molecular underpinning in biochemical reactions within the human body. This include drug absorption from the gastrointestinal tract via active and passive transport mechanisms, metabolic clearance of drugs from systemic circulation, hepatic and renal drug metabolism, and adverse effects mediated by drug interactions with off-target enzymes, ion channels, and receptors. Enzyme assays are based on the measurement of how fast a given (unknown) amount of enzyme will convert substrate to product (the act of measuring a velocity). The enzyme assay is required to determine the concentration of a product or a substrate at a given time after starting the reaction. There are several assay procedures as follows to monitor the enzyme–substrate interaction qualitatively and quantitatively. These include spectroscopic, fluorimetric, manometric, and polarimetric method (Copeland and Anderson, 2001).

- Spectrophotometric method is based on the change of absorbance due to conversion of substrate to product because of enzyme action, measured in the visible region or in the UV region. This method is mostly used in enzyme assay as it is easy, simple, and sensitive.
- The fluorescence method is applied when the molecule does not contain any UV-absorbing chromophore, but its emission wavelength changes after absorbing light of different wavelengths. This method is applicable when the enzyme catalyzed reaction follows oxidation and reduction pathway, where change in fluorescence takes place. It is a highly sensitive method to detect small quantities of samples.
- The manometric method is applicable when one of the reaction components is gas. The electrode method is suitable to follow the reactions in which change in pH of the reaction system takes place.
- The polarimetric method is suitable for detection of several isomerases, which can convert optically active isomers to inactive or vice versa. Sampling

methods are followed by withdrawing samples at intervals and estimating the substrate or product by chemical methods. This method can be used to study the enzymes that can act on carbohydrates linked with the breakage of a glycosidic link present in the structure.

Enzymes are very essential in human physiology as they are effective drug target for having their catalytic activity. Most of the pharmaceutical companies are nowadays focusing on small-molecular-weight drug molecule that can bind to the receptor (enzymes) by altering (inhibition/induction) the disease pathophysiology. This can lead to development of novel drug candidates that act through inhibition of specific enzyme targets. In fact, most of the drugs marketed by the pharmaceutical companies are of small molecule and act as specific enzyme inhibitors. Some examples of these enzymes and the disease pathogenesis they are involved with are CA in glaucoma, xanthine oxidase in gout, angiotensin-converting enzyme in hypertension, HMG-CoA reductase in cholesterol lowering, pancreatic lipase in hyperlipidemia, aldose reductase in diabetic retinopathy, dehydrogenase in inflammation, etc. The inhibitors of these enzymes are in clinical use (Copeland et al., 2007). The enzyme reactions also play a critical role in the study of drug metabolism and pharmacokinetics. The elimination of xenobiotics, including drug molecules, from systemic circulation is driven by metabolic transformations that are entirely catalyzed by enzymes. The enzyme catalyzed reactions are mainly of four types (Copeland and Anderson, 2001):

1. Oxidation reactions that involves aromatic hydroxylation, aliphatic hydroxylation, *N*-hydroxylation, *N*, *O*, *S*-dealkylation, deamination, sulfoxidation, *N*-oxidation, and dehalogenation.
2. Hydrolytic enzymes include ester, amide, and peptide hydrolysis reactions.
3. Conjugation reactions are another reaction mechanism process by which enzyme can act through glucuronidation, sulfation, acetylation, peptide conjugation, and glutathione conjugation.
4. Reductive reactions are another mechanism of enzyme action that plays a vital role in the enzyme activity.

Enzymes involved in metabolic disorder: its significance in inhibition

Pancreatic lipase

Lipases or acyl-glycerol acylhydrolases are esterases, hydrolyzing esters of glycerol with long-chain aliphatic acids. Pancreatic lipase plays a key role in conversion of triglyceride into monoacyl glycerol and free fatty acids, which has been represented in Fig. 31.4. Both the monoacyl glycerol and fatty acid are converted into mixed micelle in the presence of cholesterol and bile acids. It is then absorbed as monoacyl glycerol and further transformed to triglyceride where energy is being stored (Birari and Bhutani, 2007).

The free fatty acid is responsible in regulation of very low-density lipoprotein, low-density lipoprotein (LDL), and high-density lipoprotein (HDL), collectively called as total lipid content. Irregulation of total lipid content are considered to be a major causative factor of cardiovascular disease as well as obesity and hypertension. The excess disposition of triglyceride leads to hyperlipidemia, which is a major causative factor of developing cardiovascular diseases, atherosclerosis, hypertension, diabetes, and other functional depression of certain organs. Hyperlipidemia can also be defined as a secondary metabolic dysregulation marked by the increased levels of total cholesterol, triglycerides, and LDL cholesterol besides the decrease in HDL cholesterol. It is considered as a major life style disorder due to several life style−related irregularities. Among several causative factors, pancreatic lipase is proposed to be an important target of developing antiobesity agents. By inhibiting the pancreatic lipase action, conversion of triglyceride is being blocked which further results in decrease triglyceride reabsorption in adipose tissue and finally leads to hypolipidemia. Several drugs have been exploited clinically against obesity; however, they showed unanticipated side effects in some cases. There are several potential phytomolecules, namely, polyphenols, saponins, terpenes, obtained from natural sources provides potential pancreatic lipase inhibitory activity that can be developed as a safer alternatives compared to synthetic drugs. Therefore, the exploration of alternative hypolipidemic leads from medicinal herbs should be a rational strategy to mitigate the elevated serum/plasma levels of lipids in blood (Sharma et al., 2005).

3-Hydroxy-3-methylglutaryl coenzyme A reductase

Cholesterol is vital for various basic life processes including mammals. However, the overload of cellular and circulating cholesterol is damaging. In the 1950s and 1960s, it was clear that elevated concentrations of plasma cholesterol were a major risk factor for the development of coronary heart disease (Steinberg and Gotto, 1999). The foremost strategy to regulate cellular cholesterol homeostasis is through LDL receptor and HMG-CoA reductase. LDL receptor regulates the uptake, while HMG-CoA reductase acts in endogenous cholesterol synthesis in the liver (see Fig. 31.5) (Rudling, 1992).

Experimental studies have explained the role of these regulatory mechanisms in vitro in experimental animals and in humans treated with experimental diets or drugs.

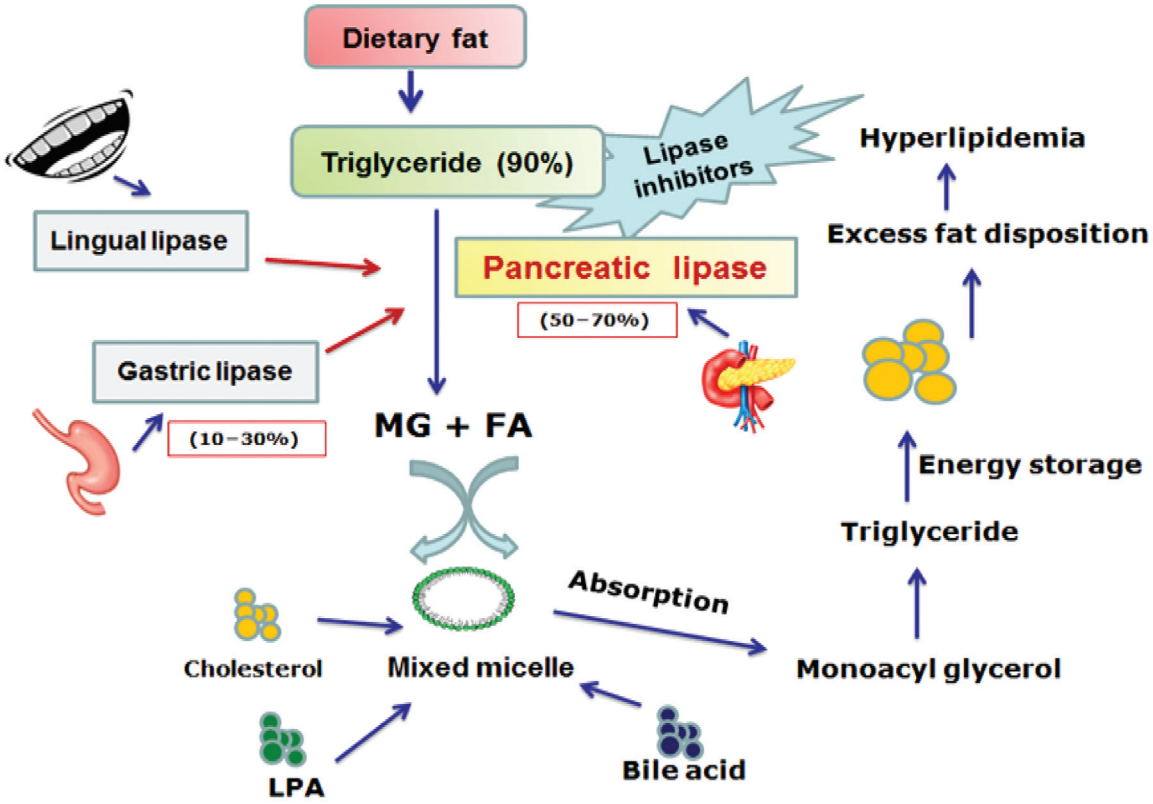


FIGURE 31.4 Pancreatic lipase inhibition.

This scientific evidence incited the exploration of drugs that could reduce plasma cholesterol improvised strategies to reduce cholesterol biosynthesis. Therefore, the rate-limiting enzyme in the cholesterol biosynthetic pathway, HMG-CoA reductase, is chosen as a natural target (Tobert, 2003). HMG-CoA reductase inhibitors are potent inhibitors of cholesterol biosynthesis having other potential additional benefits (Massy et al., 1996).

Glucosidase

kGlucosidases are the enzymes responsible for the breaking of glycosidic bonds in oligosaccharides or glycoconjugates. Glucosidases differ in the breaking of glycosidic bonds depending on the number, position, or configuration of the hydroxyl groups in the sugar molecule. Thus α- and β-glucosidases are able to break the glycosidic bonds involving terminal glucose connected at the site of cleavage, respectively, through α- or β-linkages at the anomeric center. Glucosidase activity is linked to various metabolic processes like:

1. reduction of polysaccharides to monosaccharide, which can then be easily absorbed and used by the organism;
2. catabolism of lysosomal glycoconjugate and processing of glycoprotein; and
3. biosynthesis of oligosaccharide units in glycoproteins or glycolipids.

α-Glucosidase inhibitors slow down the process of digestion and absorption of carbohydrates by competitively blocking the activity of glucosidase. As a result, the peak concentration of postprandial blood glucose is reduced, and the blood sugar level comes under control (Melo et al., 2006).

Acarbose, an α-glucosidase inhibitor provides a new direction in the management of diabetes. By competitive and reversible inhibition of intestinal α-glucosidases, acarbose increases carbohydrate digestion time and thus reduces the rate of glucose absorption. Due to this mechanism, the postprandial rise in blood glucose is decreased with respect to dose, and glucose-induced insulin secretion is decreased. Therefore acarbose treatment not only reduces an increase in blood glucose level after meals but also improves the general metabolic state. In diabetic animals, acarbose has been shown to reduce glycosuria and also concurrently resisting the decrease in the skeletal muscle GLUT4 glucose transporters due to low mean blood glucose. The amount of protein

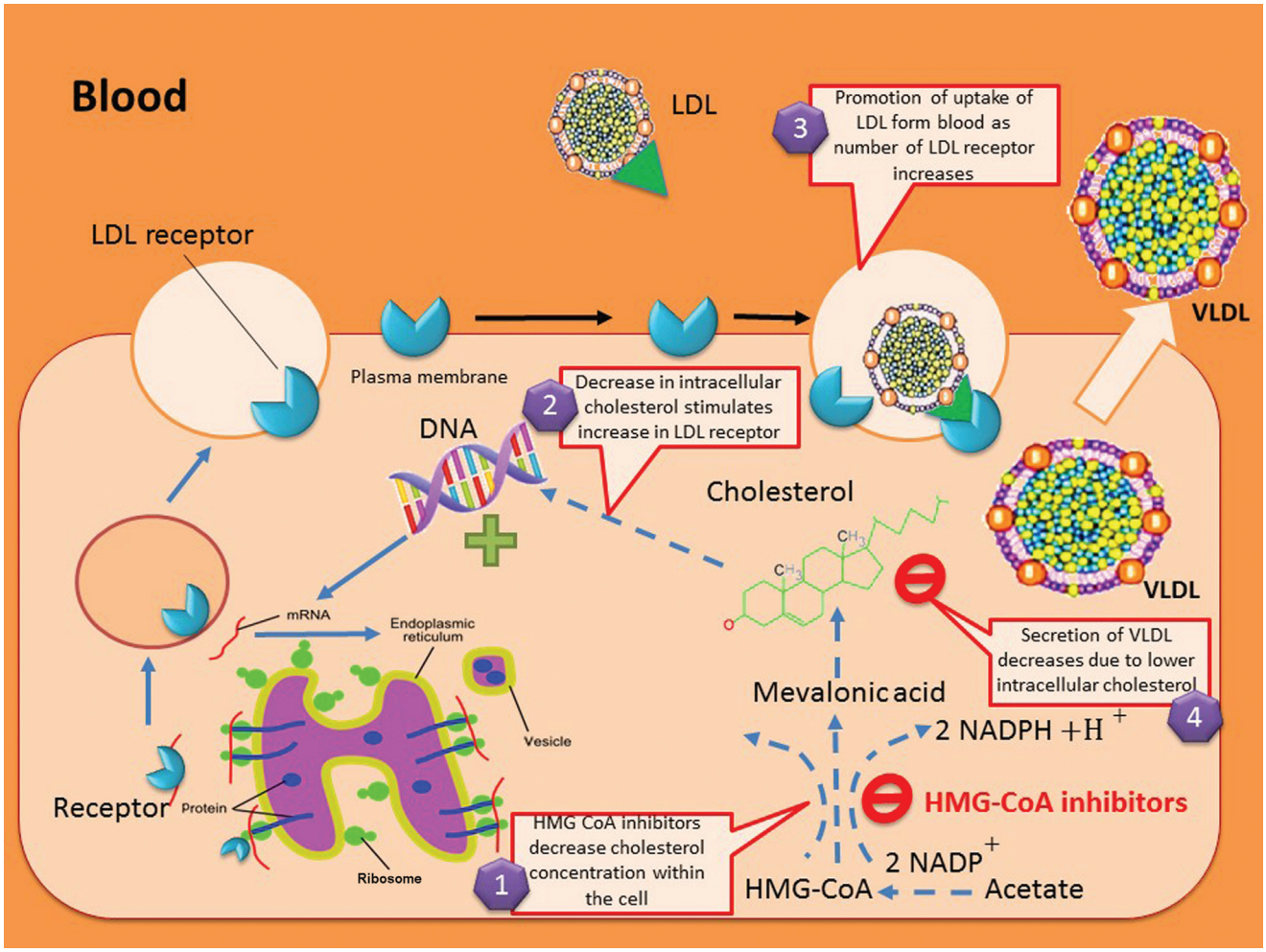


FIGURE 31.5 HMG-COA reductase inhibition.

nonenzymatically glycated is diminished and followed by reduced formation of advanced glycation end-products. The prevention of basement membrane glycation and thickening in various tissues clearly demonstrates that acarbose treatment of diabetic animals produces beneficial effects against the development of nephropathy, neuropathy, and retinopathy. Thus, the αglucosidase inhibitor acarbose have the potential to delay or possibly prevent the development of complications associated with diabetes (Bischoff, 1995).

Aldose reductase

Aldose reductase protein is a monomer comprising 315 amino acid residues. It belongs to the Aldo-keto reductase super family. Under normal blood glucose level, major part of cellular glucose is converted into glucose 6-phosphate by the enzyme hexokinase by phosphorylation. A minor part of nonphosphorylated glucose enters polyol pathway metabolism, the other route of glucose metabolism. Reduction of glucose to sorbitol mediated by the enzyme aldose reductase is the rate-limiting step of this pathway. Sorbitol is then converted to fructose by sorbitol dehydrogenase. This completes the pathway. During high blood glucose level such as in diabetes hexokinase gets saturated with the excess glucose. This in turn causes excess accumulation of sorbitol. This overproduction of the products of the polyol pathway along with depletion in reduced nicotinamide adenine dinucleotide phosphate and the oxidized nicotinamide adenine dinucleotide, which are the cofactors used in the pathway, causes various metabolic disturbances in those tissues that are involved in the insulin-independent uptake of glucose. Such metabolic disturbances are responsible for the early tissue damage in the organs such as ocular lens, retina, peripheral nerve, and renal glomerulus,

which are the major targets of diabetic complications (Nishimura, 1998).

Carbonic anhydrase

CAs (EC 4.2.1.1) are zinc-containing metalloenzymes found in higher vertebrates including humans. The major role of the enzyme is interconversion of carbon dioxide (CO_2) and water to the bicarbonate ion and protons (or vice versa). This enzyme family is mainly classified into five distinct subfamilies based on their genetic structure. Among them, α-CA II is one of the most active and cytosolic bound enzymes. It regulates several physiological and pathological processes, including transportation of CO_2 and bicarbonate ions between metabolizing tissues and lungs, thus maintaining the pH of blood and homeostasis (Supuran, 2008). It also plays a significant role in bone resorption, electrolyte secretion in various tissues, and organs and some other biosynthetic reactions, namely, gluconeogenesis, lipogenesis, and ureagenesis. The inhibitory action of different CA isoforms may exhibit several clinical applications as diuretics, antiglaucoma, antiobesity, antiepileptic agents. Sulfonamides like acetazolamide, dorzolamide, and brinzolamide are the most widely used classical CA inhibitors (mainly CA II mediated). However, they are nonspecific to isoenzymes and associated with several adverse reactions like depression, malaise, gastrointestinal irritation, metabolic acidosis, renal calculi, and transient myopia (Supuran, 2008). Several phytomolecules, namely, phenols/polyphenols, phenolic acids, and coumarins, obtained from the natural sources have been found as a CA inhibitors, which can be further explored as the novel CA inhibitors.

Medicinal plants used in metabolic disorders

MPs have been used since time immemorial for prevention and cure for various metabolic disorders. Although in most of the cases scientific validations of these MPs are missing, the therapeutic applications of the plants have provided promising results. Due to encouraging results, MPs are still continued to be used alongside modern medicine. MPs provide a wealth of phytoconstituents having diverse biological activities, which are found to be highly effective in the management of metabolic diseases. These metabolic diseases encompass wide range of disease including obesity, hypertension, hypercholesterolemia, atherosclerosis, diabetes, etc. These diseases are mainly the result of lack of physical activity and excessive stress, which results in high generation of reactive free radicals in the body, thus causing oxidative damage to cells and tissues. These damages disrupt the normal function of metabolism in the body and results into abnormal activities as metabolic disorders. Almost all the phytomedicines derived from plants are potent antioxidants that can provide an effective treatment option for metabolic disorders. Several MPs have been reported for various metabolic disorders like *A. sativum* for hyperlipidemia, *A. paniculata for* hyperglycemia, *Centella asiatica* for hypertension, *Clitoria ternatea* as diuretic, *Coffea arabica* for hypercholesterolemia, *Commiphora wightii* for hyperlipidemia, *C. longa* for obesity, *Momordica charantia* for hyperglycemia, *Terminalia arjuna* as cardioprotective, etc. Several bioactive lead compounds from MPs have been explored for management and treatment of different metabolic disorders; some of them are represented in Table 31.1. Apart from the antioxidative effects, the plants have other photochemical giving diverse range of activities, which in combination or individually help to mitigate the metabolic disorders and their symptoms. Besides MPs provides a cost-effective and safe alternative to modern medicine, which are both costly and elicits variety of side effects (Mukherjee et al., 2016, 2017a).

The catalytic power of enzymes is mostly related to the metabolic process of human health. From the historical time, the enzyme inhibitors were targeted as a potent therapeutic agent against several pathogenic conditions. The details understanding of physiochemical characteristics of enzyme-binding sites are very helpful to develop small, drug-like molecules. In this context, identification of enzyme target and identifying their inhibitor is found utmost important for early drug discovery process. The initial activity screening is thus required to gather knowledge of the molecular target and development of assay design for HTS. Herbs are a potential source of drug discovery and may lead to the development of several leads. This plays the key role in this context, which not only helps in screening the herbs but also assures the therapeutic efficacy of the natural products as being used in traditional health practices.

Ethical issues

There are no major ethical issues concerned connected to the enzyme inhibition assay as these are in vitro experiments.

Translational significance

Drug discovery and drug development from MPs have reclaimed the attention of the scientists and pharmaceutical industries. Several lead molecules have been developed from the MPs. An attempt has been

TABLE 31.1 Some medicinal plants for metabolic disorders (Mukherjee, 2015; Mukherjee et al., 2016; Mukherjee et al., 2013).

Plants	Phytoconstituents	Therapeutic activity
Allium sativum (Amaryllidaceae)	Allicin, garlicin	Hypolipidemic, antiatherosclerotic, hypoglycemic, anticoagulant, antihypertensive, antimicrobial, anticancer, antidote (for heavy metal poisoning), hepatoprotective, immunomodulator
Aloe vera (Liliaceae)	Aloe-emodin, aloin-A, barbaloin	Antiaging, antiinflammatory, hypoglycemic, gastroprotective, wound healing
Andrographis paniculata (Acanthaceae)	Andrographolide	Antihyperglycaemic, antipyretic, antiinflammatory, antileishmanial, antifertility, anti-HIV, cardiovascular, antiplatelet immunomodulatory, anticancer
Berberis aristata (Berberidaceae)	Berberine	Antidiabetic, cardiotonic, hepatoprotective, antidiarrheal
Bombax malabaricum (Bombaceace)	Mangiferin, bombasin, bombasin 4-*O*-β-glucoside, bombalin, bombamaloside	Cardioprotective, antioxidant, antiacne, antimicrobial, hepatoprotective, wound healing, anticancer
Camellia sinensis (Theaceae)	Catechin, (+)-gallocatechin, (−)-epicatechin, (−)-epicatechin gallate, (−)-epigallocatechin, (−)-epigallocatechin gallate	Antidiabetic, hypocholesterolemic, cardioprotective, antiaging, antiinflammatory, neuroprotective, antimicrobial, antioxidant, anticancer
Centella asiatica (Apiaceae)	Asiaticoside, asiatic acid, madecassic acid, brahmic acid, isobrahmic acid, centic acid and centoic acid brahmoside, brahminoside, centelloside	Antihypertensive, hypocholesteromic, antiwrinkles, anticancer, antibacterial, antioxidant, cicatrizing, stretch marks improvers
Clitoria ternatea (Fabaceae)	Taraxerol	Diuretic, antidiabetic, antimicrobial, antipyretic, antiinflammatory, analgesic, antiplatelet
Coffea Arabica (Rubiaceae)	Ferulic acid, chlorogenic acid	Hypocholesterolemic, antioxidant, anticancer, antibacterial, antiinflammatory, antimutagenic, wound healing
Commiphora wightii and *Commiphora mukul* (Burseraceae)	Guggulsterone E & Z	Antihyperlipidemic, cardioprotective, antiwrinkle, antimicrobial, antiinflammatory, analgesic, hepatoprotective
Curcuma longa (Zingiberaceae)	Curcumin, demethoxycurcumin, bisdemethoxycurcumin, turmerone, atlantone, zingiberene	Antiobesity, hypolipidemic, antihypertensive, antiinflammatory, cholagogue, hepatoprotective, blood purifier, antioxidant, antiischemic, detoxifier, and regenerator of liver tissue, antiasthmatic, antitumor, antiprotozoal, stomachic, carminative
Dillenia indica (Dilleniaceae)	Betulinic acid, betulin, cycloartenone, n-hentriacontanol, β-sitosterol	Antiobesity, hypolipidemic, antimalaria, antinflammatory, anticancer, antiviral
Emblica officinallis (Euphorbiaceae)	Vitamin C, gallic acid	Antidiabetic, rejuvenating agent, hepatoprotective, antitumor, antioxidant
Eugenia jambolana (Myrtaceae)	Gallic acid, ferric acid, chlorogenic acid	Antidiabetic, antihypertensive, hypolipidemic, antioxidant
Gingko biloba (Ginkgoaceae)	Ginkgoflavonglucosides, ginkgolic acid, ginkgolide A, ginkgolide B, bilobalide	Antioxidant, anticancer, cardioprotective, anticholinesterase (Alzheimer's)
Glycine max (Fabaceae)	Genistein, diadzein	Antiobesity, anticancer, antioxidant, antiinflammatory, antiosteoporosis, antidiabetic, antioxidant, antiaging
Glycyrrhiza glabra (Fabaceae)	Glycyrrhizin, glycyrrhetinic acid, glycyrrhizic acid	Cardioprotective, antiarrhythmic, antibacterial, antidiabetic antiviral, expectorant, antiinflammatory, antiulcer effect
Momordica charantia (Cucurbitaceae)	Momordicin, gentisic acid	Antidiabetic, antiviral, antibacterial, anticancer
Moringa oleifera (Moringaceae)	4-(4′-*O*-acetyl-α-L-rhamnopyranosyloxy)benzyl isothiocy-anate, 4-(α-L-rhamnopyranosyloxy) benzyl isothiocy-anate, niazimicin, pterygospermin, benzyl isothiocyanate, 4-(α-L-rhamnopyranosyloxy) benzyl glucosinolate, β-carotene	Hypoglycemic, antihypertensive, hypocholesterolemic, agent, antitumor, antioxidant, antiinflammatory, diuretic, antihepatotoxic, antifertility, antiurolithiatic, analgesic

(*Continued*)

TABLE 31.1 (Continued)

Plants	Phytoconstituents	Therapeutic activity
Ocimum sanctum (Lamiaceae)	Carvacrol	Antihypertensive, antioxidant, antidiabetic, antimicrobial, anticancer, antifertility, hepatoprotective, analgesic, adaptogenic
Panax ginseng (Araliaceae)	Malonylginsenoside, ginsenoside, ginsengiside	Immunomodulator, antidiabetic, hypocholesterolemic, nutraceutical
Phyllanthus emblica (Phyllanthaceae)	Ascorbic acid, gallic acid, elaeocarpusin	Antidiabetic, cardioprotective, antioxidant, antiaging, anticancer
Pinus pinaster (Pinaceae)	Caffeic acid, catechin, ferulic acid, taxifolin	Antidiabetic, antioxidant, antimycordial, antiinflammatory, antimicrobial, photoprotectant
Piper longum and *Piper nigrum* (Piperaceae)	Piperine	Hypocholesterolemic, antiinflammatory, bioavailability enhancer, immunomodulator, gastric protectant, antioxidant
Punica granatum (Punicaceae)	Ellagitannins, flavonols, anthocyanins, catechin, procyanidins, ellagic acid, gallic acid, delphinidin, pelargonidin	Antidiabetic, antioxidant, anticancer, antimicrobial, antidiarrhoeal, apoptotic, antigenotoxic, antityrosinase, antiinflammatory, antiaging
Rubia cordifolia (Rubiaceae)	Rubiadin	Diuretic, antioxidant, nephroprotective
Terminalia arjuna (Combretaceae)	Arjunic acid	Cardiprotective, antihyperlipidemic, antiatherogenic
Terminalia belerica (Combretaceae)	Gallic acid, ellagic acid	Antidiabetic, antimalarial, anti-HIV, antioxidant, antiproliferative, antimutagenic effects, antioxidant, photoprotective
Terminalia chebula (Combretaceae)	Gallic acid, ellagic acid	Antihypertensive, antidiabetic, anticancer; antimutagenic activity antioxidants, potent UV-protectant
Tinospora cordifolia (Menispermaceae)	Tinosporoside A	Hypolipidemic, anticancer, antidiabetic, antiinflammatory
Trigonella foenum- graecum (Fabaceae)	Trigonelline	Anticholinesterase, antidiabetic, antiulcer, wound healing, CNS stimulant, antiinflammatory and antipyretic immunomodulatory, antioxidant
Vitis vinifera (Vitaceae)	Resveratrol, catechins, procyanidins, epicatechins; caffeic acid, *p*-coumaric acid, cinnamic acid	Antioxidant, antihypertensive, antidiabetic, antiobesity, cardioprotective, chemopreventive, antiaging
Zingiber officinale (Zingiberaceae)	Gingerol, shagol	Antiobesity, hypocholesterolemic, antihypertensive, antithrombotic, antiviral activity, antiinflammatory antibacterial, antioxidant, gastroprotective

made to highlight some of the major aspects for drug development process from natural products. Quality assessment including authentication, phytochemical evaluation, standardization, validation processes, safety, efficacy, and clinical aspects are the major parameters for drug development from MPs. MPs have been the basis of treatment of human diseases dates back practically to the existence of human civilization. It has been the basis of modern system of medicine and will exist as one important source of future medicine and drug therapeutics. Several medicines are derived either in the form MPs. Approximately 40% of all medicines are discovered from the natural products and so it has a crucial role in pharmaceutical research and drug development.

World Wide Web resources

This chapter deals with different enzyme inhibition models useful in therapeutic evaluation of herbal drugs. Several web resources are available in this context including the following, which the readers may consult:

- Tutorial on enzyme inhibition, by Dr. Peter Birch of the University of Paisley: https://web.archive.org/web/20070228044059/http://orion1.paisley.ac.uk/kinetics/Chapter_3/contents_chap3.html
- Symbolism and Terminology in Enzyme Kinetics by Nomenclature Committee of the International Union of Biochemistry (NC-IUB): https://web.archive.org/web/20060620032006/http://www.chem.qmul.ac.uk/iubmb/kinetics/ek4t6.html#p6

- BRENDA, Database of enzymes giving lists of known inhibitors: https://www.brenda-enzymes.org/
- BindingDB, a public database of measured protein-ligand binding affinities: http://www.bindingdb.org/bind/index.jsp
- Models of enzyme inhibition: http://biochem-vivek.tripod.com/id41.html

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Glossary

Drug Natural or synthetic substance that (when taken into a living body) affects its functioning or structure and is used in the diagnosis, mitigation, treatment, or prevention of a disease or relief of discomfort.
Herb Plant with leaves, seeds, or flowers used for flavoring, food, medicine, or perfume.
Enzyme A substance produced by a living organism, which acts as a catalyst to bring about a specific biochemical reaction.
Quality control Processes of maintaining standards in manufactured products by testing a sample of the output against the specification.
Standardization Standardization is the process of developing, promoting, and possibly mandating standards based and compatible processes as per recommended guidelines.
Validation The action of checking or proving the validity or accuracy of any method or process.
Marker analysis The process for analysis of specific phytoconstituents present in the plants based on standard markers.
Phytoconstituents Chemical compounds that occur naturally in plants.
Metabolic disorder Abnormal chemical reactions in the body, which disrupt the normal process of metabolism.
Bioassay Measurement of the concentration or potency of the substance by its effect on living cells or tissues.
High-throughput screening High-throughput screening (HTS) is a drug discovery process for assaying the biological or biochemical activity of a large number of drug-like compounds.
Phytopharmaceuticals Includes purified and standardized fraction with defined minimum four bioactive or phytochemical compounds (qualitatively and quantitatively assessed) of an extract of a medicinal plant or its part, for internal or external use of human beings or animals for diagnosis, treatment, mitigation, or prevention of any disease or disorder but does not include administration by parenteral route.
New chemical entities A drug that contains no active moiety that has been approved by the FDA in any other application submitted under section 505(b) of the Federal Food, Drug, and Cosmetic Act.

Abbreviations

ACE	Angiotensin-converting enzyme
CA	Carbonic anhydrase
CE	Capillary electrophoresis
CVD	Cardiovascular disease
ELSD	Evaporative light scattering detector
GC	Gas chromatography
HDL	High-density lipoprotein
HM	Herbal medicine
HMG-CoA reductase	3-Hydroxy-3-methyl-glutaryl-coenzyme A reductase
HPLC	High-performance liquid chromatography
HPTLC	High-performance thin-layer chromatography
HTS	High-throughput screening
LC	Liquid chromatography
LDL	Low-density lipoprotein
MPs	Medicinal plants
MS	Mass spectrometry
NMR	Nuclear magnetic resonance
QC	Quality control
TC	Total cholesterol
TCM	Traditional Chinese Medicine
TLC	Thin-layer chromatography
UHPLC	Ultrahigh-pressure liquid chromatography
UV	Ultraviolet
WHO	World Health Organization

Long answer questions

1. Why drug discovery from natural resources has gained popularity these days?
2. How newer technologies have been introduced to improve and accelerate the natural product drug discovery?
3. What is the principle and types of extraction of natural products?
4. Discuss the role of TLC bioautography in natural product drug discovery.
5. Why enzyme inhibition is considered as suitable target for drug discovery?

Answers to long answer questions

1. Drug discovery from medicinal plants has reclaimed the attention of the researchers, scientists and pharmaceutical industries globally. Herbal products have been used by folklore traditions for the treatment of several diseases. Herbal medicines have the diverse source of chemical entities such as alkaloids, glycosides, terpenoids, flavonoids, and lignans. Evaluation of its phytochemistry and therapy is necessary to validate their use through various modern techniques to meet the requirements to be used in modern drug. It is quite popular among people due to their practical benefits, traditional beliefs, economical advantage, easy access, and other reasons, which have a regional, religious, and social basis. HM is time honored and are being used by the people for their own healthcare, so these systems are well rooted with profound clinical basis, where scientific

validation is some time the major constrains for their development. In spite of all these setbacks, HM remains with a constant growth in the global market. This discipline has devolved over years by exploiting natural products for numerous diseases for indigenous resources.

2. In the past, most of new drugs have been discovered as secondary metabolites and derived molecules from natural resources. Interests in NPR are being continued due to the failure of alternative drug discovery techniques to produce many lead molecules in several therapeutic areas such as immunosuppression, antiinfectives, metabolic disorders, etc. NPs have been exploited as the big source of finding new lead structures, which might be used as a model for the development of new molecules or drugs in the area of pharmaceuticals. It is obvious from the natural product research that the NPs have been and will be an important source of new drug for pharmaceutical industry. Newer techniques have been introduced to improve and accelerate the natural product drug discovery and development to find the drug targets, lead molecules, and structures elucidation. Drug discovery leading to robust and viable drug candidates is a challenging scientific task. This is the transition from a screening hit to a drug molecule, which requires expertise and experience. There are various important techniques including automated separation techniques, high-throughput screening (HTS), and combinatorial chemistry, which are used for enhancing drug discovery from NPs. These methods can be used for minimizing the inherent limitations of natural products and offers a unique opportunity to re-establish NPs as a major source for drug discovery. The present article attempts to describe the utilization of natural resources as drug candidates, with a focus on the success of these resources through in vitro enzyme inhibition assay.
3. Extraction involves the separation of medicinally active portions (phytoconstituents) of plant tissues from the inactive or inert materials by using selective solvents in standard extraction methods. For aromatic plants, hydrodistillation techniques including water and steam distillation, hydrolytic maceration followed by distillation, expression, and enfleurage (cold fat extraction) may be used. Apart from the traditional solid–liquid extraction methods, such as maceration, infusion, decoction, and boiling under reflux, a wide range of modern techniques have been introduced in the past decades. These include MAE, UAE, and SFE for plants.
4. TLC bioautography is a very useful method to find out active compounds in a crude extract or fractions. The plant sample (extract or fraction) is spotted on a TLC plate and allowed to run with an appropriate solvent system that offers a remarkable resolution of the compounds in the form of different bands. These methods involve the screening of a huge number of phytoconstituents against several targets using an automatic system. Typically, several thousands of compounds with very minute quantity can be tested simultaneously. Bioassay-guided fractionation has been the state-of-the art method for identifying bioactive natural products for many years. This technique involves repetitive preparative-scale fractionation and assessment of biological activity till the isolation of pure compounds with the selected biological activity.
5. Enzymes are considered to be an attractive target for the drug development due to their essential catalytic roles in many physiological processes in our body. The enzyme activity may be altered in various disease conditions. In most of the cases, the enzyme catalysis can be hindered by small-molecular-weight, drug-like molecules, which are known as inhibitors. The in vitro evaluation of enzyme–inhibitor interactions have been found as a key point for primary screening of new drug molecules. The development of enzyme inhibitors as therapeutic agents involves optimization of multiple pharmacologic properties beyond the affinity and selectivity of the molecule for its target enzyme. Many of these pharmacologic properties have their molecular underpinning in biochemical reactions within the human body. This include drug absorption from the gastrointestinal tract via active and passive transport mechanisms, metabolic clearance of drugs from systemic circulation, hepatic and renal drug metabolism, and adverse effects mediated by drug interactions with off-target enzymes, ion channels, and receptors. Enzymes are very essential in human physiology as they are effective drug target for having their catalytic activity. Most of the pharmaceutical companies are nowadays focusing on small-molecular-weight drug molecule, which can bind to the receptor (enzymes) by altering (inhibition/induction) the disease pathophysiology. This can lead to development of novel drug candidates that act through inhibition of specific enzyme targets. In fact most of the drugs marketed by the pharmaceutical companies are of small molecule, act as specific enzyme inhibitors. Some examples of these enzymes and the disease pathogenesis they are involved with are carbonic anhydrase in glaucoma, xanthine oxidase in gout, angiotensin-converting enzyme in hypertension, HMG-CoA reductase in cholesterol lowering, pancreatic lipase in hyperlipidemia, aldose reductase in diabetic retinopathy, dehydrogenase in

inflammation, etc. The inhibitors of these enzymes are in clinical use.

Short answer questions

1. What are the basic several assay procedures to monitor the enzyme–substrate interaction?
2. What is bioguided fractionation of extract and isolation of phytoconstituents?
3. What is high-throughput screening?
4. What are hyphenated chromatographic techniques?
5. What are the enzymes involved in metabolic disorder used as drug targets?

Answers to short answer questions

1. There are several assay procedures like spectroscopic, fluorimetric, manometric, polarimetric method to monitor the enzyme–substrate interaction qualitatively as well as quantitatively.
2. Bioassay-guided fractionation has been the state-of-the art method for identifying bioactive natural products for many years. This technique involves repetitive preparative-scale fractionation and assessment of biological activity till the isolation of pure compounds with the selected biological activity. There are several techniques such as column chromatography, flash chromatography, and other hyphenated chromatographic techniques employed for the fractionations and isolations of the plant secondary metabolites.
3. High-throughput screening (HTS) is a drug discovery process for assaying the biological or biochemical activity of a large number of drug-like compounds.
4. There are several techniques such as column chromatography, flash chromatography, and other hyphenated chromatographic techniques employed for the fractionations and isolations of the plant secondary metabolites. In addition, the analytical techniques includes mass spectrometry (MS), nuclear magnetic resonance (NMR), high-performance liquid chromatography (HPLC), gas chromatography (GC), capillary electrophoresis (CE), HPLC-NMR, HPLC-MS, GC-MS, and CE-MS are utilized for the characterization and identification of the isolated molecules.
5. Pancreatic lipase, HMG-CoA reductase, glucosidase, aldose reductase, and carbonic anhydrase are the enzymes involved in metabolic disorder used as drug targets.

Yes/no type questions

1. Does HTS can test many compounds at a time?
2. Does TLC bioautography involve columns?
3. Is supercritical fluid extraction associated with any gas?
4. Is countercurrent extraction associated with high temperature?
5. Does preparation of *asava* and *arista* involve fermentation?
6. Does percolation involve heat?
7. Is quality assessment and scientific validation of herbal medicine necessary?
8. Is there any Nobel Prize for any natural product?
9. Is HMG-CoA reductase important for metabolism?
10. Is enzyme inhibition important for drug discovery?

Answers to yes/no type questions

1. Yes—Typically, several thousands of compounds with very minute quantity can be tested simultaneously by HTS.
2. No—In TLC, a plant sample (extract or fraction) is spotted on a TLC plate and allowed to run with an appropriate solvent system.
3. Yes—Carbon dioxide (CO_2) is used as an extracting fluid in this technique.
4. No—This method is more efficient, requires less time, and pretense no risk from high temperature.
5. Yes—Medicinal preparations of Ayurveda (like *asava* and *arista*) adopt the technique of fermentation for extracting the active principles.
6. No—Percolation involve cold process involving solvents.
7. Yes—Quality assessment and scientific validation of HMs are very important parameters to be considered in assuring the quality, efficacy, and safety.
8. Yes—Prof. Youyou Tu received the Nobel Prize in 2015 for artemisinin, the antimalarial natural compound from *Artemisia annua*.
9. Yes—It is the rate-limiting enzyme in the cholesterol biosynthetic pathway.
10. Yes—Enzymes are considered to be an attractive target for the drug development due to their essential catalytic roles in many physiological processes in our body.

First Nobel Prize for traditional medicine inspired drug discovery

Prof. Youyou Tu received the Nobel Prize in Physiology or Medicine in 2015 from China Academy of Traditional Chinese Medicine, Beijing, for her discoveries concerning a novel therapy against Malaria. She was born in December 30, 1930, Zhejiang Ningbo, China. Prof. Tu Youyou worked on Chinese medical texts from the Zhou, Qing, and Han Dynasties to find a traditional cure for malaria, ultimately extracting a compound—Artemisinin—that has saved millions of lives. When she isolated the ingredient she believed would work, she volunteered to be the first human subject. She is the first mainland Chinese scientist to have received a Nobel Prize for discovering drugs from traditional medicine.

CHAPTER

32

Safety assessment of food derived from genetically modified crops

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Summary

Genetically modified foods are an essential requirement for the food demand of our ever-increasing population. Various approaches are available for assessing the safety of GM foods including the use of bioinformatics, examining the stability of protein in the gastrointestinal tract, and allergenicity testing using animal models. This chapter explores the safety-related concerns of GM foods.

What you can expect to know

These days, crops are being genetically modified by artificial insertion of the gene to meet the requirement of ever-growing population. These transgenic varieties have improved agronomic characteristics (e.g., insect resistance, herbicide resistance, disease resistance, and drought tolerance) but the safety of all new foods (including GM crops) should be ensured prior to their release onto the market. Questions of the safety concerns including toxicity, allergenicity, and environmental impact of GM crops and foods are frequently posed. Avoidance of allergens is the best preventive approach to deal with allergies as there is no permanent cure. Therefore, it becomes essential to assess the allergenic potential of genetically modified (GM) crops before their commercial release. The basic safety assessment concern includes the question of whether a newly expressed protein's allergic potential is lesser than, equal to, or higher than that of its native crop. A step-by-step approach has been used to ensure the safety of GM foods. This chapter includes the basic information regarding the protease and thermal-resistant properties shown by allergens and mechanistic aspects involved therein. Simulated gastric fluid, simulated intestinal fluid, and thermal treatment assays are described in detail to enhance the knowledge about in vitro methods of allergenicity assessment.

History and methods

Introduction

Genetically modified (GM) crops are needed to meet the requirement of ever-growing world population. Billions of people worldwide are unable to meet their daily micro-nutritional requirement, and there is need to increase food production by 70% by the year 2050. With the continued increase in population, the major challenge is how to manage the food for everyone. To some extent, GM foods may fulfill this requirement (Delaney, 2015). By the use of latest molecular biology techniques, desirable traits in plants can be introduced by artificial insertion of gene from unrelated species or sometimes from an entirely different kingdom. Development of GM crops began in the late 1980s with the advancement in the biotechnology technique for directly altering the DNA of the genome and rearrangement of DNA by using methods such as electroporation' or infection with recombinant vectors (e.g., *Agrobacterium tumefaciens)*. These GM crops have new trait(s) introduced in it as compared to native crop (e.g., insects resistance, herbicides and disease resistance, drought tolerance, or improved nutritional content). Conventional plant breeding methods were time-consuming and imprecise. However, the desired trait can be inserted into a plant with higher accuracy using genetic engineering methods (e.g., insertion of Bt

Animal Biotechnology.
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gene in corn, which offers insect resistance). This gene, isolated from a bacterium, named *Bacillus thuringiensis*, produces a protein that can kill the insect larvae. The first GM crops, tomato and soybean, were evaluated for risk assessment and approved by the United States Food and Drug Administration (USFDA). It should be ensured that GM foods are safe before their release into the market. There is a chance that an inserted gene may result in the translation of protein that has the ability to provoke an allergenic response that can sensitize the consumers. Furthermore, it is also possible that inserted gene may elicit allergenic potential by cross-reactivity in an already sensitized population. To save sensitive consumers from unwanted exposure to allergens, appropriate preventive measures should be taken. Humans are exposed to a variety of allergens present in the environment and food. Pollens, fungi, insects, and a variety of food products of animal or plant origin may be harmful to the exposed sensitized group. It is often believed that GM crop/foods may cause additional problems if effective measures are not taken. Also, people should be aware of the ill effects of allergens as no effective medical treatments are currently available for treating this health concern. Therefore, it is essential to assess the allergenic potential of GM crops and food prior to commercialization.

The 20th anniversary (1996–2015) of the commercialization of GM crops concluded recently. Accumulated hectare utilized for irrigation of the GM crops in this time duration exceeded two billion hectares, which is equivalent to twice the total land mass of China or the United States, clearly signifying that biotech crops are putting their roots strongly. The two billion accumulated hectares comprise 1.0 billion hectares of biotech soybean, 0.6 billion hectares of biotech maize, 0.3 billion hectares of biotech cotton, and 0.1 billion hectares of biotech canola. Up to ~18 million farmers benefit from biotech crops in the past 20-year period (1996–2015) and ~90% were small resource-poor farmers. In 2011, commercially cultivated GM crops were Alfalfa (*Medicago sativa*), Argentine Canola (*Brassica napus*), Bean (*Phaseolus vulgaris*), Carnation (*Dianthus caryophyllus*), Chicory (*Cichorium intybus*), Cotton (*Gossypium hirsutum* L.), Creeping Bentgrass (*Agrostis stolonifera*), Flax or Linseed (*Linumusitatissumum* L.), Maize (*Zea mays* L.), Melon (*Cucumismelo*), Papaya (*Carica papaya*), Petunia (*Petunia*), Plum (*Prunus domestica*), Polish canola (*Brassica rapa*), Poplar (*Populus nigra*), Potato (*Solanum tuberosum* L.), Rice (*Oryza sativa* L.), Rose (*Rosa hybrida*), Soybean (*Glycine max* L.), Squash (Cucurbita *pepo*), Sugar beet (*Beta vulgaris*), Sweet pepper (*Capsicum annuum*), Tobacco (*Nicotiana tabacum* L.), Tomato (*Lycopersicon esculentum*), and Wheat (*Triticum aestivum*). About 45 countries including Argentina, Australia, Bolivia, Brazil, Burkina Faso, Canada, Chile, China, Colombia, Costa Rica, Czech Republic, Egypt, Arab Republic, El Salvador, Germany, Honduras, India, Iran, Islamic Republic, Japan, Korea, Malaysia, Mexico, Myanmar, Netherlands, New Zealand, Pakistan, Paraguay, Philippines, Poland, Portugal, Romania, Russian Federation, Singapore, Slovak Republic, South Africa, Spain, Sweden, Switzerland, Taiwan, Thailand, Turkey, the United Kingdom, the United States of America, and Uruguay are taking their step ahead for the development of various GM crops. In a landmark development, India ranked first in cotton production in the world with 11.6 million hectares planted by 7.7 million small farmers. (source: http://www.isaaa.org/gmapprovaldatabase/default.asp). Herbicide and drought tolerance, insect resistance, improved nutritional characteristics of foods or feeds and altered fatty acid profiles are major choice of developers in the most GM crops, nowadays.

The first international and national provisions for the safety assessment and regulation of GM crop-derived foods were started by Organization for Economic Co-operation and Development (OECD, 1986) and the first regulatory approval of a GM crop had come after approximately one decade (in 1995). In 1996, the International Food Biotechnology Council (IFBC) and the International Life Sciences Institute (ILSI) jointly developed a decision-tree approach (Metcalfe et al., 1996) that is widely accepted by regulatory authorities all over the world. In GM foods, the inserted proteins are needed to be assessed before their insertion and it is very important to ensure that the products of novel genes introduced into GM crop are not harmful. It is also important to ensure that the process of transformation does not cause any unintended change in the characteristics and levels of expression of endogenous allergenic proteins. The safety assessment focuses on the new gene products and whole foods derived from the GM crop. Both intended and potential unintended effects of the genetic modification should be taken into account. The assessment of GM crops involves the steps like characterization of the parent crop, characterization of the donor organisms from which any recombinant DNA sequences are derived, the transformation process and the introduced recombinant DNA sequences, safety assessment of the introduced gene products (proteins as well as metabolites), and food safety assessment of whole food derived from edible part. Earlier, GM brinjal in India was suspended (for an indefinite time period) prior to its intended release due to safety-related issues (Kumar et al., 2011a,b). But recently, issue related to GM crops is gaining attention in India (Padmanaban, 2014; Warrier and Pande, 2016).

During the safety assessment of GM foods, allergenicity is one of the most important issues. Food allergy

is an increasing global health concern. It is an immune provocation in susceptible individuals, triggered by certain food proteins, including proteins derived from GM foods. Sensitization develops when a susceptible individual is exposed to an amount of protein sufficient to induce an immune response. Subsequent exposure to the same or similar allergenic protein in sensitized individuals can provoke an adverse reaction. Allergic reactions may be mild and local, but sometimes can be severe, systemic, and fatal. Severe allergic reactions with a rapid onset of symptoms are known as anaphylaxis reactions. The susceptibility of any individual is dependent on several factors, including genetic predisposition and environmental factors.

In food, every protein may not be responsible for provoking immunological reactions but certain proteins that can induce allergic complications are known as allergens. Every allergen is an antigen, but not every antigen is an allergen. In an allergenic protein, certain regions have immune reactive capacity; these regions are known as epitopes. It has been reported that certain biochemical characteristics are shared by many (but not necessarily all) food allergens; one such characteristic is the relative stability and resistant to the denaturation of proteins. Pepsin resistance is thought to be an important property of allergens because when any portion of the protein that remains intact, the chance of an immune response is higher. In the United States, each year about 30,000 people come to hospital emergency departments due to food-induced anaphylaxis, and nearly 200 people die (Sampson, 2003). The recent study in US children population demonstrates the increasing trend in the incidence of food-induced anaphylaxis. Peanuts followed by tree nuts/seeds are implicated as the major contributing factor in this increase (Motosue et al., 2018). In the United Kingdom, millions of people suffer from food-induced allergic reactions. Food and Agriculture Organization (FAO) of the United Nations and World Health Organization (WHO) expert consultation committee on allergenicity of foods derived from biotechnology documented an approach for assessing the allergenic potential of novel proteins in transgenic crops (Codex Alimentarius, 2003).

Rationale for the allergenicity assessment of genetically modified Foods

The rationale behind the allergenicity assessment of GM foods is to compare the allergenicity of GM food with equivalent non-GM food variety. It focuses on whether a newly expressed protein has allergic potential or not. To ensure the safety of inserted protein, the GM crop if found having increased allergic potential compared to its non-GM counterpart is not approved by regulatory agencies. GM food with allergenic potential may cause life-threatening allergic reactions in the already sensitized group as there may be sequence homology between known allergens and novel inserted protein. Various methods have been used in a step-by-step manner to ensure the safety of GM foods. Among these, a few important points to ponder are the following:

- The source of the introduced protein: it should be preferably from a nonallergenic source.
- A measure of the abundance of the novel protein in the food.
- Comparison of amino acid sequences of newly expressed protein with known allergens present in allergen database like SDAP and Allergen Online.
- Stability of protein in the gastrointestinal (GI) tract.
- Thermal stability of the novel proteins.
- Various animal models like mouse, rat, guinea pig, dog and pig have been developed and are being used for allergenicity assessment procedure of GM foods.
- Serum IgE-binding test to evaluate the presence of specific IgE antibody against the protein of interest in serum.

Safety assessment approaches regarding GM foods differ from country to country, but the basic principle for the allergenicity assessment is based on a common approach that is internationally acceptable. Several international organizations like FAO/WHO, OECD and ILSI have provided guidelines for the safety assessment of GM foods, a prerequisite for their release into the market.

Out of several approaches used for the assessment of allergenicity of the novel protein, stability to digestion in the GI tract is considered a primary requirement. It should be performed for all newly inserted or expressed proteins. Stability in the GI tract is the first step toward the evaluation of the allergenicity of food proteins.

Mechanism of food protein-induced allergenicity

Proteins are an integral part of food, and they provide nutritional support for the body. Food proteins may or may not be allergenic. Allergenic proteins are thought to induce immunological responses commonly via the GI tract, and due to this, digestibility and gut permeability are important factors when assessing the allergenic potential of novel proteins. Protein processing starts in the mouth where it is mixed with saliva that contains enzyme “ptyalin.” No significant enzyme is found in the mouth for protein digestion. The main digestion of protein takes place in the stomach and

small intestine. The environment of both the compartments is different. Food proteins move in the GI tract, where it gets digested with pepsin in the stomach and broken down into smaller peptide fragments. These fragments are then subjected to digestion in the intestine, and finally, amino acids or small peptides get absorbed.

It is believed that intact proteins or peptides of higher molecular weight have the ability to sensitize the individual as well as release of allergic mediators. Some portion of food remains undigested, and this may contain allergenic proteins as well. The intestinal absorption of food allergens and the immune responses to them are interrelated; the nature of the allergen can decide the type of immune responses generated. The intestinal epithelium, which is joined together with its neighbors via tight junctions and mucus produced by goblet cells, may act as a barrier, and can limit the permeation of macromolecules, ingested pathogens, parasites, toxins, and anti-nutrients by tight cell junctions; however, proteins have been reported to cross the intestinal barrier in an intact form.

The GI absorption of intact food allergens such as ovalbumin, peanut allergens, and Gly m Bd 30K from soybean using in vivo models have been well documented. Many of the food allergens are proteins having intramolecular disulfide bonds (2S) that make them stable and resistant to pepsin digestion (Kumar et al., 2012). Higher stability of proteins in the hostile GI tract for a period of time could be sufficient to elicit an immune provocation. The stable proteins or peptide fragments are internalized, processed, and presented by antigen-presenting cells (APC) like macrophages, dendritic cells (DCs), and B cells present in the outer layer of intestine (i.e., lamina propria). In the intestine, the DCs are commonly found antigen-presenting cells. DCs in intestine are generally found in the semimature forms, and once these cells come in contact with food allergens, they get activated, become mature, and finally move to the Peyer's patches. In the Peyer's patches, $CD4^+$ Th2 cells recognize these allergens. These Th2 cells secrete cytokines like IL-4 and IL-13, which along with CD40 legend, help in class switching to immunoglobulin E (IgE). The newly formed IgE antibodies move to mast cells and bind Fc epsilon receptor 1 (FcεR1). This is known as priming of IgE on mast cells or basophil cells (Kumar et al., 2012). After subsequent secondary exposure to same (or even similar) allergens, the allergens get linked between two IgE molecules (IgE−allergen−IgE). This phenomenon is also known as cross-linking of allergen. The cross-linking of these allergens initiates a cascade of reactions involving kinases like Lyn and Syk that ultimately ends in degranulation of mast cells. The degranulation results in the secretion of allergic mediators like histamine, mast cell proteases, prostaglandins, leukotrienes, serotonin, β-hexosaminidase, and cytokines. These mediators cause several disorders in the body like breathlessness, sneezing, and dilation of blood capillaries. If the reaction is very severe, then it can be life-threatening and the situation is known as anaphylaxis. A brief outline of pepsin-resistant proteins that induced food allergy is given in Table 32.1. Recently, Yu et al. have reviewed the role of Toll-like receptor (TLR) in the food allergy (Yu et al., 2016). For example, TLR7 agonist ameliorates the peanut-allergic TH2 cell-mediated response. In humans, allergic rhinitis is effectively managed by TLR9 agonist (Creticos et al., 2006) and a modified allergoid preparation including a TLR4 agonist is effective in treating ragweed pollen allergy (Patel et al., 2014). In summary, the outcomes of these studies indicate the involvement of TLR in food allergy manifestations.

Simulated gastric fluid assay

During the allergic assessment of GM foods, the resistance of proteins to proteolytic digestion has been detailed in current safety assessment guidelines from multiple studies. It is reported that proteins with resistance to pepsin digestion in SGF can induce allergenic reactions. The assumption behind the SGF assay had simple reasoning that nutritionally desirable proteins should be rapidly digested, and therefore have less opportunity to exert adverse health effects when consumed. This assumption appears to have been confirmed, in several cases, like peanut, egg, milk, soybeans, red gram, green gram, red kidney beans, and chickpeas. Therefore, the stability of a transgenic protein to pepsin digestion under acidic conditions (pH 1.2−2.0) is generally assumed a simple but effective test for assessing the allergenic risk for transgenic proteins (Astwood et al., 1996). Along with other positive evidence, there are reports indicating no link between the stability of a protein in SGF and its allergenicity (Fu et al., 2002).

Therefore, it can be inferred that the correlation between allergenicity and digestive stability is not absolute in each and every case. Since resistance to degradation by acid proteases is used to make regulatory decisions, further studies are needed to explore this correlation. Taken together, SGF assays may not equally mimic mammalian in vivo digestion conditions, but the stability of a transgenic protein or a digestion fragment in SGF may be related to its allergic potential, as pepsin resistance can provide sufficient time to interact with the GI tract's immune component and induction of allergic reactions.

TABLE 32.1 Stability of food allergens in SGF and SIF.

S. N.	Protein group	Protein source	SGF stability (min)	SIF stability (min)
Allergenic proteins				
1.	β-Lactoglobulin	Cow's milk	120	5
2.	BSA	Cow's milk	0	120
3.	α-Lactalbumin	Cow's milk	0	15
4.	Lactoperoxidase	Cow's milk	0	120
5.	Ovalbumin egg	Egg	5	5
6.	Ovomucoid	Egg	0	60
7.	Conalbumin	Egg	0	120
8.	Lysozyme	Egg	60	120
9.	*Ara h* 1	Peanut	5	15
10.	*Ara h* 2	Peanut	0.5	0.5
11.	Peanut lectin	Peanut	5	120
12.	Soybean lectin	Soybean	5	120
13.	Trypsin inhibitor	Soybean	120	120
14.	Patatin	Potato tuber	0	0.5
15.	Papain	Papaya	0	120
16.	Bromelain	Pineapple	0	120
Nonallergenic proteins				
17.	α-Lactalbumin	Human milk	0	60
18.	Zein	Corn	120	0.5
19.	Trypsin inhibitor	Bovine pancreas	120	120
20.	Red kidney bean lectin	Red kidney bean	15	120
21.	Pea lectin	Pea	5	120
22.	Lentil lectin	Lentil	0.5	120
23.	Lima bean lectin	Lima bean	5	120
24.	Jack bean lectin	Jack bean	15	120
25.	Cytochrome c	Bovine heart	0	60
26.	Rubisco	Spinach leaf	0	120
27.	Phosphofructokinase	Potato tuber	0	5
28.	Sucrose synthetase	Wheat kernel	0	0.5

Stability was measured as the last time period (in minutes) that the protein could be seen in the SDS–PAGE gel.
Source: Fu et al., 2002. J. Agric. Food. Chem. 50, 7154–7160.

How simulated gastric fluid assay works

The adult human GI tract is a long tube with a size of approximately nine meters running through the body from the oral to the anal aperture. The comparative digestibility of allergenic and nonallergenic proteins is highly significant when stability to digestion is used as the basis to predict the allergenic potential of novel proteins (Thomas et al., 2004). Pepsin is an aspartic protease generated from the autocleavage of pepsinogen under acidic conditions in the stomach. Protein degradation by pepsin or pepsinolysis is generally a very rapid reaction but this is affected by the secondary or tertiary structure of protein substrate. The most effective pH for optimum activity for pepsinolysis ranges between 1.8 and 3.2, but pepsin is

generally irreversibly denatured at pH 6–7. Due to the fact that a pH greater than 6 can irreversibly denature pepsin activity, the SGF reaction can be stopped by neutralizing aliquots of the solution at different incubation periods using bases like $NaHCO_3$ and NaOH. These incubation mixtures can be analyzed to track the digestion of substrate proteins using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The food allergen first encountered by proteolytic enzymes in the stomach and then the fragmented peptides move into the intestine from the stomach (or gets digested completely). The basic outline of the enzymatic action of pepsin on allergen is given in Fig. 32.1. Pepsin has broad substrate specificity and preferentially cleaves proteins at amino acid leucine, phenylalanine, and tyrosine from C-terminus (Schnell and Herman, 2009). A pictorial demonstration of pepsin action during SGF assay is given in Fig. 32.2.

Components of simulated gastric fluid

The SGF is a set of reagents held under specific conditions mainly comprised of 0.32% pepsin, pH 1.2, and 37°C. These conditions are designed to stimulate human gastric conditions present in the stomach (Astwood et al., 1996). Differences in pepsin concentration, pH, protein–substrate concentration, detection procedures (resolution on SDS–PAGE gel, loading quantity, and protein staining methods) are considered as major regulatory factors in the SGF assay. WHO/FAO recommend the protocol described by Astwood et al. (1996). Another multilaboratory protocol for SGF assay has been reported by Thomas et al. (2004). The laboratories involved were the ILSI; Health and Environmental Sciences Institute, Washington, DC, United States; Sanquin Research, Amsterdam, Netherlands, Monsanto Co., St. Louis, Missouri, United States; The Dow Chemical Co., Midland, Michigan, United States; Syngenta Central Toxicology Laboratory, Alderley Park, United Kingdom; Bayer CropScience, Sophia Antipolis, France; US Food and Drug Administration National Center for Food Safety and Technology, Summit Argo, Illinois, United States; DuPont Co., Newark, Delaware, United States; University of Nebraska, Lincoln, Nebraska, United States; Bayer Crop Science, Research Triangle Park, North Carolina, United States; Syngenta Biotechnology Inc., Research Triangle Park, North Carolina, United States; National Institute of Health Sciences, Tokyo, Japan. This study explored the fact that when SGF assay was performed by different researchers with aliquots of the same reagents under similar test conditions, a panel of scientists could identify a similar time point for protein bands to become undetectable on SDS–PAGE gels.

A fully validated SGF assay should be reproducible, robust, and relevant and must be largely insensitive to factors that are likely to vary among laboratories. Kinetic data analysis can be used as an interpretation tool of the SGF assay. Herman et al. (2006) incorporated kinetic concepts into SGF studies rather than using a single time point when a protein band was no longer visible on an SDS–PAGE gel or Western blot. Protein bands on SDS–PAGE gels were quantified by densitometry over a digestion time point, and the pattern of protein degradation was modeled using a negative exponential equation (pseudo-first-order decline) (Herman et al., 2006). Thus, a kinetic approach for the analysis of SGF results uses multiple data points and relative protein decline to overcome some of the shortcomings associated with observing the first time point at which a protein is no longer visible. Researchers have focused on the most persistent protein fragment

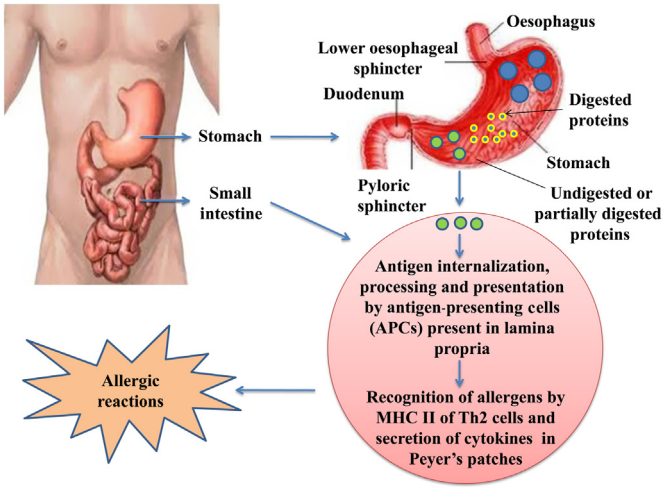


FIGURE 32.1 The basic outline of enzymatic action of pepsin on an allergen.

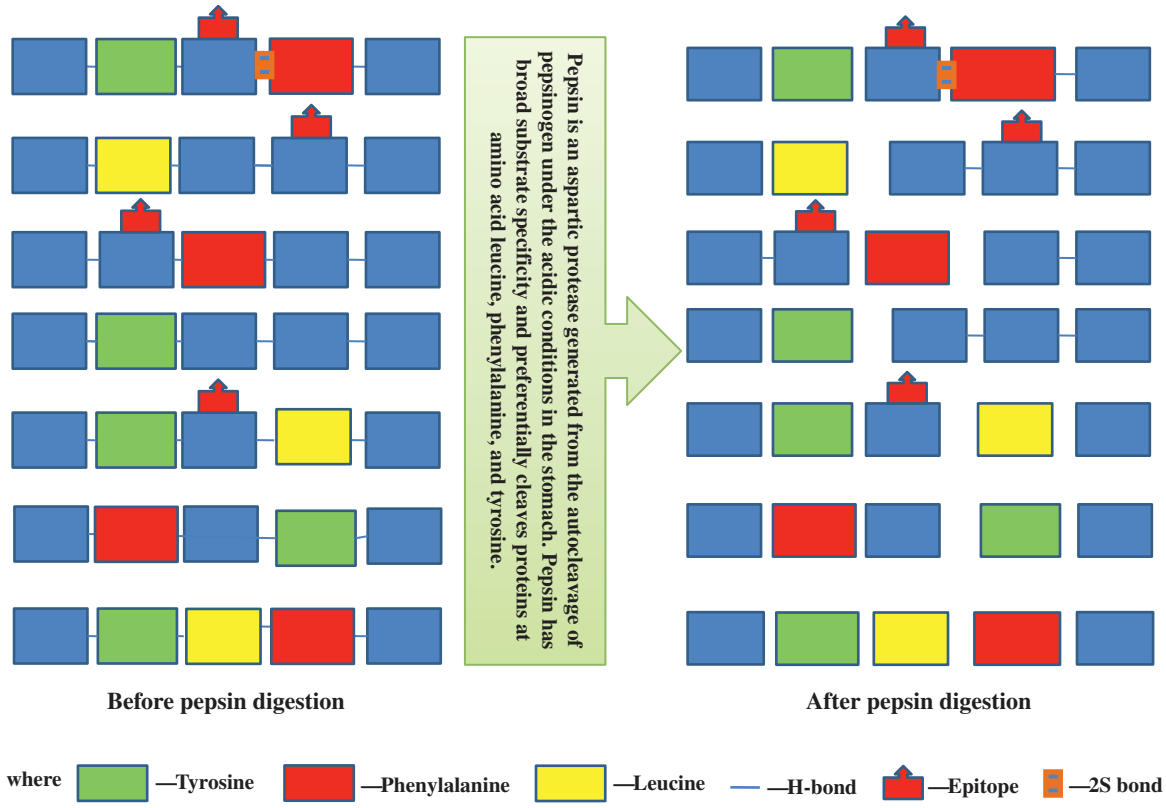


FIGURE 32.2 Mechanism of the action of pepsin on polypeptides.

when assessing allergenic risk. Variation between pepsin lots, pepsin concentration, and substrate concentration did not substantially affect estimated degradation, although low purity pepsin lots had moderately lower catalytic power.

General protocol of the simulated gastric fluid assay

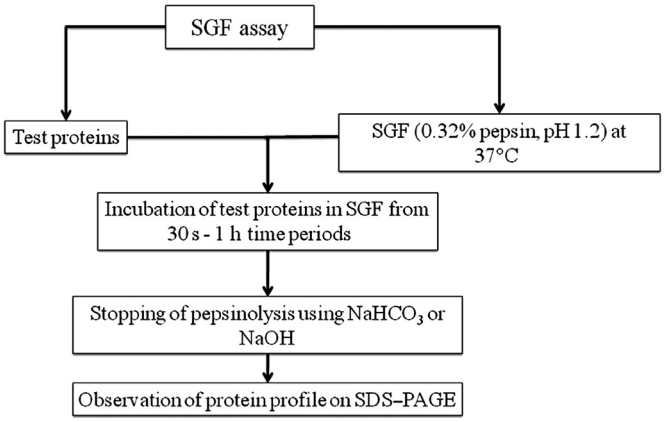
The first detailed study regarding the SGF assay was carried out in the late nineties by Astwood and his group. Since then many modifications have been suggested to enhance its utility, but in each and every case, the basics of the assay are more or less similar. The pepsin activity recommended by several groups ranged between 5000 and 20,000 units/mg of test protein. The pepsin concentration (3.2 mg/mL), the ratio of the pepsin to protein (3:1), and pH (1.2 and 2.0) along with different time point incubation (at 37°C) were almost similar everywhere (Flow Chart 32.1). The nature of substrate sometimes influences the optimum activity of pepsin, with a relatively broad pH range between 1.2 and 3.5. Nevertheless, in a study performed on codfish allergens, all proteins were degraded to small fragments within 1 minute and lost their IgE-binding capacity when the digestion was performed at physiologic conditions (pH, 2.5); however, a small pH shift from 2.5 to 2.75 hampered the pepsin digestion of several allergens including the major fish allergen parvalbumin Gad c 1. It was also indicated that food allergens are not necessarily more stable in SGF as compared to nonallergenic proteins. In the majority of cases, it was found true. Many proteins with unproven allergenicity exhibit high stability (Table 32.2).

Factors relevant to gastrointestinal digestion of allergens

The SGF was designed to mimic the mammalian GI system. However, several other factors can play a role like the buffering effect of food ingredients, mechanical breakdown of food tissue, range of stomach pH, additions of surfactants (phospholipids), and gastric lipase in physiological amounts, peristalsis, as well as possible emulsification of lipids and gastric emptying, etc.

Supportive and negative evidence of simulated gastric fluid

Several independent researchers have reported supporting data regarding the utility of SGF assay.



FLOW CHART 32.1 SGF assay protocol.

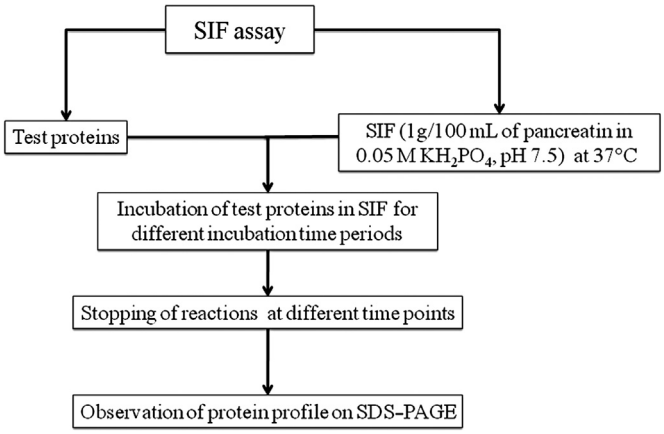
TABLE 32.2 Effect of changing the ratio of pepsin to test protein on the stability observed in a SGF assay.

S. N.	Pepsin/test protein ratio (w/w)	Stability of test protein in SGF (min)		
		10:1	1:1	1:10
Food allergens				
1.	β-Lactoglobulin B	120	120	120
2.	Ovalbumin	5	60	120
3.	Papain	0	0	0
Nonallergenic proteins				
	Zein	60	60	120
	Pea lectin	5	120	120
	Cytochrome c	0	0.5	0.5
	Sucrose synthetase	0	0	0

Stability was measured as the last time period (in minutes) that the protein could be seen in the SDS–PAGE gel.
Source: Fu et al., 2002. J. Agric. Food. Chem., 50, 7154–7160.

Although SGF assays is not exactly similar to in vivo digestion, its results are very close to the result of in vivo digestion of the mammalian system (Polovic et al., 2010). More interestingly, it has also been reported that the proteins stable to pepsin digestion have also shown Western blotting on at least one-dimensional SDS–PAGE (Misra et al., 2011). Earlier studies have also supported the fact that in the majority of cases, pepsin-resistant proteins show the IgE-binding capacity (Kumar et al., 2011a,b; Verma et al., 2012a,b). Recently, a pepsin-resistant 20-kDa protein with IgE-binding property has been observed in both GM (expressing salt tolerance *cbl* gene) and native tobacco (Verma et al., 2014). Though there are several supporting papers on SGF assay, a number of subsequent reports have indicated an elusive link between protein stability in SGF and their allergenicity.

Prediction of allergenicity using SGF assay has been supported in a number of reports and is a general requirement as a part of the allergenicity assessment of transgenic proteins expressed in food; however, the predictive power of this assay has also raised several questions. There are many reasons behind the elusive predictive capability of the SGF assay, including lack of consideration of the prevalence of the allergen in food, effects of food processing, and food–matrix interactions. The food–matrix interactions may play an important role because components of food may sequester certain proteins away from the acid and pepsin in gastric fluid. The purified kiwi allergen, Act c 2, was digested quickly in SGF, but was protected from digestion by fruit pectin both in vitro and in vivo. Similarly, in transgenic corn expressing the *Escherichia coli* heat-labile enterotoxin facilitated the association of



FLOW CHART 32.2 SIF assay protocol.

this protein with starch granules that protected it against digestion in SGF. Further, it was noticed that the digestibility of rice allergenic proteins is influenced by pH level, heat processing, starch matrix, solubility, and protein properties, on a case-by-case basis (Lang et al., 2015). Thus, evaluating the allergenicity of purified proteins in the SGF assay may not always lead to desired outcomes.

Although the value of comparing the stability of proteins in SGF for the purpose of evaluating the allergenic potential of novel food proteins is dubious, such comparisons are routinely used for this purpose. The resistance to in vivo digestion of a food protein increases its potential for causing an allergic reaction in susceptible individuals. Some peptide fragments of digested proteins can be recognizable by allergen-specific T cells. However, the amount of food protein and the condition that can trigger the allergic reaction are largely unknown.

General protocol of simulated intestinal fluid assay

There is a general belief that allergenic proteins remain unaffected in the proteolytic environment of the human GI system and can be absorbed through the intestinal mucosa (Taylor et al., 1987). If protein fragments are visible in SGF assay, then it is better to perform additional enzymatic testing known as the SIF assay. Sequential enzymatic digestion (SGF followed by SIF assay) can be useful to determine whether a protein is likely to survive in the human GI tract long enough to interact with the immune system. Pancreatin is used in preparation of SIF for the aforementioned assay.

Simulated intestinal fluid is prepared according to standard protocol as described in the United States Pharmacopoeia (1995). Briefly, 1 g/100 mL of pancreatin is dissolved in 0.05 M KH_2PO_4, pH 7.5. Aliquots of SIF are placed in microcentrifuge tubes and incubated at 37°C for 10 minutes in a water bath. The test protein at a concentration of 5 mg/mL (in 0.05 M KH_2PO4, pH of 7.5) should be added to each of the microcentrifuge tubes to start the reaction. At different time intervals such as 0, 0.5, 5, 15, 60, and 120 minutes, Laemmli buffer should be added to each tube. Samples are boiled for 10 minutes in a water bath. The SDS–PAGE analysis, in combination with densitometry, is performed for comparing the degradation at different time points (Flow Chart 32.2).

Effect of assay conditions on protein stability in simulated intestinal fluid assay

Assay conditions affect the relative digestibility of protein in SIF. The relative amount of enzyme and test protein used in SIF assay affects the results in a particular protein. Changes in the ratio of enzymes to protein or change in pH affect the digestibility of protein (Table 32.3). The ratio of enzyme-to-test protein (by weight) varies greatly in studies from a 1:250 ratio of trypsin-to-soybean β-conglycinin (Kamata et al., 1982), a 5,000:1 ratio of pancreatin-to-neomycin phosphotransferase II (Fuchs et al., 1993), a 1:10 or 1:100 ratio of trypsin-to-phaseolin (Nielsen et al., 1988), and a 25:1 ratio of chymotrypsin/trypsin-to-insecticidal crystal protein Cry1 Ab expressed in transgenic tomatoes (Noteborn et al., 1995). SIF can have a significant impact on the evaluation of the allergenic potential of a food protein derived from GM crops if the ratio of enzyme-to-test protein varies.

Supportive and negative evidence of simulated intestinal fluid assay

The stability of food proteins under the *in vitro* digestive condition is widely used as a criterion for the

TABLE 32.3 Enzyme/test protein ratios used for SIF assay.

S. N.	Protein	source	Enzyme used	Enzyme/test protein ratio	References
1.	Albumin	Beans	Trypsin	1:40	Marquez and Lajolo
2.	Phaseolin	Dry beans	Trypsin	1:10 or 1:100	Nielsen et al.
3.	β Conglycinin	Soybean	Trypsin	1:250	Kamata et al.
4.	α Lactoglobulin	milk	Trypsin	1:100	Maynard et al.
5.	Neomycin phosphotransferase II (NPTII)	Recombinant *E. coli*	Pancretin	5000:1	Fuchs et al.
6.	CRYIA(b) NPTII	Recombinant *E. coli*	Chymotrypsin/trypsin	25:1	Noteborn et al.

Source: -Fu et al., 2002. Ann. N.Y. Acad. Sci., 964, 99–110.

prediction of protein allergenicity by the agricultural biotechnology industry. Several guidelines have considered SGF or SIF digestibility as a predictive tool for the estimation of allergenic potential of proteins. However, there is a need to establish standardized assay conditions that are globally accepted. A comparative study of digestibility of food allergens and nonallergenic proteins in SGF and SIF was performed by Fu et al. (2002) to provide experimental evidence to the hypothesis. Nineteen allergenic and nonallergenic food proteins were subjected to digestion in SGF and in SIF for 2 hours and 45 minutes, respectively. However, results do not indicate that food allergens are more stable to digestion in vitro than nonallergenic proteins. Such studies raise doubts regarding the digestibility assays. Despite some criticism, digestibility assay is commonly used as a criterion for allergenicity assessment of GM food.

Some major allergens like conalbumin are stable in SIF up to 120 minutes. Plant lectins (soybean and peanut) also have stability in SIF for 120 minutes. Bromelain and papain, well-known allergens of papain superfamily, showed higher stability in SIF. Ovomucoid and lysozyme, other known allergens from egg, show higher stability in SIF, up to 60 and 120 minutes, respectively. Cow's milk allergen and lactoperoxidase is also stable in SIF for 120 minutes. There are several examples of allergens that have higher stability in SIF, as given in Table 32.1 (Fu et al., 2002). In addition, the safety assessment of Cry1C protein from GM rice showed the rapid degradation of inserted protein with SGF or SIF. The Cry1C protein did not cause any adverse effects in mice indicating the less chances to provoke allergenicity by easily digested Cry1C protein (Cao et al., 2010). Therefore, in vitro digestion assay can be used to estimate whether a novel protein has allergenicity or not. Sometimes, small fragments of degraded protein in the GI tract are sufficient to provoke allergenic responses.

Contradictory result of simulated intestinal fluid digestibility of food proteins

The stability of food protein in SIF varied, ranging from 0 to 120 minutes in the case of known food allergens and also in the case of the proteins with unproven allergenicity. Few known protein allergens rapidly degrade in SIF. The β-lactoglobulin B is stable in SGF for 120 minutes, but gets digested within 5 minutes in SIF. The Ara h 2, major allergen of peanut, is degraded within 0.5 minutes in SGF and SIF. Further, a study has shown that both rice allergenic proteins and nonallergenic proteins were rapidly digested in SIF (Lang et al., 2015).

Allergens like ovalbumin, conalbumin, and papain are stable in SIF, although instant degradation of these proteins occurs in SGF. A few nonallergens like RUBISCO and cytochrome *c* are also resistant to digestion in SIF for 120 minutes. Some allergens like α-casein, shrimp tropomyosin, Ara h 2, and patatin are labile to digestion in SIF, whereas plant lectin from red kidney bean, pea, lentil (with unproven allergenicity) show stability for up to 120 minutes in SIF.

Digestibility of peanut and hazelnut allergens was investigated by SGF and SIF methods (Vieths et al., 1999). They studied the digestion of both allergens in SGF for 2 hours and subsequent digestion in SIF for 45 minutes. Hazelnut allergens get rapidly degraded, while peanut protein allergens were found to be relatively stable. It is not necessary that food allergens with higher allergenicity are more stable in SIF as compared to allergens with lower allergenicity. For example, α-casein and Ara h2 are rapidly degraded in SIF, while minor allergens like plant lectin are stable in SIF up to 120 minutes. These studies indicate that there is no direct correlation between stability in SIF and allergenicity of novel protein, indicating that digestibility of protein in SIF does not provide satisfactory enough result to be used as a

promising method for allergenicity assessment of proteins.

In some cases, a direct correlation exists between the digestibility of a protein under in vitro conditions and its allergenicity. It is a widely accepted method for allergenicity assessment, but is not able to eliminate false results completely. It is sometimes difficult to say whether a given protein has allergenic potential or not. These methods fail to identify allergens that are labile to digestion in SGF and SIF. Moreover, allergens that provoke allergenicity through other routes like respiratory route or by skin contact cannot be identified. Despite these limitations, stability to digestion is still a relevant method for the assessment of the allergenic potential of transgenic proteins inserted into foods.

Thermal treatment assay

Heat-resistant proteins are not digested in the GI tract, so there are chances that these thermal-resistant proteins can elicit an allergenic response in an individual. Resistance to thermal treatment has been observed in the case of several food allergens, so it was logical to think that there is a direct correlation between heat stability and allergenicity. If newly expressed protein shows heat-resistant property under higher temperature, further experimental analysis is required to determine allergenicity of that protein. Therefore, heat-resistant property can be utilized as an indicator as to whether a particular protein has immune-provoking potential or not. As in the case of GM food, a newly expressed proteins or inserted protein can be checked to view its allergenicity by thermal treatment assay in combination with other known methods. The three-dimensional structure of majority of proteins correlates with its functional activity. As the thermal treatment can bring about substantial changes in the structure of protein, it can also modify the protein's allergenic nature. A better understanding of thermal processing-induced biochemical and immunological changes in food allergens of a crop/food may help in understanding whether thermal processing can be useful in reducing the allergenicity of the protein. During thermal processing, neoantigens sometimes form; this provides encouragement to develop new diagnostic tools.

Mechanism of thermal treatment assay

Thermal exposure can occur in many ways during food preparation, including baking, cooking, roasting, grilling, drying, pasteurization, and sterilization. Many factors, such as digestibility (resistance to pepsin in the GI tract), solubility, and the ability to be absorbed intact across the intestinal tract, are mainly responsible for the allergenicity of proteins. In general, heat treatment normally changes the structure of protein, so it may increase or decrease the antigenicity of protein. Prior heat treatment probably increases the digestibility of protein, so the absorption of resulting polypeptides in GI tract also increases; hence, the possibility of protein eliciting an allergenic response decreases. A recent study has noticed that heating of β-lactoglobulin at 120°C significantly improved both peptic and pancreatic digestion attributed to structural alterations that resulted in reduced antigenicity β-lactoglobulin (Rahaman et al., 2017). However, in some cases, thermal processing may reduce the digestibility of a particular allergen, or some neoantigens may be formed that were not present originally in protein (Paschke, 2009). This phenomenon may enhance the allergenic problem in sensitive patients, and these newly formed antigens or neoantigens may have ability to sensitize a new consumer group. This fact is supported by the recent study where heat processing of peanut seed enhanced the sensitization capacity of Ara h 6, a major peanut allergen (Guillon et al., 2016). Thus, the major aim of thermal processing is to examine the effect of heat treatment on the structural and immunological properties of food allergens and identification of appropriate treatment methods for the reduction/elimination of allergenic residues in food.

One major factor for the formation of neoantigens may be the Maillard reaction (e.g., the interaction of the protein component with sugar residues upon heating that generates sugar-conjugated protein derivative that causes enhancement in the allergenicity of the protein). A chemical reaction between the carbonyl group of the sugar reacts with the nucleophilic amino group of the amino acid, usually requiring heat. This is known as the Maillard reaction, and it leads to brown coloration during heating or storage. Several factors, like high temperature, intermediate moisture levels, and alkaline conditions, promote the Maillard reaction. The type of sugar involved also has an effect on the Maillard reaction. Pentose-reducing sugars are more reactive than hexoses, which are more reactive than disaccharides. Beyer et al. (2001) reported that in cooked peanuts, IgE-binding has increased due to the Maillard reaction. A brief outline of Maillard reaction is given in Fig. 32.3.

The antibodies recognize and interact with IgE-binding epitopes presented on allergenic proteins. These IgE-binding epitopes are of two types: linear epitopes and conformational. In linear epitopes, amino acids are arranged in linear order in the polypeptide chain; in conformation epitopes, amino acid that are far apart in primary sequence are brought together

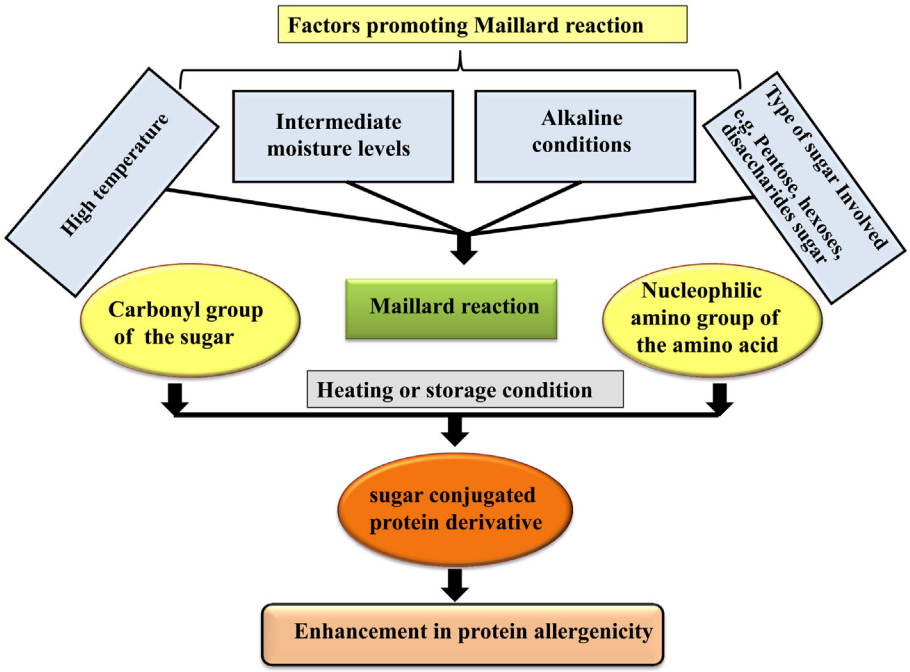


FIGURE 32.3 Maillard reaction and its relation with protein allergenicity.

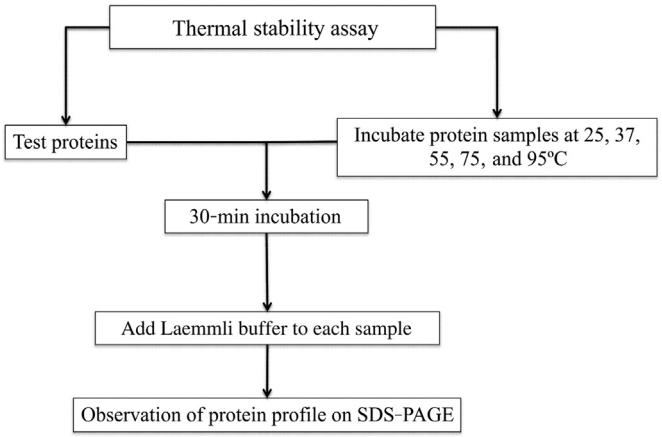
during polypeptide chain folding. Linear epitopes can be more allergenic as compared to conformational epitopes, as these are mostly resistant to heat treatment and have the ability to sensitize and provoke allergenic response. Thermal processing mostly affects conformational epitopes as the heat treatment may break the bonds. Refolding allows the formation of native conformational epitopes in most cases, but few new allergens may be formed that need further efforts to minimize the risk associated with neoantigens. Thus, thermal treatment may decrease or exacerbate or have no effect on antigenic behavior; this depends on the properties of a particular protein.

Standard protocol for thermal treatment assay

Based on the report of a joint FAO/WHO expert consultation on the allergenicity of foods derived from biotechnology (2001) and guideline to the conduct food safety assessment of foods derived from GM crops by the Codex Alimentarius Commission (2003), Indian Ministry of Science and Technology (2008) prepared a standard protocol to assess the thermal stability of proteins. During the assay procedure, protein samples (conc. 1 mg/mL) are dissolved in suitable buffer and incubated at 25°C, 37°C, 55°C, 75°C, and 95°C for 30 minutes. The samples are boiled with Laemmli buffer and protein profiles obtained at different time points are compared to determine the biological activity of the protein. The stability of the protein at a particular temperature is determined from remaining the biological activity after a 30-minute incubation at that temperature (Flow Chart 32.3). Thermally treated proteins are divided into three categories: stable, partially stable, and labile. According to DBT guidelines (2008), proteins having more than 50% biological activity remaining are considered stable at that temperature. The biological activity between 50% and 10% are partially stable and less that 10% biological activity indicates that the protein is labile. So, thermal treatment can provide a clue that newly expressed protein is stable, partially stable or labile, and hence have corresponding allergenic potential.

Functional stability of proteins and importance of thermal stability assay

Transgenic crops undergo a rigorous safety assessment procedure before the commercialization due to worldwide anti-GM movement (Kumar et al., 2011a,b). Currently available assays cannot provide exact information regarding allergenicity of the transgenic foods. For the above-mentioned reasons, the weight-of-evidence approach was introduced to assess the allergenic potential of GM crops in which thermal stability has been included as a part of the risk assessment procedure (Codex Alimentarius, 2009). It is known that cooked foods do retain their ability to cause allergic reactions. Based on these observations, it was suggested by some regulatory agencies that the heat stability should be performed to ensure additional safety of transgenic food proteins (Codex Alimentarius Commission, 2003; Indian Ministry of Science and Technology, 2008). The functional activity of the



FLOW CHART 32.3 Thermal treatment protocol.

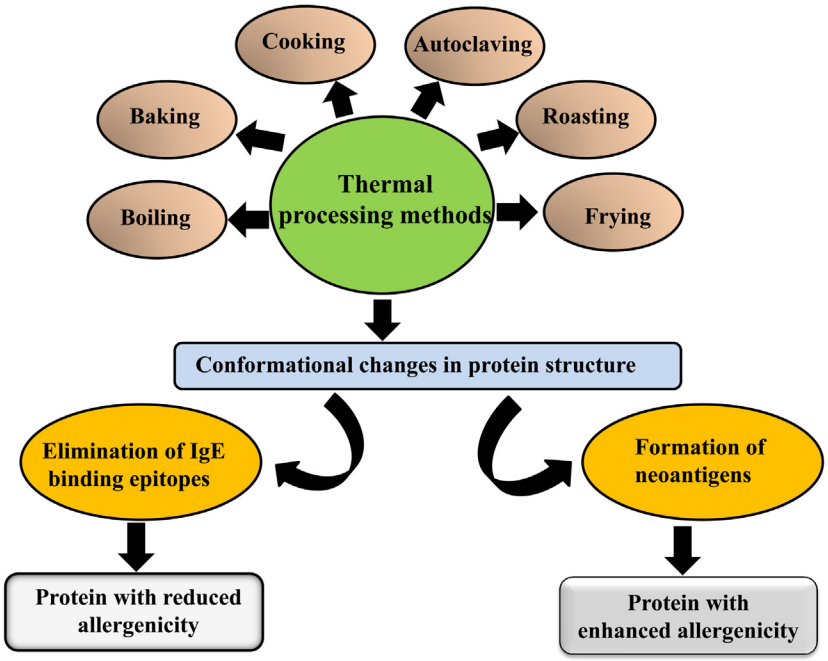


FIGURE 32.4 Thermal processing methods and their effect on protein allergenicity.

protein can be destroyed at a particular temperature. So, thermal treatment of protein is performed and biological activity is measured before and after thermal treatment. It may provide information regarding the temperature and time at which activity of the protein is destroyed by heat. Measuring biological activity of protein after exposure to heat can be one of the basic methods for risk assessment of novel proteins in transgenic crops (Indian Ministry of Science and Technology, 2008). However, it needs to be mentioned that protein denaturation does not always lead to loss of allergenicity. Thermal treatment can result in loss of epitopes originally present in their native form. It is also possible that the protein becomes more allergenic as compared to native form because hidden epitopes may get exposed following heat treatment, resulting in enhanced allergenicity (Fig. 32.4). As reported by the recent study that seven allergenic proteins are present in prawns, which are mostly heat-stable and further thermal treatment enhance antibody reactivity to prawn allergens (Kamath et al., 2014). Sometimes, heat-mediated denaturation has no effect because out of the two types of epitopes (linear and conformational), only linear epitopes are more stable and have the ability to provoke allergenicity. Conformational epitopes lose their allergenicity easily.

Several studies in the literature suggest that thermal treatment can eliminate the allergenicity of allergens such as patatin protein in potato, chitinases in fruits, the hazelnut Cor a 1.04 allergen, Kiwi fruit allergens,

and several legume allergens (Verma et al., 2012a,b). In the case of soybean, heat treatment changes the protein's profile and immunological properties, but no effect on overall allergenicity of soy protein were found (Besler et al., 2001). Some allergens are heat-stable (e.g., allergens of milk, egg, fish, and peanuts), whereas other food allergens are partially stable (e.g., soybean, cereals, celery, tree nuts, and their products) or labile (fruits of the Rosaceae family and carrots). Different thermal processing methods also have distinct effects on allergenicity. Recently, different degrees of allergic response are observed by raw, roasted, and boiled peanuts in mouse model. Compared with raw peanut, roasted peanuts showed a higher allergenicity, whereas the boiled peanuts showed a lower allergenicity (Zhang et al., 2019). Further, treatment that combined heat and pressure are able to attenuate the capacity of soybean proteins to bind IgE may contribute to the improvement of food safety (Cabanillas et al., 2018).

Major problem during the heat treatment process is the formation of new allergens, or neoallergens. These neoallergens, present in cooked food, may sensitize an individual. Neoallergens have been identified from pecans, wheat flour, roasted peanuts, lentils, almonds, cashew nuts, walnuts, soybeans, shrimp, scallops, tuna, eggs, apples, plums, milk, and potatoes. Changes in protein conformation may either have no effect on the protein allergenicity or can modulate allergenicity (Paschke, 2009). Storage conditions are also an important factor affecting the properties of proteins and may change their profile.

The food protein that is not normally allergenic may change its immunological properties during food storage. Codina et al. (1998) demonstrated that heat treatment can increase the allergenicity of soybean hull as two neoallergens of 15.3 and 10 kDa are formed. During the transport and storage conditions, heat is generated which may enhance the allergenicity of soybean hull. So, there are chances of allergenic conditions. For example, if novel transgenic crops are assessed for their allergenicity for all parameters except thermal treatment, it cannot be said with certainty what will be the behavior of that crop after prolonged storage. The chances of being allergenic will probably increase, as observed for many fruits and vegetables where the proteome changed and higher allergenicity was found (as in the case of apples). In other food crops, there is no change in immunoreactivity during storage (e.g., mangoes). However, comparison of protein profile of food crops before and after thermal treatment may provide a clue regarding the allergenicity of food crops.

Mechanical processes such as stirring may also influence the allergenic properties of food proteins. Although such treatments can cause surface denaturation of food proteins, a significant effect on allergenicity has not been demonstrated. Heat-induced denaturation of proteins can reduce the allergenic potential of the food product. Protein denaturation minimizes allergenicity by changing protein conformation, which is the result of destruction of IgE-reactive conformational epitopes. Fiocchi et al. (1995) showed that thermal treatment may reduce the allergenicity of beef and purified bovine allergens. Some food allergens may be thermostable. For example, in the case of major peanut allergen Ara h 1, the immunoreactive conformational epitopes are resistant to heat-induced denaturation. In potato allergen patatin (Sol t 1), heat treatment caused denaturation of protein; however, protein re-natured upon cooling. During the production of canned lychee fruit, thermal treatment for longer periods does not decrease the allergenicity. However, low molecular weight immunoreactive proteins were eliminated by the heat treatment.

Contradictory result in thermal treatment procedure

Retention or enhancement in the allergenicity of some foods after cooking is an important feature of some food protein allergens. Functional assays are used to measure the heat stability of newly expressed protein (Indian Ministry of Science and Technology, 2008), but functional stability is not related to allergenicity. A study carried out by Herouet et al. (2005) demonstrates that phosphinothricin acetyltransferase (PAT) protein that is safely consumed and gets inactivated at 40°C–45°C (15 minutes) or 60°C (10 minutes) but is clearly detectable even after heat treatment at 100°C. Although, the PAT protein loses its enzymatic activity, immunoreactivity is still detectable. In both heat-treated and the untreated proteins, same epitopes were recognized by IgG using anti-PAT antibodies.

Conclusively, thermal treatment of novel food proteins probably has no definite predictive value in the allergenicity assessment process. The thermal treatment assay can be used more appropriately in such conditions where toxicological responses are associated with its biological activity. It does not provide reliable information regarding the allergenic potential of a novel food protein. Although thermal stability assay provides no extra information for the safety assessment of novel protein, it can definitely be used to demonstrate the allergenicity of processed or cooked food in case some function is associated with heat stability. Various reports have indicated a reduction in the immunoreactive proteins following heat treatment. Sometimes, the thermal treatment approach generates

neoallergens that were originally absent in native variety. There is still a need to explore the utility of this method in allergenicity assessment of novel protein. As the impact of heat treatment differs from one food constituent to another, it is difficult to demonstrate the correlation of thermal treatments on protein allergenicity. Thermal treatment can provide useful information for the risk assessment of biotech crops. As this method is included in standard guidelines as a food safety approach, it must be performed for all transgenic crops with inserted gene products.

Ethical issues

Genetically modified food safety has been a controversial issue for a long time. Safety and potential risks, as well as ethical concerns associated with GM food, are still debated. There are laws in different countries focusing attention on the use and labeling requirements of GM foods. There are also social and ethical issues regarding GM food production worldwide. A gene inserted from an animal to a plant food may create ethical or religious problems in certain cases. For example, eating traces of genetic material from pork could be a problem for certain religious or cultural groups, although genetic material is DNA or RNA from any source and has only A, T(U), G, and C nucleotides. There is also fear that the distribution of GM seeds may be taken over by large multinational companies, affecting the socially equitable distribution of benefits. There is also an important issue associated with the protection of the natural environment and biological diversity, as GM food can affect the wild varieties. However, most of these fears are unfounded, lack evidence and can be considered as "fear of the unknown."

Clinical correlation

Food-induced adverse reactions promotes a variety of clinical symptoms resulting from immunologic and nonimmunologic reactions due to food intakes. In food allergy patients' symptoms can be mild, moderate, life-threatening, and sometime extremely fatal, despite the fact that the actual scenario of food allergy prevalence is still elusive. Interestingly, approximately 25% of general population has conception that they may have experienced food allergy. However, actual prevalence of food allergy can only be determined by clinical tests such as a skin or blood test to determine if someone has a food allergy. Based on these clinical diagnoses, it is speculated that around 1.5%−2% adult population and 6%−8% of children are suffering from food allergy worldwide. Although in developing and undeveloped countries, data regarding food allergy are not frequent. The effective allergy treatment is potentially dependent on accurate diagnosis of food allergy. Apart from treatment, allergic patients should also be aware with basics knowledge of allergic reactions induced by foods. Because of elaborative and descriptive nature, this book chapter can be used as a reference for clinicians and food allergy patients. For clinicians, this chapter will be an in-depth understanding of molecular mechanisms behind IgE-mediated food allergic reactions; however, allergic patients will be able to understand the basics of food allergy.

Translational significance

Genetically modified foods may be useful for solving the world hunger problem as they offer improved products with desirable qualities. Adoption of GM crops is increasing worldwide, but risks associated with the environment and human health still exist. It raises the issue that pre- and post-market surveillance of GM-derived food should be performed in order to avoid unintended effects. Among the various methods, SGF, SIF, and thermal stability assay are the few that are performed first for any newly expressed protein. SGF, SIF, and thermal stability assays are included in the standard guidelines for addressing the safety concerns of GM foods. Even though the SGF and SIF have some contradictory evidence, overall these assays give primary information regarding the probability of allergenic potential of food proteins. The thermal assay is not a commonly used parameter for safety assessment of GM-derived foods, but can add some value to the safety assessment. The addition of recent advancement of targeted proteomic technologies like multiple-reaction monitoring (MRM) mass spectrometry (MS) coupled with isotope-labeled internal standard (or AQUA peptides) is effective on the quantitation of food allergens even at 10-ppb level in a multiplex fashion although pre- and post-MS criteria is highly important in the overall process (Ahsan et al., 2016). Together, the use of SGF, SIF in crude foods, and identification of allergens may lead to elaborate allergenic aspects of GM foods and its safe use.

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World Wide Web resources

1. http://www.foodhaccp.com/online.html
   This is a comprehensive food safety information website and contains periodic newsletters, journals, and updates regarding the food safety concerns.
2. http://www.who.int/foodsafety/publications/biotech/20questions/en/
   This site presents very useful information regarding the safety of GM foods and the possible pros and cons.
3. http://www.gmo-compass.org.
   This database contains information about every genetically modified plant that has been approved or is awaiting approval in the EU. Information on the food and feed produced from the respective GM plant is also available.
4. http://www.centerforfoodsafety.org/campaign/genetically-engineered-food/crops/
   Centre for Food Safety (CSF) seeks to halt the approval, commercialization, or release of any new genetically engineered crops until they have been thoroughly tested and found safe for human health and the environment.
5. http://ec.europa.eu/food/food/intro/white_paper_en.htm

This site present white papers describing the modernizing legislation into a coherent and transparent set of rules, reinforcing controls from the farm to the table and increasing the capability of the scientific advice system, so as to guarantee a high level of human health and consumer protection related to the food safety.

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Glossary

GM crops Crops derived from insertion of desired gene (s) for high yield, salt tolerance, disease resistance, and other improvement.
GM food Food derived from GM crops.
Safety assessment Observation of adverse effect induced by GM food on biotic and abiotic system.
Allergy Immune provocation by certain harmless environment substance to susceptible individuals.
Allergens Responsible for protein induction of allergy having epitope as immunogenic site.

Abbreviations

APC	Antigen-presenting cells
DCs	Dendritic cells
FAO	Food and agriculture organization
GI tract	Gastrointestinal tract
GM crops	Genetically modified crops
IFBC	International Food Biotechnology Council
ILSI	International Life Sciences Institute

OECD	Organization for Economic Co-operation and Development
SGF	Simulated gastric fluid
SIF	Simulated intestinal fluid
USFDA	United States Food and Drug Administration
WHO	World Health Organization

Long answer questions

1. Explain the basic mechanism and methodology of SGF assay?
2. Describe the methodology of SIF assay and its mechanism?
3. What is the principle, basic mechanism, and methodology of thermal stability assay?
4. How can thermal treatment affect the allergenicity potential of foods? Describe the mechanistic aspect of the Maillard reaction.
5. What are the major lacunae in SGF, SIF, and thermal stability assays?

Short answer questions

1. What are GM foods?
2. Why are GM foods produced?
3. Which parameters are commonly analyzed for safety assessment of GM crops?
4. Give three in vitro assays for allergenicity assessment of GM foods?
5. What are the components of simulated gastric fluids?
6. What are the components of simulated intestinal fluids?

Answers to short answer questions

1. Genetically modified (GM) foods are foods derived from organisms whose genetic material (DNA) has been modified in a way that does not occur naturally, for example, through the introduction of a gene from a different organism.
2. Genetically modified crops derived food (GM Food) is an essential requirement for fulfilling the food demand for our fast-growing population. These GM crops have improved agronomic character, for example, insect resistance, herbicide resistance, disease resistance, and drought tolerance property. Further, more nutritious crops with higher yield as compared to its natural counterpart can be produced by this technology.
3. The safety concerns including toxicity, allergenicity, and environmental impact of GM crops and foods are frequently asked questions. The safety assessment of GM foods generally investigates (1) toxicity assessment; (2) potential to elicit an allergic reaction (allergenicity); (3) the stability of the inserted gene; (4) nutritional effects associated with genetic modification; and (5) any unintended effects which could result from the gene insertion. Traditional food crops are bred using plant breeding method, and they have genes only from the same species.
4. Three commonly used in vitro methods for allergenicity assessment include simulated gastric fluid assay, simulated intestinal fluid assay, and thermal treatment assay.
5. The simulated gastric fluid is a set of reagents held under specific conditions mainly comprised of 0.32% pepsin, pH 1.2, and temperature 37°C, which is designed to stimulate human gastric conditions present in the stomach.
6. For SIF assay, the simulated intestinal fluid is prepared according to the standard protocol as described in the United States Pharmacopoeia (1995). Briefly, 1 g/100 mL of pancreatin is dissolved in 0.05 M KH_2PO4, pH 7.5, and temperature 37°C. Test protein at a concentration of 5 mg/mL is used for assay.

Yes/no type questions

1. Of the foods we eat, contains less than 5% the genetic material DNA.
2. Most foods derived from genetically modified crops contain one or two additional genes.
3. Foods made from genetically modified crops required to pass human testing.
4. Foods from genetically engineered plants are less nutritious than comparable foods.
5. In an allergenic protein, certain regions have immune reactive capacity; these regions are known as epitopes.
6. Several international organizations like FAO/WHO, OECD, and ILSI have provided guidelines for the safety assessment of GM foods, a prerequisite before release into market.
7. Pancreatin is used in simulated gastric fluid (SGF) assay.
8. Heat-resistant proteins are not digested in the GI tract, are there chances that these thermal- resistant proteins can elicit an allergenic response in an individual.
9. One major factor for the formation of neoantigens may be the Maillard reaction.
10. Different thermal processing methods also have distinct effects on allergenicity.

Answers to yes/no type questions

1. No—All plant and animal cells contain DNA, so nearly all food contains genetic material regardless of whether the food has been genetically modified.
2. Yes—Genetically modified crops contain one or two additional genes than either conventional or hybrid crops.
3. No—There are currently no regulations that require human testing of these crops.
4. No—Nutritional assessments for foods from GM crops that have been evaluated by FDA through the consultation process have shown that such foods are generally as nutritious as foods from comparable traditionally bred plants.
5. Yes—An epitope, also known as antigenic determinant, is the part of an antigen that is recognized by the immune system, specifically by antibodies, B cells, or T cells.
6. Yes—Safety assessment approaches regarding GM foods differ from country to country, but the basic principle for the allergenicity assessment is based on a common approach that is internationally acceptable. GM food with allergenic potential may cause life-threatening allergic reactions in the already sensitized group. Various methods are used in a step-by-step manner to ensure the safety of GM foods.
7. No—Pepsin enzyme is used in SGF Assay. It is assumed that proteins with resistance to pepsin digestion in SGF can induce allergenic reactions. The assumption behind the SGF assay had simple reasoning that nutritionally desirable proteins should be rapidly digested, and therefore have less opportunity to exert adverse health effects when consumed.
8. Yes—Resistance to thermal treatment has been observed in the case of several food allergens, as there is a direct correlation between heat stability and allergenicity. As the thermal treatment can bring about substantial changes in the structure of protein, it can also modify the protein's allergenic nature.
9. Yes—Major problem during the heat treatment process is the formation of new allergens, or neoallergens and one major factor is Maillard reaction (the interaction of the protein component with sugar residues upon heating that generates sugar-conjugated protein derivative that causes enhancement in the allergenicity of the protein). A chemical reaction between the carbonyl group of the sugar reacts with the nucleophilic amino group of the amino acid, usually requiring heat.
10. Yes—Different heat treatment methods have different effect on the allergenicity. For example, different degrees of allergic response are observed by raw, roasted, and boiled peanuts in a mouse model. Compared with raw peanut, roasted peanuts showed a higher allergenicity, whereas the boiled peanuts showed a lower allergenicity.

CHAPTER

33

Correlating Ayurveda and biotechnology: approaches for the 21st century and beyond

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Summary

Lifestyle disorders are emerging as epidemic globally. Importance of the ancient knowledge system like Ayurveda has recently drawn the attention especially due to their perceived benefits in prevention of lifestyle disorders. Ayurveda has enunciated clear-cut recommendations of lifestyles to achieve 100 years of active life. Ayurveda has given priority to maintain the health of a healthy person or preventive aspect over curing the disease on treatment aspect.

In the ancient times, Ayurveda enjoyed the status of full-fledged knowledge system having eight distinct branches like *Shalya tantra*, *Kayachikitsa*, *Agadatantra*, *Rasayana Chikitsa*, *Shalakayatantra*, *Kauma-bhritya tantra*, *Grahachikitsya/Bhoota vidya*, and *Vaajikarana Chikitsa* focusing on required specializations. Ayurveda has its own concept of physiology, pharmacology, and pathology, which together may be termed as Ayurvedic Biology. Basic tenets of Ayurvedic Biology include *Panchamahabhut*, *Doshas*, *Dhatu*, *Agni*, *Prakriti*, *Aahar*, and *Vihar*.

Prakriti is a unique concept of Ayurveda providing foundation of individualistic treatment. The recent scientific inquisitive efforts have successfully correlated the association of *Prakriti* with the individual genetic makeup validating this concept. This chapter deals with various aspects of Ayurvedic Biology as well as provides insight into the scientific efforts to validate the concept of *Prakriti* with current advancement of science.

What you can expect to know

This chapter introduces the readers to the basic concepts of Ayurveda, which is a science of life. A browse through this chapter would prove to be an important stepping stone for the uninitiated. Subsequently, it would prod the reader to explore application of lifestyle-related recommendations to lead long disease-free and active life. It arms the readers with an insight into the concept of individual's *Prakriti* (psychosomatic constitution). This would provide scientific insights into personalized medicine, behavior, and lifestyle recommendations. This chapter also provides a peek into validation of concepts of *Prakriti* by genomic studies in India.

Introduction

Traditional medicines, herbal, and natural products have undergone tremendous resurgence in the last two decades. Earlier, interest was limited to the products only, but lately, *yoga* has also attracted some level of attention. Ayurveda, Traditional Chinese medicines, Tibetan medicine, Kempo medicine, and so on are some of the world's oldest systems. Since Ayurveda is distinct from them, it would be wrong to assume it to be a system of medicine; on the contrary, the word Ayurveda is made of two words *Ayur* + *Veda*, meaning knowledge of life. The scope of Ayurveda goes much beyond the aspects of traditional medicine. It recommends a lifestyle suitable to achieve 100 years of active life. To achieve this, according to *Charak Samhita*, Ayurveda has two objectives—the first objective is to preserve the health of the healthy persons and the second one is to alleviate the disease in the patients.

Therefore, major focus of Ayurveda has remained to ensure healthy long and active life and recommends tripod of life to achieve it. The tripod of life includes *Aahar* (diet), *Nidra* (sleep), and *Brahmacharya* (celibacy). Some ancient sages have described this tripod as

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consisting of *Aahar* (diet), *Vihar* (behavior), and *Rasayana* (antiaging and adaptogenic).

Principles of Ayurveda

Ayurveda means knowledge of life. It deals with all aspects of life two objectives: first to preserve the health of the healthy person (health promotive/disease prevention) and second to treat a person when he falls ill (treating the disease). Both the objectives are aimed at providing 100 years of healthy, disease-free, and active life. As per Ayurveda, this can be achieved by lifestyle recommendations related to *Ahar* (diet), *Nidra* (sleep), and *Brahmacharya* (controlled sex). These recommendations are further specified into daily and seasonal routines to achieve the objective of long and healthy life.

Ayurveda is a complete science and has eight branches, which are *Shalya tantra* (surgery), *Kayachikitsa* (general medicine), *Agadatantra* (toxicology), *Rasayana Chikitsa* (rejuvenation therapy), *Shalakayatantra* (ophthalmology, ENT), *Kaumar-bhritya tantra* (pediatrics), *Grahachikitsya/Bhoota vidya* (psychotherapy), and *Vaajikarana chikitsa* (aphrodisiac therapy).

Ayurveda has its own principles of physiology, pathology, anatomy, and other related areas for health promotion and disease alleviation. The basic principles and tenets of Ayurveda are described in this chapter.

Ayurvedic physiology (Sharir kriya)

Ayurvedic physiology broadly deals with the following:

1. *Panchamahabhuta* (primordial elements)
2. *Doshas—Sharirika* and *Manasika* (physical and mental)
3. *Dhatu* (tissue) and *Mal* (waste)
4. *Agni* (energy)
5. *Prakriti* (psychosomatic constitution)
6. *Aahar* (diet)
7. *Vihar* (behavior modalities)

Panchamahabhuta

A body is primarily considered to be made up of *Phanchamahabhuta*, which are the five primordial elements. In Ayurveda, all things, both living and nonliving, are made up of *Panchamahabhuta*—which are *Prithvi* (earth), *Jal* (water), *Teja* (energy), *Vayu* (air), and *Akash* (space). Human body is composed of five primordial elements along with mind and soul and is called *Rashipurush* and also as *Chikitsyapurush* (the entity amenable to treatment).

According to Ayurveda, life consists of four components—an integration of *Atma* (soul) with *Panchamahabhuta* (primordial elements), which distinguishes between living and nonliving.

According to Ayurveda, life consists of four compounds, *Sarir* (body), *Indriya* (sense organs), *Atma* (soul), and *Mann* (mind). The presence of all these four components constitutes life; if any of them is absent, life does not exist.

None of these five elements can exist individually. Actually *Prithvi* means ½ of *Prithv*i and 1/8th of *Jal*, *Teja*, *Vayu*, and *Akash*. Similarly, the following is self-explanatory for the other elements.

For Jal, ½ of *Jal* and 1/8th of *Prithvi*, *Teja*, *Vayu*, and *Akash*. For Teja, ½ of *Agni* and 1/8th of *Jal*, *Prithvi*, *Vayu*, and *Akash*. For Vayu, ½ of *Vayu* and 1/8th of *Jal*, *Teja*, *Prithvi*, and *Akash*. For Akash, ½ of *Akash* and 1/8th of *Jal*, *Teja*, *Vayu*, and *Prithvi*.

These five *Panchamahabhuta* represent as *Tridoshas*, five senses and five sense organs in humans. Their relationship is depicted in Fig. 33.1.

Doshas

Ayurveda has described that health of a person depends on balance of *doshas*, which constitutes of three *sharirik doshas* affecting the body and are *Vata*, *Pitta*, and *Kapha* and three *manasik doshas* affecting the mind and are *Satwa, Rajas*, and *Tamas*.

Manasik doshas

As per Ayurveda, there are three *manasik doshas*, which are *Swatwika*, *Rajasika*, and *Tamasika* and defines the third process and behavior of a person. A person with *Swatwika* mind would be very religious, a person with *Rajasika* mind would be royal and aristocratic, and the person with *Tamasika* mind may be involved in socially unacceptable behavior practices.

Ayurveda has its root from the Vedas especially the *Atharva* Veda, and it follows its own principles of biology, which includes physiology also. Basic physiology concept of Ayurveda includes the following.

Vata, in its normal state, protects the body bestowing enthusiasm, expiration and inspiration, all activities (of the body, mind, and speech), initiation of the urges, maintenance of the *dhatus* in their normalcy, and proper functioning of the sense organs (Vagbhatta, 2001a).

Pitta, in its normal state, attends to digestion, maintenance of body temperature, vision, production of hunger, thirst, appetite, complexion, intelligence, courage, valor, and softness of the body (Vagbhatta, 2001a).

Kapha confers stability, lubrication, compactness of the joints, forbearance, and such others (Vagbhatta, 2001a).

Types of vata (Vagbhatta, 2001b)

Prana vayu is located in head; moves in the chest and throat; supports the mind, the heart, sense organs, and intelligence; and attends to expectoration, sneezing,

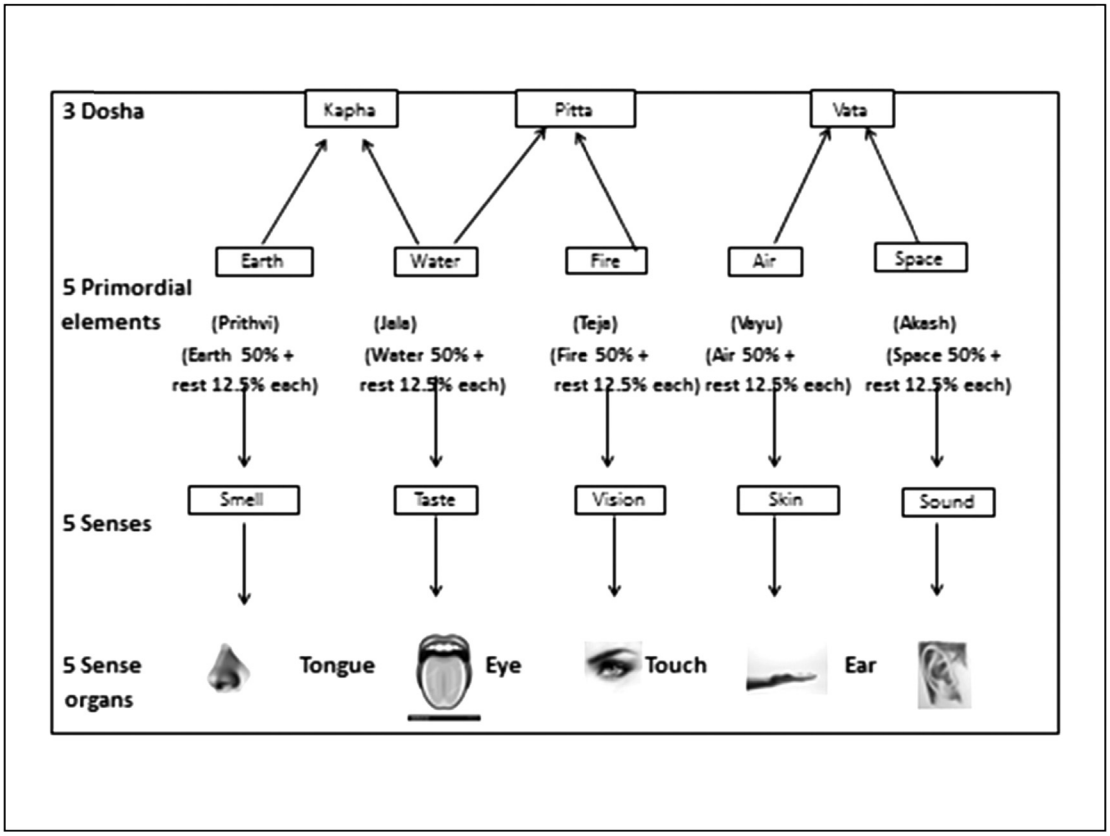

FIGURE 33.1 Panchamahabhuta and correlates.

belching, inspiration, and swallowing of food. *Udana vayu* is sited in the chest and moves in the nose, umbilicus, and throat; its functions are initiation of speech, effort, enthusiasm, strength, color, and memory. *Vyana vayu* is found in heart, moves all over the body at a great speed, and attends to functions such as walking, bringing the body parts downward, lifting the body upward, opening and closing of the eyes. *Samana vayu* is located near the digestive system, moves in the alimentary activity and abdominal viscera, withholds the food in the system, cooks, separates the essence and wastes, and eliminates. *Apana vayu* resides in the large intestine and moves in the waist, bladder, penis, and thighs and attends to the functions—elimination of the semen, menstrual fluid, feces, urine, and fetus.

Types of pitta

Pachaka pitta (Vagbhatta, 2001b) is located in the digestive system and helps in digestion. *Sadhaka pitta* is found in hrdaya and attends to mental function such as knowledge, intelligence, self-consciousness, etc., thereby helping the purposes of life. *Alocaka pitta* is sited in the eyes and helps in seeing the form. *Bhrajaka pitta* is spread throughout the skin and helps in exhibition of color and complexion. *Ranjaka pitta* is located in the stomach and imparts red color to rasa and converts it to rakta.

Types of kapha

Kledaka kapha (Vagbhatta, 2001b) is located in the stomach and moistens the hard masses of food. *Bodhaka kapha* is having a seat in tongue and helps in perception of taste. *Tarpaka kapha* is located in the head and nourishes the sense organs. *Slesaka kapha* is found in the joints and bones and lubricates the joints. *Avlambaka kapha* is sited in the chest and helps in lubrication and nourishment.

Dhatus and *Mala* (tissues and waste)

Ayurvedic physiology elaborates that there are seven types of *Dhatus*, which may be loosely translated as tissue and three major actions of waste production after transformation of *Dhatus* or after digestion of food. After appropriate digestion of food, the

remaining part is divided into Sara (Prasad, nutrient fraction) and Kitta (waste product) bhaga in our human body.

The seven tissues are *Rasa*, *Rakta*, *Mamsa*, *Meda*, *Ashti*, *Majja*, and *Shukra*.

There are three major wastes, which are fecal matter, urine, and sweat. The food after ingestion gets converted into Rasa, which further gets converted to *Rakta*, *Mamsa*, *Meda*, *Asthi*, *Majja*, and finally *Shukra*. Different kinds of *Teja* (energy) are required for transformation from one to other *Dhatus*. These transformations follow from *Rasa dhatu* to *Shukra dhatu*. *Rasagni* acts on *Rasa dhatu* (lymph) transforming it to next *Dahtu*, *Rakta* (blood) and forming a waste part (*mal*) as *Kapha*. The *Raktagani* works on *Rakta* dhatu transforming it to next *dhatu Mamsa* (muscles) and forming a waste product urine. Similarly, *Mamsagni* acts on *Mamsa Dhatu* and transforms it to next *dhatu Meda* (fat) forming ear wax as a waste matter. *Medagni* transforms *Medadhatu* to *Asthidhatu* (bone) with waste product as hair and nails. *Asthyagni* changes *Asthi dhatu* to *Majja dhatu* (bone marrow) along with forming waste product of oiliness in skin and feces. Similarly, *Majja agni* transforms *Majja dhatu* to *Sukra Dhatu* (sperm and ovum), and finally *Oja* (immunity—essence of all *dhatus)* is formed by virtue of *sukraagni* acting on *Sukradhatu*. This transformation from earlier *dhatu* to the next *dhatu* takes place only if the earlier *dhatu* is properly and fully nourished. Therefore, until the earlier *dhatu* is not fulfilled, the transformation to the next level gets disrupted. This theory of transformation is explained in Ayurveda as *Kedarikulya nayay*. The transportation or flow of nutrients and movement from one part to another part of body takes place through the microchannels of the body known as *Srotas*.

Food gets transformed into seven *Dhatus*. Their resultant *Mala* and the *Agni* are represented in Fig. 33.2.

Characteristics of Dhatus

Rasa dhatu (lymph) is a nutrient and helps in fluid nutrition. *Rakta* (blood) is the blood medium with primary function of oxygenation. *Mamsa* (muscles) are the muscular tissues required for movement, *Meda* (fat) is having lubricating property and helps in lubricating fat deposits. *Asthi* (bone) supports and provide body framework and helps supporting and accommodating bony structures. *Majja* (bone marrow) supports the tissues inside the bone. *Shukra* (sperm and ovum) is primarily responsible for reproductive functions.

Agni (energy)

Agni (energy) occupies the major position of life sustaining in Ayurveda. *Agni* means energy, which is required to run various physiological processes inside

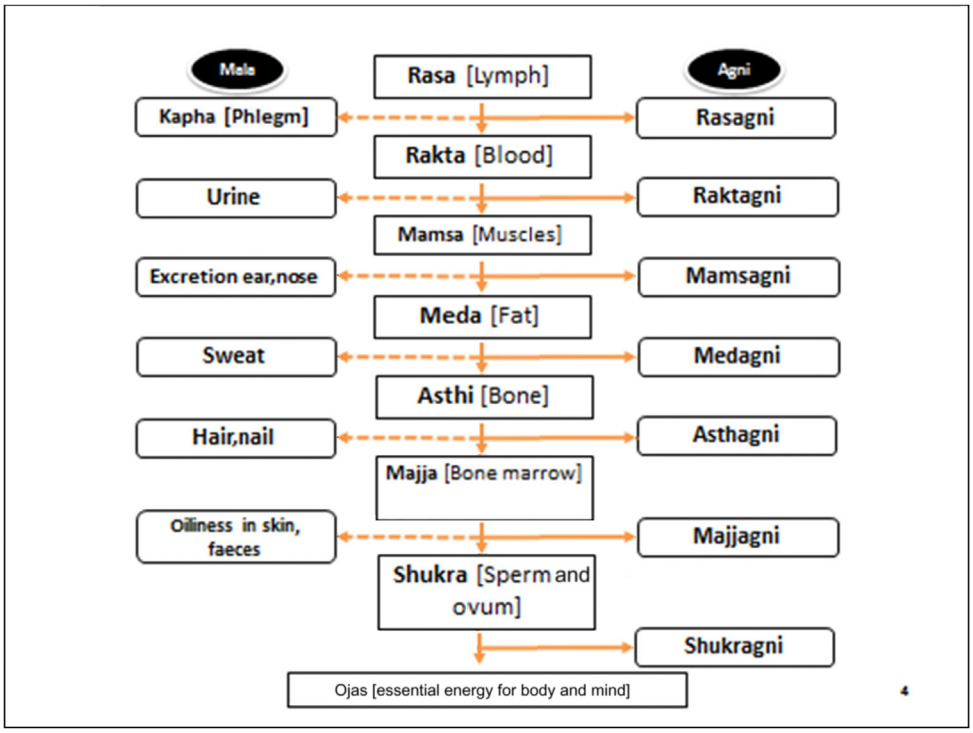

FIGURE 33.2 Transformation of food.

the body. As per Ayurveda, these *Agni* are of three types as follows:

1. *Jataragni* [1]—The digestive fire to digest food.
2. *Dhatvagni* [7]—Digestive power of tissue. This energy is the enzymatic system required to transform one *Dhatu* in another like *Rasa* to *Rakta* and *Rakta* to *Mamsa*, etc.
3. *Bhutagni* [5]—Fire for digestion at an elementary level. Five primordial elements *Prithvi* (earth), *Jal* (water), *Tejas* (energy), *Vayu* (air), and *Akash* (space).

Prakriti (psychosomatic constitution)

Prakriti is a unique contribution of Ayurveda providing first ever reference leading to customized prescription to different patients with same disease. *Prakriti* is a unique contribution of Ayurveda and explains multiple puzzles like why few people have tendency to gain weight, while some of them do not gain weight at all, when some people have extremely dry skin and while some people have moist skin, why some people take 20 glasses of water in a day and some of them take only two glasses of water a day, and why some people respond to a particular drug to which other patients do not respond and multiple other puzzles like these.

Ayurveda has provided these concepts of *Prakriti*, while there are three basic *Prakriti—Vata*, *Pitta*, and *Kapha*. There are seven more combination and permutation, which may be 10. Therefore, total population can be divided into 10 types having feature unique to them.

The particular class or trends of individual *Prakriti* are described in this chapter. Let us look at each of them individually.

Traits of Vata Prakriti *person*

Physical traits: Dry, wavy hair: thinly distributed; small eye balls with curious looks; brown stained, irregular, and small denture; tall, lean built; thin musculature; dark complexioned and dry skin; brittle nails; inconsistent appetite; fast physical movements and unsteady gait.

Mental traits: Creative, lack consistency; excellent grasp and a low memory; poor at calculation and inconsistent decision-making; love for fun, novelty and good learning aptitudes; artistic, moody and work in bouts; self-motivated, speed oriented; good initiative for relations and poor at maintenance, sentimental and sensitive, generally timid and impulsive behavior.

Traits of Pitta Prakriti *person*

Physical traits: Soft and straight hair with tendency of graying/baldness; bright eye balls with sharp looks; yellow stained, regular denture; medium in height and body build; well-defined musculature; fair complexion and sensitive skin; pink colored and soft nails; good appetite and digestion; fast physical; movements with a steady gait.

Mental traits: Aggressive, dictating, and uncompromising; medium in grasp and memory; well calculated and purposeful always and in all respects; obsession with success and material gains; competitive, may cost values; performance oriented, motivated by incentives; adventurous and preference for luxurious lifestyles; prone to stress eating; aggressive, dictating, and uncompromising; medium in grasp and memory; well calculated and purposeful always and in all respects; obsession with success and material gains.

Traits of Kapha Prakriti *person*

Physical traits: Oily, curled hair—thickly distributed; large, attractive with dreamy looks; white, regular denture; short in height and heavy body build; tendency to gain weight; pale complexion, oily and thick skin; white colored and soft nails; low appetite and digestion; slow physical movements with a steady gait.

Mental traits: Decisive, lacks initiative; good memory, low at grasping; poor learning aptitudes; conservative, reluctant to change; quality oriented, low motivation; measured decisions; simple living and spiritual orientation; deeply sentimental and emotionally involved in relations; prone to depression

Aahar (diet)

Food, according to Ayurveda, is essential for life, energy, growth, strength, complexion, clarity of sense organs, intellect, good voice, happiness, and cognition (Sharma P V, 2004a).

It even lays down broad guideline as to when and how to consume food, which is as follows (Sharma P V, 2004b):

- One should eat timely.
- When you feel hungry.
- After taking bath, after washing hands, feet, and face.
- Served by faithful and clean persons.
- Take warm wholesome food.
- Proper quantity in a comfortable place.
- Do not take water till 1 hour after food.
- After the previous food is digested to avoid formation of *Ama*.
- Avoid harmful combination of food.
- With all the favorable accessories.
- Not too fast, not too slow.
- Not while talking or laughing.
- With full concentration after due consideration to the self.

Interestingly, Ayurveda also talks about the eight factors influencing diet (Sharma P V, 2004d):

- *Prakriti* (*nature*)
- *Karana* (processing)
- *Sanyog* (*combination*)
- *Rashi* (*quantity*)—Atiyog, Heenyog, Mithyayog, Samyakyog
- *Desh* (*place*)
- *Kal* (*time*)
- *Upyog Sansatha* (*rules for use*)
- Consumer of food

As per Ayurveda, there can be four types of uses for anything like *Heenyog* (deficient or less), *Atiyog* (excessive use), *Mithyayog* (incompatibility or inappropriately used), and *Samyakyog* (proper use). About 3000 years ago, *Charak Samhita* described as "Do it yours taste" using which anyone can know whether the activity he has been involved in can be quantified under *Heenyog*, *Atiyog*, *Mithyayog*, or *Samyakyog*. The same is treated for food also. Given below is the science of consumption of proper food:

- no obstruction in heart;
- no pain in sides;
- no excessive heaviness in abdomen;
- saturation of sense organs;
- cessation of hunger and thirst;
- ease in standing, sitting, lying down, movement, inspiration laughing, and gossiping; and
- easy digestion in evening and morning.

However, one can still have indigestion and other problems even after consuming the appropriate and compatible food. This could be due to the presence of underling factors—anxiety, fear, grief, anger, and sleeplessness.

Needless to say, it also talks about the nature of inappropriate food (Sharma P V, 2004c).

Inappropriate food

Deficient food (Heena yoga): Loss of strength; unsaturation, upward movement of vâyu; complexion and development; harm to life span, virility, and immunity; damage to mind, body, intellect, and sense organs.

Excessive food (Ati yoga): Vomiting, anorexia, colic pain; hardness of belly, body ache; dryness of mouth, fainting; indigestion, stiffness in sides; heaviness of body, fever with cold; vomiting, anorexia, colic pain; narcosis, delirium.

Ahead of its times, it even talks about incompatible food (mithya yog/virudhahaar) (Sharma P V, 2004e).

- honey and rain water in equal quantity;
- honey and ghee taken together in equal quantity;
- hot water after taking honey;
- fruits like matulunga, jambu, narikela, amalaka—these and similar other substances, all sour liquids or nonliquids are antagonistic to milk;
- fish with milk;
- meat of aquatic animals with honey;
- mango with milk (mango shake);
- milk and buttermilk; and
- pigeon fried in mustard oil with honey.

As mentioned earlier, food affects human physiology in multiple ways, including *Doshas* as well as *Dhatus*. The relationship of *Panchamahabhuta* with the taste and effect of taste on individual *Dohsas* is depicted in Fig. 33.3.

Since both living and nonliving things are made up with five elements, these elements represent six as taste in food and these taste affects the *Tridoshas* inside

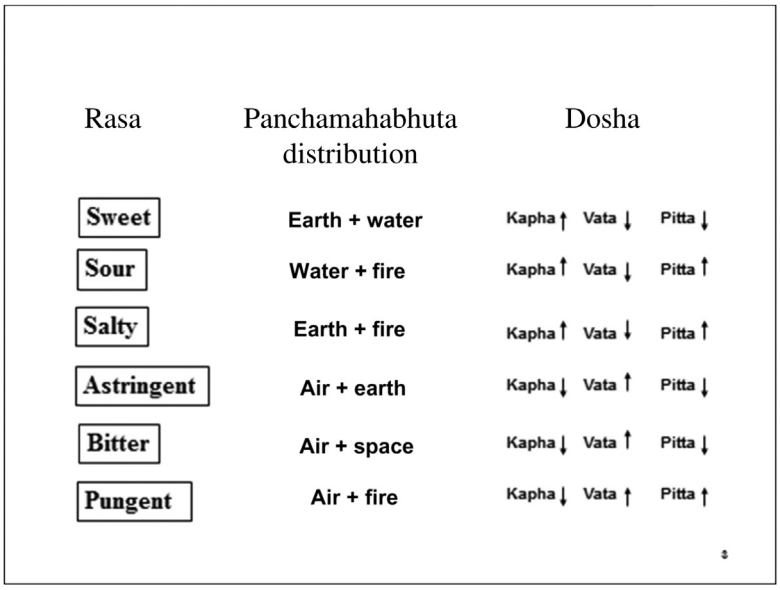

FIGURE 33.3 Tastes and doshik effect.

the body. Relationship between taste, *Panchabhuta* and *Doshas* are as follows: Sweet taste is made up of earth + water and affects tridoshas as *Kapha* ↑, *Vata* ↓, *Pitta* ↓. Sour taste is composed of water + fire and have effects as *Kapha* ↑, *Vata* ↓, *Pitta* ↑. Salty taste contains earth + fire and affects tridoshas as *Kapha* ↑, *Vata* ↓, *Pitta* ↑. Astringent taste is made up of air + earth and have effects as *Kapha* ↓, *Vata* ↑, *Pitta*. Bitter taste contains air + space and affects tridosha as *Kapha* ↓, *Vata* ↑, *Pitta* ↓. *The* pungent taste comprises air + fire and affects *Kapha* ↓, *Vata* ↑, *Pitta* ↑.

Based on the individual *Prakriti* of a person, Ayurveda has prescribed the following diet.

Diet of a Vataj Prakriti *person*

Recommended food items: fruits such as oranges, bananas, peaches, plums, lemons, limes, grape fruit, strawberries, pineapples, papayas, mango. Vegetables such as potatoes, tomatoes, corn (fresh), peas, mustard greens, chilies, sweet potatoes, carrots, beets, radish, onions (cooked). Grains such as oats, basmati rice, wheat. Dairy products such as cheese, milk, yogurt, sour cream, butter, buttermilk, ghee. Animal products, namely, chicken, fish, eggs, and spices such as turmeric, black pepper, mustard, ginger, cloves, coriander, cinnamon, rock salt, garlic, cardamom, asafoetida, fennel.

Not recommended: fruits such as apples, melons, dry fruit. Vegetables such as cauliflower, cucumber, spinach, sunflower sprouts, onions (raw), mushrooms, and cabbage. Grains such as corn, millet, barley, Dried grains. Dairy products such as ice cream and animal products—lamb and pork.

Diet of a Pittaj Prakriti *person*

Recommended food items: Fruits such as oranges, mango, plums, pineapple, melons, dates, grapes, apples. Vegetables such as potatoes, corn (fresh), squash. Cabbage, mushrooms, green beans, peas, cucumber, cauliflower, and sunflower sprouts. Grains such as brown rice, blue corn, millet, basmati rice, barley, wheat. Dairy products such as cheese, milk, cream. Animal products such as chicken and egg white and spices such as cardamom, turmeric, fennel, coriander.

Not recommended: Fruits such as lemons, bananas, cherries, peaches, papaya, strawberries, grape fruit. Vegetables such as onions (well cooked and raw), carrots, beets, spinach, sweet potatoes, radishes, tomatoes, chilies. Grains such as brown rice (short grains), corn, buck wheat. Dairy products such as kefir, buttermilk, yogurt, sour cream, ice cream. Animal products such as pork, fish, lamb, shell fish, and spices such as cinnamon, rock salt, ginger, cloves, asafoetida, salt, garlic, black pepper, mustard.

Diet of a Kaphaj Prakriti *person*

Recommended food items: Fruits such as pomegranate, dry fruit, apple. Vegetables such as potatoes, bell peppers, cauliflower, spinach, carrots, green beans, peas (fresh), mushrooms, beets, radish, mustard greens, chilies, cabbage. Grains such as corn, millet, buck wheat, barley, dry or popped grains. Dairy products such as goat milk, buttermilk. Animal products such as chicken and spices such as fennel, mint, cinnamon, garlic coriander, asafoetida, fenugreek, black pepper, mustard, ginger, turmeric, cloves, cardamom.

Not recommended food items: Fruits such as papaya, lemon, lime, grape fruit, pineapple, oranges, pineapple, plums, grapes, mango, strawberry, bananas, dates. Vegetables such as tomatoes, squash, corn (fresh), sweet potatoes, cucumber. Grains such as basmati rice, brown rice, wheat, white rice. Dairy products such as kefir, ghee, milk, yogurt, ice cream, cheese, butter. Animal products such as fish, lamb, pork and eggs and spices such as rock salt and sea salt.

Dietary recommendations according to *Prakriti* are summarized in Table 33.1.

Vihar (behavioral suggestions)

Ayurveda is a practical science and preaches how to live for 100 years of active life. To achieve this, it recommends *tripod of life*, which are *Aahar* (diet), *Nidra* (sleep), and *Brahmacharia* (celibacy). A few ancient sages have also described it as *Aahar* (diet), *Vihar* (behavior), and *Rasayayna* (adaptogens).

Ayurveda has prescribed particularly lifestyle and practices to be followed on a daily basis and also to be followed in different seasons. Both these practices are enunciated below as *Dinacharya* and *Ritucharya*.

Dinacharya

It is the specific daily routine advocated by Ayurveda for maintaining good health (Vagbhatta, 2001c; Kanjilal et al, 2018).

1. *Brahmamuhurta-jagarana* (wake-up before sunrise)
2. *Darpanena mukhasayavalokana* (to see the image of self in mirror)
3. *Malotsarga* (defecation and urination)
4. *Achamana* (washing of hands)
5. *Danta-dhavana* (tooth brushing)
6. *Jihva-nirlekhana* (tongue cleaning)
7. *Sneha gandusha-dharana* (retaining oil in mouth)
8. *Mukha-netra prakshalana* (washing of face and eyes)
9. Sugandhita dravya dharana and tambula sevana (use of mouth freshener and betel leaves)
10. *Anjana* (application of collyrium)
11. *Nasya* (oily nasal drops)

TABLE 33.1 Diet according to Prakriti types.

	Vata		Pitta		Kapha	
Types of food	**R**	**NR**	**R**	**NR**	**R**	**NR**
Fruits	Oranges, bananas, peaches, papayas, mango	Apples, melons, dry fruit	Oranges, mango, plums, pineapple, melons, dates, grapes, apples	Lemons, bananas, cherries, peaches, papaya	Pomegranate, dry fruit, apple	Papaya, lemon, lime, grape fruit, pineapple, plums, oranges, grapes, mango
Vegetables	Potatoes, tomatoes, corn (fresh), peas, mustard, greens, chilies, sweet potatoes	Cauliflower, cucumber, spinach, sunflower	Potatoes, corn (fresh), cabbage mushrooms, green beans, peas, cucumber, cauliflower	Onions (well cooked and raw) carrots, beets, spinach, sweet potatoes	Potatoes, bell peppers, cauliflower, spinach, carrots, green beans, peas (fresh)	Tomatoes, squash, corn (fresh), sweet potatoes, cucumber
Grains	Oats, basmati rice, wheat	Corn, millet, barley	Brown rice, blue corn, millet, basmati rice, barley, wheat	Brown rice (short grains), corn, buck wheat	Corn, millet, buck wheat, barley, dry or popped gains	Basmati rice, brown rice, wheat, white rice
Legumes			Mudga, Rajamsa		Mudga, Masa	
NonVegetarian	Chicken, fish, Egg	Lamb, pork	Chicken, egg white	Pork, fish, lamb, shell fish	Chicken	Fish, lamb, pork, eggs

12. *Dhumapana* (inhalation of medicated smoke)
13. *Vyayama* (physical exercise)
14. *Kshaura-karma* (regular cutting of hair, nail, etc.)
15. *Abhayanga* (body massage with oil)
16. *Sharir-parimarjana* (body cleansing)
17. Snana (bathing)
18. Vastra-dharana (dressing)
19. Anulepana (deodorants, perfumes, face pack, etc.)
20. Gandhamala-dharana (garlanding)
21. Ratna and abhushana dharana (use of precious stones and metals in the form of jewelry)
22. Sandhyopasana (worship and prayer with surya namaskar)
23. Paduka-chhatra-dandadi dharana (use of shoes, umbrella, stick, etc.)
24. Jivikoparjana upaya (to indulge in occupation)

Ritucharya

Ayurveda classifies the six seasons into different categories (Vagbhatta, 2001d; Kanjilal et al, 2018):

1. Uttarayana/Adankala (northern solstice)
   The following seasons fall under this category and are considered unhealthy:
   - *Shishir Ritu* (dewy season)
   - *Vasant Ritu* (spring season)
   - *Grisham Ritu* (summer season)
2. Dakshinayana/Visargakala (southern solstice)
   The following seasons fall under this category and are considered healthy:
   - *Varsha Ritu* (rainy season)
   - *Sharad Ritu* (autumn season)
   - *Hemant Ritu* (winter season)

In *Shitakal* (winter—*hemanta*, *sisira*)—the strength of the people will be maximum.

During *Vristi* and *Grishma* (rainy and summer)—the strength of people will be poor.

In the remaining seasons—the strength will be minimum.

Hemanta ritucharya *(mid-November to mid-January)*

- The people have strong digestion and are powerful.
- the use of sweet, sour, and salt is suggested.
- During morning, mild massaging of the body and wrestling with skilled wrestlers using half the strength is recommended.

Shishir ritucharya *(mid-January to mid-March)*

The same regimen as used in *Hemanta* is recommended to be adopted with more intensity since cold is severe and dryness exists.

Vasanta ritucharya *(mid-March to mid-May)*

- Kapha becomes liquefied by the heat of the sun and diminishes the digestive power.
- Hence, it is recommended to consume food, which are easily digestible and free from moisture.
- Mild exercises are also suggested, and sleeping at day time is to be avoided.
- It is suggested to spend time engaged in pleasant games and past times.

Grishma ritucharya *(mid-May to mid-July)*

- Physical exercises and exposure to sunlight are to be avoided.

- Food that are sweet, easily digestible, fatty, cold, and liquid are to be consumed.
- Wine is to be avoided and if taken it should be taken in minimal quantity diluted with water.

Varsa ritucharya *(mid-July to mid-Sep)*

- The digestive system is weakened.
- All those measures that enhance the digestive power are to be adopted.
- One should use old grain for food, meat of animals living in desert like conditions, wine prepared from grapes, and fermented decoction.
- Water should be boiled before drinking.

Sharad ritucharya *(mid-Sep to mid-Nov)*

One should take food that is easily digestible and which is of bitter, sweet, and astringent taste.

Quite interestingly, *Ritucharya* is intrinsically linked to the *Tridoshas* as follows:

Seasonal variations of *Vata*, *Pitta*, *Kapha: Vata gets accumulated in Grishma* (mid-May to mid-July), aggravated in *Varsha* (mid-July to mid-September) and normalizes in *Sharad* (mid-September to mid-November). *Pitta gets accumulated in Varsha*, aggravated in *Sharad and* normalizes in *Hemanta* (mid-November to mid-January). *Kapha gets accumulated in Shishir* (mid-January to mid-March), aggravated in *Vasanta* (mid-March to mid-May), and normalizes in *Grishma*.

Lifestyle modification according to *Prakriti* is summarized in Table 33.2.

Additional recommendation related to behavior: Ayurveda has recommend that 13 urges should never be suppressed and they are even called *Adharaniya Vega* as follows:

Urges never to be suppressed (Adharaniya Vega): *Suppressing these urges leads to diseases* such as micturition—pain in bladder; defecation—colic pain; seminal ejaculation—pain in genital organ; flatus—constipation; vomiting—itching, urticaria; sneezing—torticoliosis; eructation—hiccup; yawning—convulsions; hunger—emaciation; thirst—dryness of throat and mouth; tears—rhinitis; sleep—yawning; breathing—fainting

Ayurveda has also recommended urges, which should always be suppressed and they are even called *Dharniya Vega* (*urges always to be suppressed*): greed, grief, fear, anger, vanity, shamelessness, envy, excessive attachment, desire of taking another's property.

Ageing process

Based on observation of thousands of years and millions of persons, ancient rishis like *Vagbhata* have observed the features a person loses in every decade of life, which may also be called the "mile stones of ageing" as given below:

Features of "decades of life" lost at the end of the period (Ashtanga Hridyam)

In the first decade (0–10 years), human loses *Balya* (infancy). In the second decade (11–20 years), *Vriddhi* (growth) is lost. In the third decade (21–30 years), human being loses *Prabha* (luster). In the fourth decade (31–40 years), *Medha* (intellect) is lost. In the fifth decade (41–50 years), *Tvak* (Skin health) gets degraded. In the seventh decade of life (61–70 years), *Drishti* (vision)

TABLE 33.2 Lifestyle modifications according to *Prakriti*.

	Vata	Pitta	Kapha
Sleep	8–9 h of sleep, avoid night schedules	7–8 h	6–7 h, may work late night
Exercise	Avoid exhausting exercise. Pranayama and yoga are recommended	Moderate exercise is recommended but avoid in hot seasons	Can enjoy vigorous exercise
Work place environment	Avoid chill and dry weather	Prefer cool and moist environment	Prefer warm and dry environment
Job nature	Job where multitasking is required and sedentary job is suitable	Management, intellectual skills, discipline, defense, etc.	Field work, strategic management
Sports	Indoor sports, gymnastics, games that require flexibility and quickness	Outdoor and indoor sports that requires speed, quick reflexes, and intelligence	Outdoor games that requires stamina and stability
Sex	Over indulgence in sex should be avoided	Sex in moderation is good, and it makes them relaxed when they are in stress	May enjoy sex when they desire as the people are having good stamina and endurance
Dosha factors	Avoid factors that vitiate *vata*	Avoid factors that vitiate *pitta*	Avoid factors that vitiate *kapha*

gets diminished. In the eighth decade (71–80 years), *Srotrendriya* (hearing) is lost. The ninth decade (81–90 years) witnesses the diminished *Manah* (psyche), and in the tenth decade of life (91–100 years), *Sparsendriyas* (psychomotor functions) degrades.

Prakriti and genomics

Impressed with the basic guidelines leading to personalized or customized medications, several groups of scientists started scientific exploration on the concept of *Prakriti* in Ayurveda. Foremost amongst them has been the group of CSIR led by Dr. Samir Brahmachari and Mitali Mukherjee. Another is the school of Life Sciences, Pune, led by Prof. Bhusan Patwardhan. A few other groups have also started working to explore genomic rationale behind the *Prakriti* concept and its association with basic metabolism rate (BMR). While Mitali Mukherjee et al (Tav et al., 2011) have confirmed certain selective biochemicals in the blood, Prof. Patwardhan has confirmed the association with human leukocyte antigen (HLA), single-nucleotide polymorphisms (SNPs), and gene polymorphism. A summary of some of these studies are given below.

In a study (Tav et al, 2011) conducted on 96 subjects by Mitali Mukherjee et al and published in the *Journal of Translational Medicine*, it emerged that the phenotypic expressions of *Prakriti* have genotypic correlations, especially that of EGLN1 and VWF, which modulate thrombotic outcome in hypoxic conditions. Her objective was to try and integrate Ayurveda with genomics and to check whether a particular individual is more prone toward a specific disease. Subsequently, the results of the study helped her to identify specific genes for high-altitude illness among people and its adaptability, who were earlier identified on the basis of their *Prakriti*. These genes seem to match with the *Prakriti* of the subjects on the basis of the study results.

In yet another study (Patwardhan et al., 2005; Ghodke et al, 2011) conducted by Bhusan et al., the results are suggestive of positive correlation between *Prakriti* types and gene polymorphism associated with metabolic variability. The researchers in this study used Ayurvedic wisdom to hypothesize that individuals having different *Prakriti* have different metabolic rates owing to drug metabolism enzyme polymorphism. They performed CYP2C19 genotyping in 132 subjects using polymerase chain reaction—restriction fragment length polymorphism technique. The study results were suggestive of significant association between CYP2C19 genotype and different *Prakriti* categories with fast or slow metabolism being the distinguishing characteristics.

Prior to conducting this study, Prof. Bhusan had concluded a pilot study (Patwardhan et al., 2005) to determine the association between *Prakriti* and HLA DRB1 typing in a group of 76 subjects. A statistically reasonable correlation was found in this pilot study—HLA type and *Prakriti* type. While the observations from both these studies are likely to have significant impact on phenotype–genotype correlation, drug discovery, pharmacogenomics, and personalized medicine, the authors of this study are of the view that a large sample size would prove to be of statistical importance and validation and pave the way for modern approach toward drug development and not only help Ayurveda but also contemporary medicine.

Luckily, a lot of scientists and researchers have undertaken serious studies to observe the correlation between the *Prakriti* of an individual and his metabolism, his genomic associations, etc. A study result (Shirolkar, A. et al, 2018) was suggestive of altered metabolic pathways for each of the different *Prakriti*. Using a standard questionnaire and physical examination, 38 healthy volunteers were categorized in three categories of *Parkriti—Vata*, *Pitta*, and *Kapha*. The BMR varied among these individuals—less than 20 basal metabolic rate [basic metabolism rate (BMR)] was observed in *Vata* category, while 24.0–25.0 and >25.0 were observed for *Pitta* and *Kapha*, respectively. Highest level of high density lipoproteins (HDL), triglycerides (TG), and total cholesterol (TC) were found in the *Kapha Prakriti* individuals. Metabolites are the terminal products in human body metabolism and signaling molecules to initiate metabolic and catabolic pathways. Metabolomics deals with the levels of these metabolites in the body fluids and tissues in different conditions and also the physical characteristics may depend on these.

Govindaraj et al (2015) studied the correlation between genomic variations with the classification of Prakriti. A genome-wide SNP analysis was done on 262 males belonging to three Prakritis, namely, Vata, Pitta, and Kapha. 52 SNPs were significantly different between Prakritis, without any confounding and effect of stratification. Principal component analysis of these SNPs classified 262 individuals into their respective groups (Vata, Pitta, and Kapha) irrespective of their ancestry, which represent its power in categorization. Furthermore, 297 Indian population samples with known ancestry were validated, and it was observed that PGM1 correlates with the phenotype of Pitta as described in the ancient text of Caraka Samhita. Ayurveda defines characteristics of Pitta including digestion, metabolism, and energy production. *PGM1* gene is in the center of many metabolic pathways. The

finding suggests that the phenotypic classification mentioned in Ayurveda has a genetic basis.

Phenotypes of individuals mainly depend on the body metabolism. What emerges from this study is that plasma level of the simple metabolites can determine the *Prakriti* of a person. This can be the basis of diagnosis and individualized treatment in the near future. The study is suggestive of *Kapha Prakriti* individuals being predisposed to respiratory problems, slow lipid metabolism leading to lipid storage, and subsequently weight gain. In *Pitta Prakriti*, metabolism of xenobiotics and toxins was observed through cytochrome P450. In this group, disorders related to beta-oxidation were found to be more common due to increased lipid absorption. Melatonin that maintains the circadian rhythm, basal metabolism rate were found to be dominant—all these indicative of a high BMR. In *Vata Prakriti*, cellular detoxification process along with neurotransmitters, i.e., dopamine and glutamate was found to be dominant. These processes are important to excrete waste and cognition. Hence, *Vata* individuals may be more prone to having neurological problems and insulin resistance.

World Wide Web resources

There are several websites providing information on Ayurveda, Ayurvedic products, education, and research on Ayurveda. Few of the important websites are as follows:

Ministry of AYUSH, Government of India: www.ayush.gov.in

The website has multiple sections and covers education, research, AYUSH Health Care infrastructure etc. in India in a comprehensive manner.

Central Council for Research in Ayurvedic Sciences: www.ccras.nic.in

There are multiple Research Councils under the Ministry of AYUSH and Central Council for Research in Ayurveidc Sciences is one of them. There are about 30 Research Institutions under the Council, which are involved in multiple aspects of researches including multidisciplinary projects. Readers can visit the website for details on multifarious research activities of CCRAS.

AYUSH research portal: www.ayushportal.nic.in

This website provides comprehensive information about research on Ayurveda, both published and unpublished. The data on this site covers 5073 clinical trials, 11,835 preclinical studies, 7252 papers on drug research, and 4128 papers on fundamental research on Ayurveda. It also includes a section on Extra Mural Research Projects, where complete reports of the projects funded by Ministry of AYUSH are available.

3tandai

In addition to the aforementioned sites, there are multiple sites on *Prakriti* evaluation and lifestyle–related guidance as per the *Prakriti* profile. There is one interesting website, www.3tandai.com, which provides comprehensive information about one's *Prakriti*, relationship with DNA profile. This information is supported with comprehensive guidelines on lifestyle. However, a person needs to send his biological sample like sputum for DNA analysis and this service is paid.

Etha

www.ethalife.com: this website is from Europe. It provides simple tools to identify one's *Prakriti*. Also provides lifestyle-related guidance with simple stories linking nature with Ayurveda. The contents on this website have been customized to European population.

Way forward

Ayurveda is knowledge of life and should be accepted and practiced as such and should not be reduced to traditional system of medicine. Ayurveda lays major emphasis on preservation of health and prevention of disease; unfortunately, this aspect of Ayurveda has been neglected for a long time. At a time when people are looking for alternate lifestyle practices for healthy and long living the fundamental preachings of Ayurveda would come handy and may actually be called *Ayurvedic lifestyle practices*. A lot of research is required in the transformation of *Dhatus* (tissues) starting from *Rasa* to *Shukra*. Unfortunately, the scientists with their background in biochemistry and biotechnology are not even aware of such beautiful descriptions of Ayurvedic physiology.

Let us hope some of the readers of this book may pick up relevant topics for research from journey from *Rasa* to *Shukra* as well as develop simple tests to identify the *Prakriti* of individual to help and recommend appropriate customized lifestyle to meet the objective of "Jeevem sharadah shatam." (Let us live for 100 years.)

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Glossary

Ayruveda Ayurveda is made up of two words Ayu + Veda, meaning knowledge/science of life. It originated from India almost 3000 years ago.

Prakriti As per Ayurveda, everybody is born with individual psychosomatic characteristics, which are known as Prakriti. Knowledge of Prakrtiti was used to provide personalized medicine right from ancient times in India. This is an unique contribution of Ayurveda.

Panchamahabhuta There are five basic elements *Prithvi* (earth), *Jal* (water), *Tejai* (energy), *Vayu* (air), and *Akash* (space). Whole world is made up of these five priomoridal elements only and are common between living and nonliving things.

Tridoshas Ayurvedic physiology and concept disease is based on three physical (*sharirik*) and three mental *doshas* (*Manasika*). Physical doshas include *Vata*, *Pitta*, and *Kapha* and mental include *Satwika*, *Rajasika*, and *Tamasika*. Health and diseases depend on the balance of *doshas*.

Dinacharya Ayurvedic lifestyle recommended for daily routines.

Ritucharya Ayurvedic lifestyle recommends for seasonal routines.

Long answer questions

1. What are the eight branches of Ayurveda?
2. What are the various types of Vata, Pitta, and Kapha?
3. What are the various traits of Vata, Pitta, and Kapha types of persons?
4. What is Ritucharya and what is the significance in Ayurveda of each of these seasons?
5. Is there any scientific correlation between Prakriti and Genomics, please elucidate?

Short answer questions

1. What two words is Ayurveda made of?
2. What is considered as incompatible food in Ayurveda?
3. What is the *tripod of life* facilitating long and healthy life span?
4. What are the 7 *Dhatus* (tissues) in sequence?
5. What are the genomic studies which have validated Ayurvedic concept of Prakriti (individual psychosomatic constitution)?

Yes/no type questions

1. There are five types of Prakriti according to Ayurveda.
2. Ayurveda deals only with the cure of a disease.
3. Theory of *Panchamahabhuta* is deeply impregnated in all aspects of Ayurveda.
4. Teja is made up of ½ of *Agni* and 1/8 of *Jal*, *Prithvi*, *Vayu*, and *Akash*.
5. There are three *Doshas* in Ayurveda.
6. There are seven *Dhatus* (tissues) in Ayurveda.
7. As per Ayurveda, tongue is the seat of sense of hearing, eye is that of taste, and ear is that of touch.
8. As per Ayurveda, there are seven sense organs.
9. As per Ayurveda, mind is the seat of memory.
10. Charaka is considered the Father of Medicine and Sushruta is considered the Father of Surgery.

Answers to yes/no questions

1. There are three types of Prakriti—Vata, Pitta, and Kapha.
2. Ayurveda has a holistic approach to preserve the health of the healthy person (health promotive/disease prevention) and to treat a person when he falls ill (treating the disease).

3. This is one of the basic principles/tenets of Ayurveda.
4. This is true. Likewise, all elements exist in various combinations.
5. Two types of doshas—Sharirika and Manasika (physical and mental).
6. Yes. The seven tissues are Rasa, Rakta, Mamsa, Meda, Ashti, Majja, and Shukra.
7. Evidently no. Tongue is the seat of taste, eye is that of sight/vision, and ear is that of hearing.
8. According to Ayurveda, the five senses: hearing, sight, smell, touch, and taste are the organs used for perception of reality.
9. Yes.
10. Yes.

CHAPTER

34

Nanoparticle synthesis harnessing benign green routes

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Summary

Nanotechnology is a promising and emerging field of science that deals with synthesis, control, and applications of materials with one of the dimensions in range of 1−100 nm size. Chemical methods of nanoparticle synthesis often make use of hazardous and toxic chemicals that cause environmental pollution. In recent years biogenic routes of nanoparticles synthesis using bacteria, fungi, algae, plants, and biomolecules have been extensively studied and have emerged as viable and sustainable alternative. In this chapter we have described the biogenic methods available for nanoparticle synthesis with emphasis on ethical issues and resources available for the researchers and students.

What you can expect to know

In the initial sections of this chapter we shall understand the basic concepts in nanotechnology with its historical development. In the following sections we shall explore the latest developments in green synthesis of nanoparticles using various organisms and biomolecules. Various methods and relevant protocols for biogenic nanoparticle synthesis have been discussed with proper examples. In the later sections the translational and clinical significance and ethical issues related to nanoparticle research shall also be discussed. Some interesting and important discoveries and inventions in nanotechnology have also been discussed.

Introduction

Nanotechnology involves manipulation of individual atoms, molecules, or compounds into structures to produce materials and devices with at least one dimension between size 1 and 100 nm. Nanomaterials have special mechanical, electrical, thermal, or biological properties. Nanoparticles have become exceedingly important in the recent years due to its diverse applications in areas of computing, electronics, textile, agriculture, and medicine (Singh et al., 2017). In the biomedical field nanoparticles have found immense applications as an antimicrobial, anticancer, and inflammatory agent. Other applications include targeted drug delivery, tissue repair, tumor targeting, cell labeling, and magnetic resonance imaging (Singh et al., 2017).

Nanomaterials can be synthesized utilizing two broad approaches. The first approach is a top-down approach where process starts from a macroscopic bulk material gradually reduced to nanometer scale using processes such as etching and ball milling. The disadvantages of this approach are that the process is expensive and not suitable for large-scale production. The second approach called the bottom-up approach nanomaterials are generated starting from atoms. The latter is much cheaper and suited for large-scale nanomaterial synthesis. One of the widely used bottom-up strategy is the chemical reduction method where metal oxides are reduced using a suitable reducing agent. Three major classes of chemical species required in

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TABLE 34.1 Chemicals used in synthesis of nanoparticles (Duan et al., 2015).

Chemical	Role in nanoparticle synthesis	Toxic effects
Polypropyleneimine	Capping agent	Harmful
Cetyltrimethylammonium bromide	Capping agent	Harmful, dangerous for the environment
Ethylene glycol	Reducing agent	Harmful
Polyethylene glycol	Capping agent	Irritant
Trioctyl-phosphine oxide	Capping agent	Irritant
Polyacrylic acid	Capping agent	Toxic
Sodium borohydride	Reducing agent	Toxic and corrosive
Formaldehyde	Reducing agent	Toxic
Hydrazine	Reducing agent	Toxic and highly flammable
Dodecylamine	Capping agent	Dangerous for the environment
Ethanol	Solvent system	Harmful, highly flammable, toxic
Dimethylformamide	Solvent system	Toxic
Toluene	Solvent system	Harmful and highly flammable

this process are reducing agents, capping agents, and the solvents. Generally synthetic reducing agents, capping agents, and stabilizers that are used in this process are toxic in nature and pose considerable threat to the environment (Table 34.1). It was described by Anastus and Warner in their 12 principles for green chemistry (Anastas and Warner, 1998; Duan et al., 2015) that processes should aim at minimizing the uses of hazardous chemicals and solvents, if not completely eliminate them. On the other hand atom economy, energy efficiency, use of renewable feedstocks, and derivative reduction should also be given prime importance when considering a synthesis process. Biological processes or green processes using microorganisms, plants, or biomolecules mostly meet these criteria (Fig. 34.1). Biological synthetic processes are mostly carried out at ambient temperature eliminating the need for harsh conditions and high-energy requirements. In recent years green synthesis of nanoparticles has drawn significant attention of the scientific community reflected by the increasing number of publications. Green synthesis mostly presents a single-pot approach that eliminates the need of separate agents for reduction, stabilizing, and capping. Moreover, this approach is efficient, fast, economic, and eco-friendly and thus poses least threat to the environment. The end products or byproducts are generally biodegradable and hence does not pose any environmental threat. Bioprocesses use feedstocks that are renewable and sustainable in nature. Biological agents such as bacteria (Hulkoti and Taranath, 2014), fungi (Kitching et al., 2014), algae (Sharma et al., 2016), plants (Mittal et al., 2013), and biomolecules have shown immense potential for assembly of nanoparticles. Bacteria have shorter growth cycles and can be used industrially in nanoparticle synthesis both in an intracellular or a extracellular mode using fermentation process under controlled environment of bioreactors. Due to their shorter life cycles significant amount of nanoparticles can be synthesized in short span of time. In fungi the nanoparticle synthesis process is mostly extracellular and faster due to higher secretion of reducing proteins, thus making fungi a good a good choice for green nanoparticle synthesis. Similarly plants and algae also are good agents for green synthesis. Even crude extract from plants and microorganisms can quickly convert metal salts to nanoparticles bypassing any need for refined chemicals. In this chapter biogenic synthesis has been elucidated with the help of examples. Other than this, methods and protocols have been provided as guidance for novice researchers.

History

Nanotechnology is a relatively recent term to describe the understanding and control of matter in dimensions of 1–100 nm. Although the term nanotechnology was not there, nanomaterials have been around for several centuries. Earliest evidences of nanotechnology can be found from ancient India around 600 BCE where nanostructures like carbon nanotubes were found in wootz steel, a product that was exported throughout the world in those ages. The pottery from

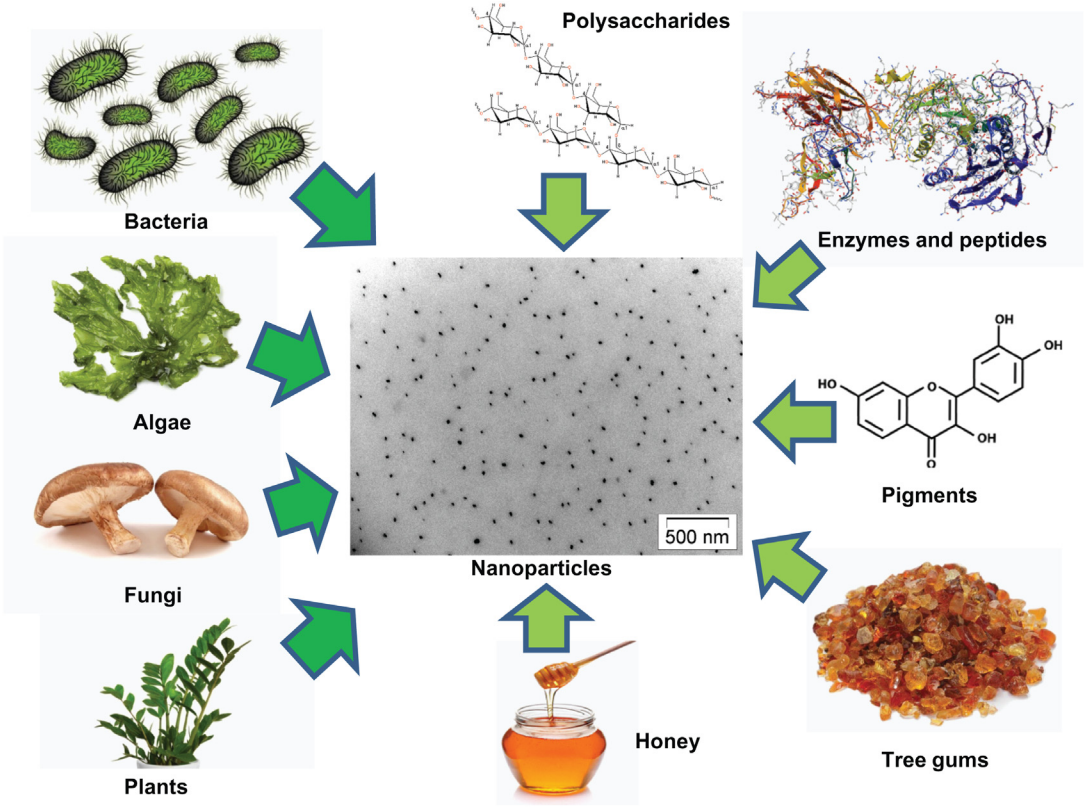


FIGURE 34.1 Biogenic routes of production of nanomaterials.

the middle ages showed extensive use of silver, copper, and gold nanoparticle to bring glaze and luster. In ancient Mesopotamia around CE 900, artisans were using some form of nanotechnology to bring glittering effect on the surface of pots. Romans also had a good knowledge of creating colloidal metal nanoparticle around the same age as evident from their pottery. In modern ages Michael Faraday discovered colloidal gold in 1857 and demonstrated its property of producing different colored solutions. Later in 1912 the concept of a nanometer was first proposed by Nobel laureate Richard Zsigmondy for measuring the particle size of colloidal gold. Father of modern nanotechnology Richard Feynman during a lecture entitled "There's Plenty of Room at the Bottom" in 1959 described the concept the manipulating matter at the nanometer level. Norio Taniguchi in 1974 coined the term "nanotechnology" to describe the process in semiconductors at the nanometer level. Eric Drexler in his book "*Engines of Creation: The Coming Era of Nanotechnology*" published in the year 1986 described the futuristic concept of a nanoscale assembler that can make copies of itself. The next major breakthrough was the development of carbon nanotubes by Japanese scientist Sumio Iijima of NEC Corporation in the year 1991. Following these developments the subject of nanomaterials started to show its impact on national policy making procedures. This was started in the year 2000 when US President Bill Clinton first advocated the research funding in nanotechnology. This was followed by enacting the 21st Century Nanotechnology Research and Development Act in year 2003. The National Nanotechnology Initiative (NNI), an US government R&D initiative, was formed. It comprised 20 departments and independent agencies involved in nanotechnology-related activities (Hulla et al., 2015). In India the government initially launched Nanoscience and Technology initiative followed by Mission on Nano Science and Technology (Nano Mission) launched in the year 2007. Recognizing the success of Nano Mission, the Union Cabinet accorded the approval for continuation of the Nano Mission in its Phase-II during the 12th Plan period with an allocation of INR 650 crores. Government funding agencies such as Department of Information Technology (DIT), Defence Research and Development Organisation (DRDO), Council of Scientific and Industrial Research (CSIR), and Department of Biotechnology (DBT) have provided the funding to researchers, scholars, and projects under this initiative where Department of Science and Technology (DST) is the nodal agency responsible for implementing the Nano Mission.

Principle

One of the widely used methods in the bottom-up approach is through the reaction of metal salts with suitable reducing agents. The basic principle is the reduction of metal salts to their respective zerovalent state yielding pure metal nanoparticles as end products. This basic reduction process occurs in all processes involving either chemical or biological agents. In biologically mediated processes the reduction reactions occurs intracellularly or extracellularly with single or multiple biomolecules capable of donating electrons and thus act as reducing agents much like chemical reducing agents. In microorganism such as bacteria, the nanoparticle production is the outcome of a mechanism in which the bacteria tries to remove or neutralize toxic compounds present in its vicinity. To ensure its survival the bacteria transforms the toxic metal compounds to lesser harmful forms using redox chemistry. Intracellular synthesis comprises transportation of ions into the bacterial cells to form nanoparticles, while extracellular synthesis involves trapping metal ions on the surface of the cells and reducing the ions in the presence of enzymes and other metabolites produced by the bacteria. In algae conversion of metal salts to the corresponding pure nanoparticles occur due to reducing actions of enzymes such as reductases and other biomolecules like carbohydrates or polyphenols present on the surface and inside the algal cells. In commercial aspects the extracellular synthesis of nanoparticles is generally a method of choice due to the ease of downstream processing in contrast to the intracellular synthesis where processes such as cell lysis and subsequent purification steps make the process quite cumbersome. The production of nanoparticles is also linked with processes like bioremediation and bioleaching. In case of fungi the location of nanoparticle synthesis is generally extracellular making the downstream processing easier. The fungal secretome, which is rich in reducing enzymes, facilitates the reduction process in fungi-mediated nanoparticle synthesis (Kitching et al., 2014).

Methodology

Nanoparticles synthesis using biological materials is a bottom-top approach where living organisms or biological molecules extracted from them provide the reducing power. In this section we have described some generalized methods and protocols used for synthesis of nanoparticles using various microorganism, plants, or biomolecules (Flow Chart 34.1). Researchers working on such synthesis may use these methods and protocols as a guideline and make the required changes specific to their requirement.

Materials required

Glasswares/plasticwares: Erlenmeyer flasks, beakers, measuring cylinders, glass pipettes, test tubes, screw cap bottles

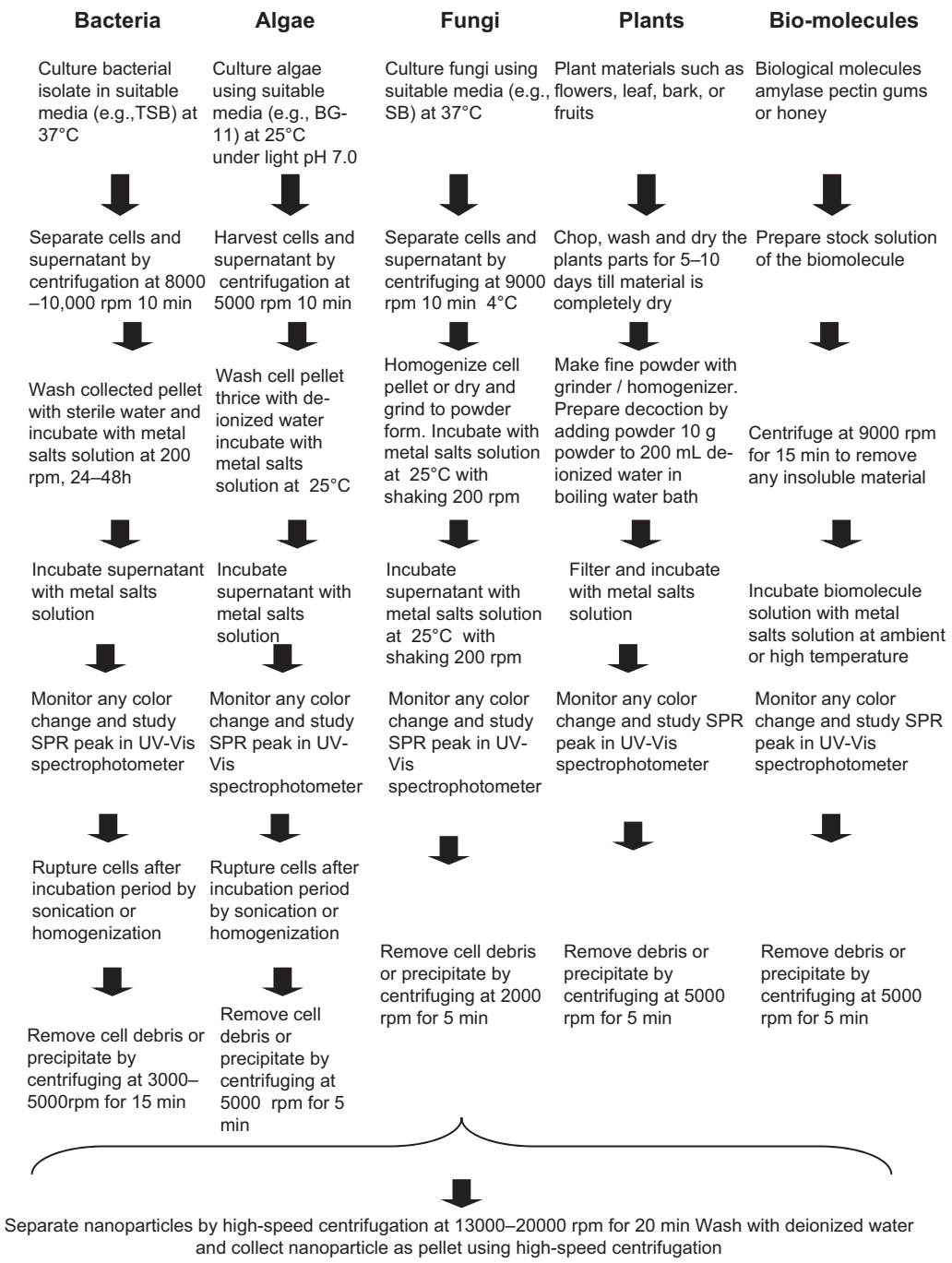
Instrument/equipment: Micropipettes, magnetic stirrer with heating/hot plate, microwave oven, refrigerated centrifuge, weighing balance (milligrams), mixer grinder /hand blender/homogenizer.

Chemicals/reagents: Silver nitrate ($AgNO_3$; MW, 169.87), chloroauric acid ($HAuCl_4$; MW, 339.78), zinc nitrate hexahydrate ($ZnNO_3.6H_2O$; MW, 297.49), neem gum, pectin, amylase, sodium hydroxide (NaOH; MW, 39.99), hydrochloric acid (HCl; MW, 36.4), tryptic soy broth (TSB), Sabouraud dextrose broth (SDB), BG11 (blue green) Broth, and deionized water.

Methods

Nanoparticle production using bacteria: Nanoparticles can be produced by using either bacterial cells or the culture supernatant. In both these cases the bacterial metabolites such as proteins, peptides, and the other small molecules reduce metal oxides to nanoparticles. While using cell pellet the synthesis takes place inside cell and nanoparticles have to be extracted using cell disruption methods such as ultrasonication or homogenization. Using culture supernatant is the straightforward method as no cells are involved in this process, and downstream processing is much easier. Briefly bacterial cultures are incubated in sterilized bacterial culture media like TSB usually at 37°C to attain sufficient cell density. Cell pellet is washed and incubated with metal salt solution. Postincubation for 1−2 days the cell pellet is obtained and ultrasonicated to rupture cells. Following this the cell debris are removed and nanoparticles are purified through centrifugation. When culture supernatant is used for nanoparticle synthesis, the bacterial cells are discarded by centrifugation. The cell-free supernatant is mixed with metal salt solution and incubated. Nanoparticle synthesis is monitored through change in the color of the reaction mix and more precisely through measurement of surface plasmon resonance (SPR) peak through UV-Vis spectrophotometer. Postsynthesis the nanoparticles are purified and collected through centrifugation (Singh et al., 2015, 2016).

Nanoparticle production using fungi: Synthesis using fungi may be done using two different approaches. In the first approach the fungal culture supernatant is used for nanoparticles synthesis. In second approach the biomass is homogenized, and the extract is used for the nanoparticles synthesis. Fungal cells may be wet homogenized or dried to obtain the biomass powder. This extract either in a liquid form or a powdered form may be used for nanoparticle synthesis. Fungal cells are inoculated into a suitable media such as SDB



FLOW CHART 34.1 Nanoparticle synthesis by biogenic routes.

and incubated at 30°C for 3–4 days to obtain sufficient biomass. After sufficient growth is obtained, the fungal biomass is separated and the cell-free supernatant is used for nanoparticle synthesis (Velhal et al., 2016).

Nanoparticle production using algae

Algae are photosynthetic organisms and hence require light for growth. Hence the algal cultures are maintained in a media such as BG11 broth under optimal light conditions. Log-phase algal cells are generally harvested for the production of nanoparticles as these have high concentration of enzymes and other biomolecules of reducing nature. Algal extract-based synthesis is the most widely used method for the preparation of nanoparticles. Algal extract is obtained either through homogenization of the wet and dried algal cells or even through simple boiling procedure.

Alternatively algal biomass or culture supernatant may also be used using a procedure similar to bacterial or fungal cells (Patel et al., 2015; Ramkumar et al., 2017).

Nanoparticle production using plants

Nanoparticle extraction using plant materials is relatively a simple approach in which plant extract or decoction of the plant part is generally prepared. Plant parts are rich in small organic molecules such as alkaloids, terpenoids, and flavonoids apart from high-molecular-weight molecules. When a plant part such leaf, flower, stem, or fruits are homogenized or boiled, these compounds gets extracted, which can be used for nanoparticle production. Usually plant part like flowers, leaves, stems, or fruits are dried before preparing a decoction. Rest of the procedures for purification of nanoparticles are similar to those used for bacteria, fungi, and algae (Sathishkumar et al., 2016; Singh et al., 2016).

Nanoparticle production using biomolecules

Nanoparticles synthesis using bacteria, fungi, plants, and algae takes advantage of the reducing power of thousands of biomolecules present in them. However as the reduction process is the result of several molecules, the nanoparticles synthesized also has the tendency to vary in terms of size, shape, and other properties. There is an option to use commercially available purified biomolecules such as alginate (Vijayakumar et al., 2016), pectin (Umasuganya et al., 2016), or amylase (Mishra and Sardar, 2012) or extract these molecules and use them to synthesize nanoparticles. In case of biomolecules the synthesis method is fairly straightforward involving simple incubation at ambient or high temperatures. Rest of the procedures for purification of nanoparticles is similar to those used for bacteria, fungi algae, or plants.

Examples

Bacteria-mediated synthesis of nanoparticles

The microbial life on the earth started approximately four billion years ago. Among microorganisms, bacteria have the most remote history of origin inhabiting some of the most hostile environments on the earth. One of these adaptations related to survival is their ability to convert toxic metal compounds into pure metal. The early studies on the production of nanoparticles using bacteria started early in 1984 with studies using *Pseudomonas stutzeri* AG259 that was isolated from a silver mine and showed deposition of silver on the cell membrane (Haefeli et al., 1984). Since then bacteria such as *Aeromonas*, several *Bacillus* species such as *Bacillus amyloliquefaciens*, *Bacillus licheniformis*, *Bacillus methylotrophicus*, *Bacillus subtilis*, *Brevibacterium* (Singh et al., 2015), *Corynebacterium*, *Desulfovibrio*, *Enterobacter*, *Escherichia*, *Klebsiella*, *Lactobacillus*, *Pseudomonas*, *Rhodobacter*, *Streptomyces*, and *Weissella* (Singh et al., 2016) have been reported to produce nanoparticles either extracellularly or intracellularly from metal precursors. The nanoparticles synthesized by bacteria have been found to have antimicrobial action and cytotoxic effect against cancer cell lines.

Algae-mediated synthesis of nanoparticles

Algae are diverse group of autotrophic unicellular or multicellular organisms, which are photosynthetic like plants, but unlike plants, they lack vascular elements such as xylem and phloem tissues. Although algal biosynthesis of nanoparticles is a less explored route compared to plants or bacteria, in recent years, algae have been used to synthesize different metal and metal oxide nanoparticles such as silver, gold, iron, and palladium. Recent studies using green algae such as *Enteromorpha*, *Chlorella*, *Parachlorella*, *Chlamydomonas*, *Coelastrum*, *Scenedesmus* and *Botryococcus*, *Spirogyra*, *Caulerpa*, *Pithophora*, and *Euglena* in nanoparticles biosynthesis (Patel et al., 2015; Ramkumar et al., 2017). Brown algae like *Sargassum*, *Turbinaria*, and *Ecklonia* have also shown a good potential for the synthesis of metal and metal oxide nanoparticles. Likewise blue green algae (bacteria) like *Calothrix*, *Anabaena*, *Aphanizomenon*, *Cylindrospermospsis*, *Lyngbya*, *Limnothrix*, *Synechocystis*, and *Synechococcus* (Patel et al., 2015) also reduces metals to respective nanoparticles. Red algae like *Gracilaria*, *Laurencia*, and *Chondrus* have been used in nanoparticles synthesis. Similarly, diatoms that are unicellular algae enclosed within a cell wall and are made up of silica like *Amphora* has also shown their potential in silver nanoparticles biosynthesis.

Fungi-mediated synthesis of nanoparticles

The production of nanoparticles using fungal system has several advantages over their bacterial counterparts. Fungi in general have higher metal tolerance, which means they can resist higher metal salt concentrations, which lead to higher NP yields. Fungal strains such *Arthroderma*, *Aspergillus*, *Macrophomina*, *Neurospora*, *Penicillium*, *Rhizopus*, and *Trichoderma* have been reported to produce nanoparticles. Other than these fungal species, edible mushrooms such as *Agaricus* and *Pleurotus* also have been found promising

in green synthesis of nanoparticles. Baker's yeast *Saccharomyces* has also been found to synthesize nanoparticles. In most of the cases the mode of nanoparticles synthesis was predominantly extracellular. The resulting nanoparticles showed a wide range of biological activities (Velhal et al., 2016).

Plant-mediated synthesis of nanoparticles

Plants have shown a great potential for detoxification and bioremediation of the toxic metals by conversion of toxic soluble metal compounds to lesser toxic and insoluble forms. For example the synthesis of gold nanoparticles have been demonstrated by *Medicago sativa* and *Avena sativa* biomass. Similarly, alfalfa (*Medicago sativa*) biomass has been reported to accumulate magnetite nanoparticles. *Sesbania*, a leguminous plant, have been demonstrated to accumulate high concentrations of Au inside its cells in the form of monodisperse gold nanoparticles. Nanoparticles formed intracellularly needs to be extracted in their intact active form, which leads to a relatively cumbersome downstream processing. On the contrary, nanoparticles synthesis using plant extract is a much simpler and straightforward approach requiring minimum purification steps, hence the most extensively used method. Various plant parts such as stems, leaves, seeds, fruits and roots (Sathishkumar et al., 2016; Singh et al., 2016), and flowers have been used for this purpose and the extract of these has been used as a biological reducing agent. Other than these plant by-products such as plant husk, peel, and bark also have been successfully used for nanoparticles biosynthesis. Intracellular and extracellular synthesis of metal nanoparticles becomes possible due to the presence of enzymes and other organic molecules like pigments present inside plants cells as well as its extracts.

Biomolecule-mediated synthesis of nanoparticles

In case of microorganisms or plants the production of nanoparticles is either by living organisms or their extracts. In both the cases the conversion of metal precursors to nanoparticles was affected by a crude mix of various biomolecules where no single molecule can be attributed to the reduction process. Contrary to this nanoparticles can also be produced using pure biomolecules extracted from living systems. The advantages of using this approach are improved ease of purification and better control of the production parameters. Several types of biomolecules such as plant- and microbial-based polysaccharides, plant and microbial enzymes, tree gums, and small molecules such as biosurfactants and pigments have been found to be effective in nanoparticle synthesis.

Alginate-mediated synthesis

Synthesis of metal nanoparticles has also been established using alginic acid, an anionic polysaccharide, obtained from the cell wall of brown algae, generally available in the form of its sodium salt. Aqueous sodium alginate is transformed into a hydrogel when it comes in contact with multivalent ions such as Ca^{2+}, Fe^{2+}, and Zn^{2+}, etc. having a typical egg box−like structure. It can be used for the green synthesis of nanoparticles because of its mild reducing activity and biocompatibility. In a recent study silver nanoparticles with an average size less than 30 nm were produced using sodium alginate as a stabilizer (Vijayakumar et al., 2016).

Pectin-mediated synthesis

Pectin a structural heteropolysaccharide obtained commercially from citrus and apple although it has ubiquitous in cell wall of plants. It is nontoxic and hence widely used in food and pharmaceutical industry for various applications. The presence of functional groups such as carboxyl and hydroxyl groups makes pectin suitable for chelating with various metals. Due to its properties like good biocompatibility, lower toxicity, and biodegradability, it has been actively evaluated as an alternate source for nanoparticle synthesis. Recently pectin isolated from *Musa paradisiaca* (banana) has been reported to be successfully employed in synthesis of gold nanoparticles at ambient conditions (Umasuganya et al., 2016). Rapid synthesis of gold nanoparticles was carried out at 55°C using this pectin sample, which reduced the chloroauric acid to spherical gold nanoparticles with an average size of 8 nm.

Microbial exopolysaccharide-mediated synthesis

The reducing and stabilizing capacity is not restricted to plants polysaccharides but also extends to microbially derived exopolysaccharides that have displayed their tremendous potential in recent studies. Xanthan gum (XG), another extracellular polysaccharide produced by strains of *Xanthomonas campestris*, has been used for green synthesis of nanoparticles. For example in a recent study by Muddineti et al. XG-stabilized PEGylated gold nanoparticles have been produced where XG acts as a stabilizer in the presence of ascorbic acid as a reducing agent. The synthetic procedure involved the reaction of the aqueous solutions of $HAuCl_4$ and XG followed by the addition of ascorbic acid, which led to the synthesis of stabilized XG-

AuNPs. The XG-AuNPs had a mean hydrodynamic diameter of 80 nm where particle size showed a gradual increase on increasing the concentration of $HAuCl_4$ (Muddineti et al., 2016).

Pullulan is a polysaccharide produced from starch substrate by the fungus *Aureobasidium pullulans*. It consists of maltotriose units in which glucose are connected via α-1,4 glycosidic bond, whereas the maltotriose units are interconnected by α-1,6 glycosidic bond. Pullulan is edible polysaccharides and has E number of 1204. This microbial polymer has been extensively evaluated for green synthesis of nanoparticles, for example, in a recent study pullulan was used as both a reducing agent and a stabilizer for silver nanoparticles. The synthesis procedure involved reaction of the aqueous solution of pullulan and silver nitrate under autoclaving conditions (121°C for 15 minutes) followed by its storage in dark for 3 months. The resulting AgNPs were having various shapes such a rod-shaped, hexagonal, oval, and spherical with sizes ranging from 2 to 40 nm (Kanmani and Lim, 2013). Another study describes pullulan and its oxidized derivative, 6-carboxypullulan for synthesis of silver nanoparticles (Coseri et al., 2014).

Similarly the high molecular mass exopolysaccharides from diazotrophic *Bradyrhizhobium japonicum* 36 also converted silver salts to silver nanoparticles (Rasulov et al., 2015). The total exopolysaccharide from this species consisted of two fractions, one high molar fraction (HMW) insoluble in ethanol and another low molar mass fraction (LMW) soluble in ethanol. Both these fractions were able to produce spherical NPs with a difference in their respective plasmon resonance peaks at 420 and 450 nm, respectively for LMW NPs and HMW NPs. The LMW NPs had a size distribution in the range of 5–20 nm, while HMW NPs were bigger with size ranging 20–50 nm.

Nanoparticle synthesis based on tree gums

Gums are heterogeneous polysaccharides found in plants, animals, and many bacteria having complicated structures and are hydrocolloids by nature. These are hydrophilic in nature due to a large number of hydroxyl groups, arranged in a regular manner along the backbone of the molecule. Gum hydrocolloids are effective water adsorbents, and this property is the key to their use in foodstuffs and enhanced oil recovery.

Gum ghatti

Gum ghatti (GG) is a naturally occurring water complex polysaccharide derived as an exudate from the bark of *Anogeissus latifolia* (Combretaceae family), a native tree of the Indian subcontinent. This biopolymer has high-arabinose content and is an acidic heteropolysaccharide occurring in nature as mixed calcium, magnesium, potassium, and sodium salt. This gum was used as both a reducing and a stabilizing agent for synthesis of silver nanoparticles. The synthesis was done by autoclaving solution of GG with silver nitrate at 121°C. By varying the concentration of the GG and autoclaving time, the size of the nanoparticles could be controlled. Using this method spherical and monodispersed AgNPs with average diameter of 5.7 nm could be obtained (Kora et al., 2012).

Gum arabic (gum acacia)

Gum arabic (GA) also known as acacia gum is a branched, neutral, or slightly acidic, complex polysaccharide obtained from the stems and branches of *Acacia senegal* and *Acacia seyal*. It is widely used in food and pharmaceutical applications. GA has mostly been used as capping agent for nanoparticles. In a study gold nanoparticles were produced by using a novel approach using GA and synchrotron X-ray radiation. Here an aqueous solution of $HAuCl_4$ and GA was irradiated with X-rays from a synchrotron (4–30 keV, 105 Gy/s) at room temperature for 5 minutes with no other chemical reducing agents or catalysts. Using this method very small nanoparticles (Au-SNPs) of average diameter of 1.6 nm were produced. GA capped particles (Au-SNPs@GA) of diameter 40 nm were also produced (Liu et al., 2013).

Neem gum

Neem gum (NG) is naturally occurring water-soluble phytoexudate derived from the bark of *Azadirachta indica* (family Meliaceae) containing complex polysaccharides. This efficacy of this gum to produce nanoparticles was studied recently with silver nanoparticles. The silver nanoparticles (AgNPs) were produced by the reaction of aqueous solutions of NG and silver nitrate under autoclaving conditions (121°C, 15 psi) for varying time of 10–60 minutes (Velusamy et al., 2015). Another study describes the production of zinc oxide nanoparticles using NG as both a reducing agent and a stabilizer. Like the earlier studies here the aqueous solution of zinc nitrate was allowed to react with varying concentrations of NG under alkaline conditions at room temperature (Geetha et al., 2016).

Microbial biosurfactant–mediated synthesis of nanoparticles

Biosurfactant are surface-active molecules produced by variety of microorganisms. Like chemical

surfactants these too can reduce surface and interfacial tension of water. Due to these interesting properties they find application in several industrial processes and biomedical fields (Mukherjee et al., 2006). In the recent years the ability of the biosurfactants to stabilize metal nanoparticles has been harnessed evident through several studies. One property of biosurfactants that has been readily used to synthesize is the use of reverse micelles. In an organic solvent the biosurfactant form micelles where the nonpolar group faces the solvent, while the polar group faces inside creating a microenvironment where the metal salt and reducing agent can react and give rise to size-controlled nanoparticles. One such study involves the use of rhamnolipid type of biosurfactant obtained from *Pseudomonas aeruginosa* for the synthesis silver nanoparticles (Ganesh et al., 2010).

Enzyme-mediated synthesis of nanoparticles

Enzyme-based synthesis of nanoparticles has been extensively investigated in the recent years. Investigators have evaluated multiple enzymes such as α-amylase, diastase, cellulase, reductases, laccase, and Bromelain for their efficacy in reduction and stabilization of various nanoparticles. The use of enzyme α-amylase was reported for the synthesis of silver nanoparticles. The synthesis procedure involves the incubation of alpha-amylase solution with a solution of $AgNO_3$ in the ratio of 2:3 at 25°C with periodical monitoring. The nanoparticle synthesis was complete by 36 hours evident from saturation in the absorption peak. The resulting nanoparticles were found to be triangular and hexagonal shaped in transmission electron microcopy with size ranging from 22 to 44 nm (Mishra and Sardar, 2012). Likewise gold nanoparticles of 2–20 nm were also synthesized by the same group using alpha-amylase. Excellent catalytic activity was shown by these AuNPs in complete conversion of *p*-nitroaniline and *p*-nitrophenol to *p*-phenylenediamine and *p*-aminophenol, respectively (Mishra and Sardar, 2014).

Pigment-mediated synthesis of nanoparticles

Biological pigments are the organic substances produced by the living organism and obtain color due to selective absorption of certain wavelengths of light. They are present in bacteria, algae, plants, and animals and have specific functions. Industrially they find application in food industry and as dyes. Recently pigments have been evaluated for their ability to synthesize metal nanoparticles, for example, flexirubin extracted from *Chryseobacterium artocarpi* was utilized in the synthesis of silver nanoparticles (Venil et al., 2016).

Honey-mediated synthesis of nanoparticles

Simple agro products like honey have been evaluated for green and facile synthesis of cerium oxide nanoparticles in a recent study. The synthetic process included initial reaction of aqueous solution of Ce $(NO_3)_3 \cdot 6H_2O$ with honey at 60°C and then at higher temperatures up to 800°C leading to dry CeO_2-NPs. Spherical CeO_2-NPs were synthesized having an average diameter of 23 nm although particles up to 35 nm were detected (Darroudi et al., 2014). The use of common agro-based material like honey as a reducing and stabilizing agent has certain added advantages like easy availability and cost economy.

Protocol

Nanoparticle production using bacteria

Synthesis using bacterial cell pellet (Singh et al., 2016):

- Inoculate bacterial isolate into 100 mL of sterilized TSB in 250 mL Erlenmeyer flasks.
- Incubate in a rotating shaker set at 37°C, 120 rpm for 24 hours.
- Collect the bacterial pellet by centrifugation at 10,000 rpm for 10 minutes.
- Wash the collected pellet thoroughly with sterile deionized water
- Dissolve in 20 mL of sterile deionized water, with a final concentration of 1 mM of filter-sterilized solution of $AgNO_3$.
- Incubate the reaction mixture for 1–2 days in an orbital shaker at 200 rpm and 25°C.
- Monitor the synthesis by color change by visual inspection.
- At the end of the incubation period, centrifuge at low speed (3000 rpm) for 5–10 minutes to remove the cell debris.
- Rupture the bacterial cells by repeated cycles of ultrasonication, followed by washing with sterile deionized water, and centrifugation.
- Collect silver nanoparticles by high-speed centrifugation at 13,000–20,000 rpm for 10 minutes.
- Wash the obtained product thoroughly with methanol and water to remove the undesired components.
- Perform high-speed centrifugation at 13,000–20,000 rpm for 10 minutes to collect nanoparticles as pellet.

Synthesis using bacterial culture supernatant (Singh et al., 2015):

- Inoculate bacterial isolate into 100 mL of sterilized TSB in 250 mL Erlenmeyer flasks.

- Incubate in a rotating shaker set at 37°C, 120 rpm for 24 hours.
- Centrifuge the culture at 8000 rpm for 10 minutes to remove the bacterial pellet.
- Mix the supernatant with filter-sterilized $AgNO_3$ solution (1 mM final concentration) and incubate in an orbital shaker at 200 rpm and 25°C.
- Monitor the synthesis of silver nanoparticles by visual inspection for a change in the color of the culture medium. Alternatively withdraw samples for detection of any characteristic SPR peak (400–450 nm) using a spectrophotometer.
- After the completion of the incubation period, centrifuge the mixture at 2000 rpm for 5 minutes to remove any medium components.
- Perform high-speed centrifugation at 16,000 rpm for 20 minutes to collect silver nanoparticles.
- Wash the obtained product several times by centrifugation and redisperse in water to remove the unconverted silver ions and any medium components.
- Finally perform a high-speed centrifugation at 16000 rpm for 20 minutes to collect the AgNPs form of a pellet.

Synthesis using fungal culture supernatant

Nanoparticle production using fungi (Velhal et al., 2016):

- Inoculate fungal isolate into 100 mL of sterilized fungal media in 250 mL Erlenmeyer flasks.
- Grow cells aerobically at 30°C under shaking at 120 rpm for 72 hours.
- After growth centrifuge the culture containing medium at 9000 rpm for 10 minutes at 4°C.
- Mix supernatant with filter-sterilized $AgNO_3$ solution (1 mM final concentration) and incubate in an orbital shaker at 200 rpm and 25°C.
- Monitor the synthesis of nanoparticles by visual inspection for a change in the color of the culture medium. Alternatively withdraw samples for detection of any characteristic SPR peak using a spectrophotometer.
- After the completion of the incubation period, centrifuge mixture at 2000 rpm for 5 minutes to remove any medium components.
- Collect nanoparticles by high-speed centrifugation at 16,000 rpm for 20 minutes.
- Wash the pellet several times by centrifugation and resuspend in deionized water to remove the unconverted metal ions and any other medium components.
- Finally collect AgNPs in the form of a pellet using high-speed centrifugation.

Synthesis using fungal biomass

- Inoculate fungal isolate into 100 mL of sterilized fungal media in 250 mL Erlenmeyer flasks.
- Grow cells aerobically at 30°C under shaking at 120 rpm for 72 hours.
- After growth centrifuge the culture containing medium at 9000 rpm for 10 minutes at 4°C.
- Filter the grown biomass was and wash three times in the sterile deionized water.
- Ground the biomass was 1:1 in deionized water (w/v) using an agate mortar.
- Centrifuge the mixture for 5 minutes at 6500 rpm and filter through a 0.2-μm microfilter.
- Alternatively wash the fungal biomass thrice with one volume of the sterile deionized water.
- Vacuum dry the biomass at 50°C and grind to fine powder when completely dry.
- Use the dry fungal biomass or the fungal extract to synthesize nanoparticles.
- Mix fungal biomass or extract with $AgNO_3$ solution (1 mM final concentration) and incubate in an orbital shaker at 200 rpm and 25°C.
- Monitor the synthesis of nanoparticles by visual inspection for a change in the color of the culture medium. Alternatively withdraw samples for the appearance of any characteristic SPR peak using a spectrophotometer.
- After the completion of the incubation period, centrifuge mixture at 2000 rpm for 5 minutes to remove any medium components.
- Collect nanoparticles by high-speed centrifugation at 16,000 rpm for 20 minutes.
- Wash the pellet several times by centrifugation and resuspend in deionized water to remove the unconverted metal ions and any other medium components.
- Finally collect Ag nanoparticles in the form of a pellet using high-speed centrifugation.

Nanoparticle production using algae (Patel et al., 2015; Ramkumar et al., 2017):

- Maintain algal cultures through usual subculturing techniques under laboratory conditions at 25°C, under optimal cool white fluorescent light, in algal medium such as BG11 (generally at pH 7.0).
- Harvest the log-phase cultures of microalgae and cyanobacteria by centrifugation at 5000 rpm for 10 minutes at 20°C and wash three times with sterile deionized water.
- Suspend biomass of each culture in 20 mL of 1 mM aqueous $AgNO_3$ solution, pH 7. Alternatively use the cell-free culture liquid obtained in the previous centrifugation. Solution of $AgNO_3$ should be added

to cell-free culture liquid to make up final concentration of 1 mM.
- Incubate at 25°C under cool white fluorescent light or dark (provided by wrapping the flasks with aluminum foil) for 72 hours.
- Keep fresh algal medium with addition of $AgNO_3$ as a control.
- Take samples at every 12-hour intervals (0, 12, 24, 48, and 72 hours) in 1 mL aliquots samples, centrifuge, and take the absorbance using a spectrophotometer.
- Stop incubation when a prominent SPR peak is detected (between 400 and 450 nm in case of AgNPs).
- Centrifuge mixture at 5000 rpm for 5 minutes to remove any medium components or algal biomass.
- Collect nanoparticles by high-speed centrifugation at 13,000–20,000 rpm for 20 minutes.
- Wash the pellet several times by centrifugation and resuspend in deionized water to remove the unconverted metal ions and any other medium components.
- Finally collect AgNPs in the form of a pellet using high-speed centrifugation.

Nanoparticle production using plants (Sathishkumar et al., 2016):

- Chop the plant part finely into small pieces. Any unwanted part that may interfere with the extraction process, for example, fibrous peels should be removed.
- Wash the chopped plant parts thoroughly thrice with deionized water and dry in shade for 5–10 days.
- Make fine powder by grinding with commercial electrical stainless steel blender. Use this fine powder to prepare a simple decoction.
- Take 10 g of plant part powder and mix with 200 mL of deionized water and keep in boiling water bath for 10–20 minutes. This time has to be optimized for each plant part under investigation.
- After cooling, filter the extract through Whatman No. 1 filter paper and store at 4°C for further use.
- Prepare 1 mM substrate ($HAuCl_4$ for gold nanoparticles and $AgNO_3$ for silver nanoparticles) in deionized water. Adjust the extract volume so that the substrate remains 1 mM. Standardize the nanoparticle synthesis by adjusting the extract volume.
- Optimize parameters such pH, temperature, and reaction time to improve nanoparticle quality and yield.
- Check the reaction mixture for the development of color change and SPR maxima should be monitored using a spectrophotometer.
- Postsynthesis centrifuge mixture at 5000 rpm for 5 minutes to remove any plant extract components or precipitates.
- Wash the pellet several times by centrifugation and resuspend in deionized water to remove the unconverted metal ions and any other medium components. Collect AgNPs or AuNPs in the form of a pellet using high-speed centrifugation at 13,000–20,000 rpm for 20 minutes.
- Alternatively perform dialysis of the reaction mixture repeatedly by using dialysis membrane of suitable cut-off against 1000 mL of deionized water for 9 hours at 30°C.
- Finally collect nanoparticles in the form of a pellet using high-speed centrifugation.

Nanoparticle production using biomolecules

Synthesis using enzyme (Mishra and Sardar, 2012):

- Enzymes like alpha-amylase can be used for the synthesis of gold and silver nanoparticles.
- Prepare chloroauric acid $HAuCl_4$ solution (1 mM) and Tris-HCl buffer (pH 9.0).
- Prepare alpha-amylase working solution 4 mg/mL in previously prepared Tris-HCl, pH 9.0. For this add 240 mg pure alpha-amylase enzyme in 40 mL of deionized water. After the enzyme dissolves completely make up the volume to 60 mL with deionized water.
- Incubate 40 mL of pure alpha-amylase solution (4 mg/mL) and 60 mL of aqueous solution of chloroauric acid (1 mM) and incubate with or without shaking at 25°C.
- Withdraw samples at regular intervals and monitor using spectrophotometer.
- Keep one control in which only $HAuCl_4$ solution is taken without the enzyme alpha-amylase and record the spectra after regular intervals of time.
- On appearance of the characteristic peak of AuNPs at 525 nm, centrifuge the solution at 9000 rpm for 25 minutes at 4°C.
- Discard the clear supernatant and wash the pellet five times with distilled water.
- Air dry the pellet and use for further studies and use.

Synthesis using tree gums (Velusamy et al., 2015; Geetha et al., 2016):

- Use commercially available tree gums such as guar gum, gum arabic (GA) or use locally available gums such as NG.
- Collect and dry NG exudates of *Azadirachta indica* and dry for 2–3 weeks until completely dry.
- Use a kitchen mixer grinder/blender to obtain a fine powder of the dried gum.

- Use a fine sieve to collect the gum in a powdered form and store at room temperature for further use.
- Prepare a homogenous 0.5% (w/v) gum stock solution by adding the fine powder of sieved gum to amber bottle containing deionized water with constant stirring on a magnetic stirrer at 60°C for 2 hours.
- Centrifuge this solution to remove the insoluble materials use the supernatant for preparing various metal nanoparticles by variation of the method described for silver or zinc oxide nanoparticles.
- Add 2.97 g zinc nitrate hexahydrate to 0.1%–0.5% (w/v) gum stock solution to obtain 0.1 M solution. Keep under constant stirring to dissolve zinc nitrate.
- Add 0.1 N NaOH dropwise and stir for about 2 hours. Check the production of ZnO nanoparticles by detecting any absorption peak 350–380 nm.
- Allow the reaction mixture to settle for overnight, centrifuge, and discard the supernatant.
- Wash the nanoparticles by centrifugation and redispersing several times with deionized water to remove the impurities.
- Dry nanoparticles at 80°C overnight in hot air oven for complete conversion of zinc hydroxide into zinc oxide to obtain ZnONPs.
- For preparing silver nanoparticles using NG prepare 1 mM $AgNO_3$.
- Mix 1 mL (0.1–0.5% w/v) of the gum extract to 7 mL solution of 1 mM aqueous silver nitrate ($AgNO_3$).
- Autoclave the reaction mixture at 121°C and 15 psi for 20 minutes. Nanoparticle synthesis can be monitored using UV-vis spectra for SPR band between 400 and 430 nm.
- Purify the AgNPs by repeated centrifugation at 12,000 rpm for 20 minutes followed by resuspension of the pellet in deionized water.
- Finally pellet AgNPs by centrifuging at 12,000–16,000 rpm for 20 minutes and dry at 80°C overnight.

Synthesis using plant polysaccharide (Umasuganya et al., 2016):

- Plant polysaccharide such as pectin can be either extracted from plant material or purified commercial pectin can be used for synthesis of nanoparticles.
- Generally pectin can be extracted from plant materials by hot water extraction method.
- Suspend dried plant cell wall material such as fruit peels in deionized water in a solid–liquid ratio of 1:25 (w/v) and stir the suspension at 60°C for 2 hours.
- Filter the resulting slurry using Whatman No. 1 filter paper and resuspend the residue again in deionized hot water with stirring.
- Collect the filtrate from both the processes and precipitate with four volumes of ethanol and then dried at 40°C for 48 hours and store for nanoparticle synthesis.
- Prepare stock solutions of pectin (0.5%) and $HAuCl_4 \cdot 3H_2O$ (1×10^{-3} M) using deionized water.
- Add pectin solution to $1 \times 10^{-}$ M aqueous $HAuCl_4 \cdot 3H_2O$ solution and heat in a water bath at 55°C.
- Notice any change in color to ruby red, indicating the formation of AuNPs. Alternatively withdraw samples at regular intervals for detection of SPR peak near 525 nm.
- Postsynthesis centrifuge mixture at 5000 rpm for 5 minutes to remove any plant extract components or precipitates.
- Collect AuNPs by high-speed centrifugation at 13,000–16,000 rpm for 20 minutes.
- Wash the pellet several times by centrifugation and resuspend in deionized water to remove the unconverted metal ions and any other medium components. Collect AuNPs in the form of a pellet using high-speed centrifugation.

Ethical issues

Nanoparticles have raised concern due to their potential toxic nature toward living organisms. Although humans and other organism have been exposed to natural particles like pollens, fine sand and dust, volcanic ash, ocean spray, and biological material like viruses since ancient times, the concern about nanoparticles is a recent one owing mainly to anthropogenic/synthetic/engineered nanoparticles. Body parts like skin, lungs, and the gastrointestinal tract are always exposed to the environment and are natural entry routes for any kind of nanomaterial. Among these the lungs and gastrointestinal tract are more vulnerable to NPs compared to skin, which is to some extent impermeable. Postentry NPs can translocate from the entry ports into the circulatory and lymphatic systems and ultimately to body tissues and organs. Although many types of nanomaterials are nontoxic and even beneficial to health, the concern is regarding a few types of nanoparticles, which cause irreversible damage to cells by oxidative stress or organelle injury. It has been observed that extent of such damage depends on the size and composition of the NPs. The toxic effect of metallic nanoparticles can be caused due to various factors such as size, dose, concentration, chemistry, surface area of nanoparticles, crystalline structure, and surface coating and functionalization. It is therefore important to emphasize the study of toxicology of each material so as to obtain accurate

information to aid future policy and regulatory processes (Das et al., 2017). Toxicity evaluation of nanomaterials is complicated as the methods used for toxicity assessment of drugs and other toxic materials cannot be applied to evaluate nanomaterials. The reasons behind this are that nanomaterials behave differently than bulk materials due to their extremely small size and unique surface properties. Due to their small size new routes of exposure may emerge. Surface properties of nanomaterials affect the toxicokinetics and hence different from a bulk material. Also the mass of the nanomaterials scale with surface area and hence calculations based on mass often do not work out. Hence due to the unavailability of reliable physical data like shape, composition, surface area, surface properties, and agglomeration state, it is difficult to obtain reliable and reproducible exposure and toxicity data (Hulla et al., 2015). It has been observed that similar nanoparticles produced using different reducing agents display variable biological activity. This phenomenon is more predominant among biogenic nanoparticles where toxicity levels may vary drastically according to the biological agent used for synthesis. Various factors including composition, size, shape, surface charge, and capping molecules affect the properties of nanoparticles (Venil et al., 2016). For example silver nanoparticles synthesized using flexirubin showed significantly higher cytotoxicity against MCF-7 breast cancer cell-line compared to chemically synthesized AgNPs. In studies with pullulan and its oxidized counterpart 6-carboxypullulan, it was seen that AgNPs synthesized with 6-carboxypullulan were smaller (8−25 nm) compared to pullulan (50−55 nm). 6-Carboxypullulan-mediated AgNPs exhibited better antimicrobial action because of their higher negative values of zeta potential (Coseri et al., 2014). In another interesting study it was found that AgNPs synthesized by green tea extract (GT-AgNP) and coffee extract (C-AgNP) showed excellent antimicrobial actions. However C-AgNPs were nontoxic to mammalian cells, while GT-AgNPs were toxic to mammalian cells, thus restricting their use in antimicrobial chemotherapy (Ronavari et al., 2017). In recent years standardized protocols have also been generated for this purpose. Reference materials for nanotoxicity testing have been made available by the UK Nanotechnology Research Coordination Group and the US National Nanotechnology Characterization Laboratory. The International Alliance for Nano Environment, Human Health and Safety Harmonization started developing test protocols for nanotoxicity testing. In the light of the toxicity testing in the 21st century proposed by the US National Research Council (NRC) high-throughput screening of nanomaterials seems promising and not too distant goal. Meanwhile Organisation for Economic Co-operation and Development (OECD) has developed guidelines for toxicity testing of nanomaterials. In India the government's Nano Mission constituted a nanoregulatory task force comprising eminent experts to frame regulations governing safe practices in handling nanoparticles. Due to their superior UV protection properties, the cosmetic industry uses nanomaterials like titanium dioxide or zinc oxide in sunscreens and other cosmetic formulations. New cosmetics regulation (EC) No. 1223/2009 which comes into effect in the EU on July 11, 2013 has considered nanomaterials for use in cosmetics. Under this ruling any nanomaterial intended to be used in cosmetics must pass through the assessment and any cosmetic product containing nanoparticles have to be labeled properly with label "nano" in the ingredient list.

In India CKMNT (Centre for Knowledge Management of Nanoscience & Technology) in association with nanoregulatory task force developed "Guidelines and best practices for safe handling of nanomaterials in research laboratories and industries." This comprehensive document published by CKMNT is based on published reports by regulatory bodies like International organization for standardization (ISO), OECD, The National Institute for Occupational Safety and Health (NIOSH), Occupational Safety and Health Administration (OSHA) and other reports available in the public domain. Availability of standard toxicity testing methods for nanomaterials shall ensure their screening and utilization in translational medicine and diagnostics. This is more important for biogenic nanomaterials as their toxicity levels vary according to the nature of the biologic materials.

Translational significance

Nanoparticles have become exceedingly important in the recent years due to its diverse applications in areas of computing, electronics, textile, agriculture, and medicine. In the biomedical field nanoparticles have found immense applications as an antimicrobial, anticancer, and antiinflammatory agent. Other applications include targeted drug delivery, tissue repair, tumor targeting, cell labeling, and magnetic resonance imaging. Nanoparticles prepared through biogenic routes sometimes show enhanced activities compared to their chemically synthesized counterparts (Table 34.2). For example silver nanoparticles synthesized using flexirubin showed significantly higher cytotoxicity against MCF-7 breast cancer cell-line compared to chemically synthesized silver nanoparticles. Hence translational value of the biogenic nanoparticles has been evaluated in a number of studies. Silver nanoparticles have been established as excellent

TABLE 34.2 Biogenic nanoparticles and their biomedical potentials.

Sl. No	Nanoparticle	Particle size (nm)	Reducing and stabilizing organism/biomolecule	Biological activity	Reference
1	Silver	50–150 nm (mean, 97 nm)	*Brevibacterium frigoritolerans* DC2 culture (Bacteria)	Antimicrobial	Singh et al. (2015)
2	Silver	150 nm	*Weissella oryzae* DC6 culture (bacteria)	Antimicrobial and biofilm inhibition	Singh et al. (2016)
3	Silver	14–32 nm	Several species of microalgae and cyanobacteria	antibacterial action	Patel et al. (2015)
4	Silver	4–24 nm	*Enteromorpha compressa* (algae)	Antimicrobial action and cytotoxic against cancer cell lines	Ramkumar et al. (2017)
5	Silver	2–13 nm	*Aspergillus terreus* MTCC632 (fungi)	Antibacterial action	Velhal et al. (2016)
6	Gold	19–31 nm	Cannonball fruit (*Couroupita guianensis*) extract	Antioxidant activity	Sathishkumar et al. (2016)
7	Silver	21–25 nm	Sodium alginate	Antibiofilm activity	Vijayakumar et al. (2016)
8	Gold	Mean 8 nm	Pectin	Cytotoxic against cancer cell lines	Umasuganya et al. (2016)
9	Gold	Mean 80 nm	Xanthan gum	Cytotoxic against cancer cell lines	Muddineti et al. (2016)
10	Silver	2–40 nm	Pullulan	Antimicrobial action against MDR strains	Kanmani and Lim (2013), Coseri et al. (2014)
11	Silver	2–50 nm	Bacterial exopolysaccharides	Antimicrobial action	Rasulov et al. (2015)
12	Silver	Mean 5.7 nm	Gum ghatti	Antimicrobial action	Kora et al. (2012)
13	Gold	1.6 nm	Gum arabic	Photothermal cancer therapy	Liu et al. (2013)
14	Silver	12–30 nm	Neem gum	Antibacterial action	Velusamy et al., 2015
15	Zinc oxide	30–60 nm	Neem gum	Antimicrobial action	Geetha et al. (2016)
16	Silver	Mean 15 nm	Biosurfactant	Antimicrobial action	Ganesh et al. (2010)
17	Silver	Mean 49 nm	Flexirubin (pigment)	Anticancer activity	Venil et al. (2016)
18	Cerium oxide	Mean 23 nm	Honey	Cytotoxic activity	Darroudi et al. (2014)

antimicrobial agents. AgNPs synthesized using alginate displayed significant antibiofilm activity against pathogens like *Listeria monocytogenes* and *Vibrio parahaemolyticus*. Toxicity of these nanoparticles was evaluated on freshwater crustacean *Ceriodaphnia cornuta* where the AgNPs showed much lower toxicity even at 50 μg/L compared to silver nitrate, which does considerable damage at concentration less than 5 μg/L in compared to silver nitrate itself (Vijayakumar et al., 2016). Studies have shown that pullulan-synthesized AgNPs had significantly high antimicrobial action against both bacteria and fungi and displayed in vitro killing of multidrug-resistant strains in a dose-dependent manner (Kanmani and Lim, 2013). In another study it was found that the antimicrobial action was higher in case of AgNPs synthesized with 6-carboxypullulan compared to those produced by pullulan (Coseri et al., 2014). Similarly high molecular mass exopolysaccharides from diazotrophic *Bradyrhizhobium japonicum* 36 were used for silver nanoparticle synthesis. Silver nanoparticles synthesized by low-molecular-weight fraction had better activity than those produced by high-molecular-weight fractions (Rasulov et al., 2015). Silver nanoparticles produced using GG had significant activity against both Gram positive and Gram negative microbes such as *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* (Kora et al., 2012). Ag-NP s synthesized using NG displayed potent antibacterial activity against *Salmonella enteritidis* and *Bacillus cereus* evident through live/dead assay using confocal microscopy and was further confirmed using DNA damage studies (Velusamy et al., 2015). The antimicrobial action is not restricted to silver nanoparticles, but zinc oxide (ZnO) nanoparticles using NG showed good antimicrobial action against Gram positive

bacteria *S. aureus* and Gram negative *E. coli* (Geetha et al., 2016). Silver nanoparticles produced using rhamnolipid biosurfactant from *P. aeruginosa* displayed profound antimicrobial activity against various bacterial and fungal test strains with minimum inhibitory concentration as low as 7.81 μg/mL (Ganesh et al., 2010). Other than these reports nanoparticles synthesized using bacteria, fungi, or algae also display significant antimicrobial action. Other major activity shown by biogenic nanoparticles is their cytotoxic effect against cancer cells. Gold nanoparticles synthesized using pectin induced apoptosis in the mammary adenocarcinoma cell lines MCF-7 and MDA-MB-231 and hence opens up new avenues for its prospective use in cancer therapy (Umasuganya et al., 2016). Gold nanoparticles produced using XG, an extracellular polysaccharide produced by strains of *Xanthomonas campestris*, displayed profound cytotoxic effect on murine melanoma (B16F10) cells confirmed that the curcumin-loaded nanoparticles produced in this study could translocate into the cytoplasm more efficiently than free curcumin and showed better cytotoxicity compared to free curcumin. Hence XG and ascorbic acid stabilized NPs may open up new possibilities in targeted drug delivery (Muddineti et al., 2016). Flexirubin, a pigment extracted from *C. artocarpi*, was utilized in the synthesis of silver nanoparticles (Venil et al., 2016). The resulting silver nanoparticles displayed anticancer activity investigated with MCF-7 human breast cancer cell-line showed 34%, 93%, and 99% efficiency with flexirubin alone, silver nanoparticles alone, and flexirubin-mediated silver nanoparticles, respectively. Higher numbers of apoptotic cells were observed in cells that were treated with flexirubin-mediated silver nanoparticles than in cells treated with flexirubin or silver nanoparticles alone. These studies demonstrate the efficiency of nanoparticles to deliver the drug to the target cells. A similar mechanism of action is seen in Food and Drug Administration–approved chemotherapy drug Abraxane where paclitaxel is coupled to albumin nanoparticles. This makes the drug more potent and less toxic compared to the native drug. GA capped gold particles of diameter 40 nm showed excellent thermal stability and strong photothermal effect required to kill cancer cells. Bioclearance studies demonstrated that these can be gradually excreted through renal and hepatobiliary systems minimizing their long-term toxic effects in vivo and ensuring their safety for photothermal cancer treatment (Liu et al., 2013). Spherical CeO_2 nanoparticles were synthesized using honey as a reducing agent. In vitro cytotoxicity studies were performed in animal cell culture on neuro2A cells using MTT assay, which displayed dose-dependent cytotoxicity and nontoxic nature of these NPs below 25 μg/mL (Darroudi et al., 2014). Other areas where biogenic nanoparticles may be used are targeted drug delivery, tissue repair, tumor targeting, cell labeling, and magnetic resonance imaging. These may display enhanced activity since nanoparticles created through biogenic methods often display unique properties different than those created from chemical agents.

World Wide Web resources

Research in nanotechnology is a relatively recent and growing field where novice researchers and students often need to access resources useful for them. In this section we have enlisted some important nanotechnology-related resources from around the globe (Table 34.3). Researchers can explore these online resources to seek answers to their research queries. The resources enlisted here cater both novices and researchers already involved in advanced nanotechnology.

Turning point

Graphene is a carbon allotrope and is world's first two-dimensional nanomaterials. It is much stronger than steel but incredibly flexible and lightweight. This transparent material is electrically and thermally conductive. Although this material is a component in the structure of other carbon allotropes such as charcoal, graphite, fullerenes, and carbon nanotubes but was first isolated in year 2004 by Andre Geim and Kostya Novoselov for which they shared the Nobel Prize in 2010. Graphene is disruptive powerful technology having potential of even replacing existing materials and technologies. The current methods for graphene synthesis are chemical vapor deposition (CVD) and exfoliation of graphite. Although the quality of CVD produced graphene is typically high, the real challenges are its industrial scale up. One promising technology for scalable production is the oxidation and exfoliation of graphite to graphene oxide (GO), followed by its subsequent reduction to graphene. The reduction process of GO to graphene often involves the usage of harsh chemistry such as hydrazine. In a recent published article (Lehner et al., 2019) a promising method of preparing graphene by bacteria *Shewanella oneidensis* has been described. This bacterial reduction approach increases the conductance of single graphene oxide flakes as well as bulk graphene oxide sheets by 2.1–2.7 orders of magnitude, respectively, while simultaneously retaining a high surface-area-to-thickness ratio. This method yields conducting flakes with differing functionalization on the top and

TABLE 34.3 Key Internet resources available for researchers in nanotechnology.

Sl. No	Resource	Description and utility	URL
1	National Nanotechnology Initiative	U.S. Government research and development initiative involving the nanotechnology-related activities of 20 departments and independent agencies. Useful sources and links can be found here.	https://www.nano.gov
2	CORDIS	Stands for Community Research and Development Information Service. It is European Commission's primary source of results from the projects funded by the EU's framework programs for research and innovation. This includes works funded on nanotechnology.	https://cordis.europa.eu/en
3	Organization for Economic Co-operation and Development	It is an international economic organization with 36 member countries. It is entrusted with the responsibility of publishing testing guidelines for toxic materials and chemicals. Guidelines on toxicity testing of nanomaterials can be found here.	http://www.oecd.org
4	American National Standards Institute	ANSI-NSP is the coordinating body facilitating the development of standards in the area of nanotechnology such as nomenclature/terminology; health, safety and environmental aspects; materials properties; and testing, measurement, and characterization procedures.	https://www.standardsportal.org
5	Nanowerk	Nanowerk is an online resource for information on nanosciences, nanotechnologies, and other emerging technologies. It delivers useful, entertaining, and cutting-edge information from all nano-related matter.	https://www.nanowerk.com
6	Nano Mission	Nano Mission is a Government of India program for capacity building in nanotechnology. Researcher can obtain funding and related information.	http://nanomission.gov.in
7	National Institute of Standards and Technology	NIST supports accurate and compatible measurements by certifying and providing over 1300 Standard Reference Materials including nanomaterials with well-characterized composition or properties.	https://www.nist.gov/srm
8	Nature Nanotechnology	It has a high impact factor monthly journal publishing the best research from across nanoscience and nanotechnology.	https://www.nature.com/nnano
9	Nanomedicine: Nanotechnology, Biology and Medicine	This is an international, peer-reviewed journal presenting novel, significant, and interdisciplinary theoretical and experimental results related to nanoscience and nanotechnology in the life sciences.	https://www.journals.elsevier.com/nanomedicine-nanotechnology-biology-and-medicine
10	Journal of Biomedical Nanotechnology	This is a peer-reviewed multidisciplinary journal providing broad coverage on the applications of nanotechnology in medicine, drug delivery systems, infectious disease, biomedical sciences, biotechnology, and all other related fields of life sciences.	http://www.aspbs.com/jbn.html

bottom faces and hence enables the development and scaling of new types of graphene-based materials and devices for nanocomposites, conductive inks, and biosensors, while avoiding usage of hazardous, environmentally unfriendly chemicals. In future biological method may emerge as the predominant method for graphene synthesis. Graphene has huge possibilities in biomedical applications such as drug delivery, tissue engineering, antimicrobials, biosensors, and advanced medical devices. This makes this discovery a major turning point in the history of nanomaterials because of its potential to revolutionize technology in near future.

Dawn of the nanomachines era

The 2016 Nobel Prize in Chemistry was awarded to three scientists—Jean-Pierre Sauvage (University of

Strasbourg, France), J. Fraser Stoddart (Northwestern University, Evanston, IL, USA), and Bernard L. Feringa (University of Groningen, the Netherlands) for their work on world's smallest mechanical devices called the "nanomachines." These machines are one billionth of a meter in size and about one thousandth the width of a strand of human hair. Jean-Pierre Sauvage in 1983 succeeded in linking two ring-shaped molecules together to form a chain, called a *catenane*. For a machine to be able to perform a task, it must consist of parts that can move relative to each other. The two mechanically interlocked rings created by Jean-Pierre Sauvage fulfilled this requirement paving the first stepping stone toward nano mechanization. The second stepping stone was created when Fraser Stoddart in 1991 developed a *rotaxane*. He was successful in threading a molecular ring onto a thin molecular axle and demonstrated that the ring was able to move along the axle. The most important part of his invention was that he had full control of the machine movement unlike chemical systems where the movements are random. Bernard Feringa lay the final stepping stone into the era of nanomachines. The first molecular motor was developed by Bernard Feringa in 1999 where he made a molecular rotor blade to spin continually in the same direction. Using molecular motors, he has rotated a glass cylinder that is 10,000 times bigger than the motor and also designed a nanocar. Although the technology is still in its infancy, its enormous potential has been acknowledged by a Nobel Prize. This reminds us of the Eric Drexler's book *Engines of Creation: The Coming Era of Nanotechnology* published in the year 1986 where he envision the concept of nanoscale assembler that can make copies of itself. Be it space travel, nanobots or quantum computers in coming years nanotechnology shall make it all possible. Imagine billions of small machines are floating in your blood. If you met with an accident, the wound spontaneously heals in hours. Nanobot transport pharmaceutical agents directly to the injury. Science fiction? Yes. But for how long?

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Glossary

Nanoparticles Nanoparticles are metallic or nonmetallic particles below 100 nm in at least one dimension.

Biomedical Biomedical refer to the knowledge, interventions, or technology that are of use in healthcare or public health.

Drug delivery Drug delivery refers to the approaches, formulations, technologies, and systems for transporting a pharmaceutical compound in the body as needed to safely achieve its desired therapeutic effect.

Nanometer A nanometer is a unit of spatial measurement that is 10^{-9} m or one billionth of a meter.

Reduction Reduction is a chemical reaction that involves the gain of electrons by one of the atoms, ion, or molecule involved in the reaction between two species.

Redox reaction Redox is a type of chemical reaction, which involves a transfer of electrons between two species.

Reducing agent Reducing agent is an element or compound that donates electrons to other chemical species in a redox chemical reaction. The reducing agent loses electrons and hence become oxidized.

Capping agent A capping agent is an amphiphilic molecule consisting of polar head group and a nonpolar hydrocarbon tail in which nonpolar tail interacts with surrounding medium, whereas polar head coordinates to the metal nanoparticles.

Solvent Solvent is a substance that dissolves a substance called solute resulting in a solution. Solvents are usually liquid, for example, water, hexane, acetone, and ethanol.

Biomolecule Molecules produced by living organisms and are essential to one or more of biological processes such as growth, development, and reproduction. Examples of biomolecules are carbohydrates, proteins, lipids, hormones, and pigments.

Biogenic Substances, metabolites, or secretions by life forms such bacteria, fungi, plants, or animals.

Byproduct Secondary products derived from a biological process, which are not the primary product of the metabolism, which can be used for other for other useful purposes, for example, Vanillin and essential oils.

Feedstock Feedstocks are bulk raw materials constituting the principal input for an industrial process, for example, molasses or corn steep liquor in industrial fermentation.

Colloid A mixture in which one substance of microscopically dispersed insoluble particles is suspended throughout another substance such that the mixture does not settle or take a very long time to settle appreciably. The dispersed-phase particles have a diameter between approximately 1–1000 nm.

Carbon nanotubes (CNT) Allotropes of carbon and cylindrical nanomaterials that consist of two-dimensional hexagonal lattice of carbon atoms.

Secretome Secretome includes all proteins secreted by living cells into the extracellular space including enzymes, factors, chemokines, cytokines, and adhesion molecules.

Downstream processing Refers to the recovery and the purification of biosynthetic products like biomolecules and pharmaceuticals obtained from bacteria, fungi, plants, or animals through fermentation or cell culture.

Graphene Graphene is an allotrope of carbon in the form of a two-dimensional, atomic-scale, hexagonal lattice. It is the basic structural element of other allotropes, including graphite, charcoal, carbon nanotubes, and fullerenes.

Surface plasmon resonance Surface plasmon resonance (SPR) is the resonant oscillation of conduction electrons at the interface between negative and positive permittivity material stimulated by incident light.

Ultrasonication Ultrasonication is a process that uses sound energy at high frequencies to break apart particle agglomerates by cavitation, the expansion and implosion of bubbles. It is used in biology to rupture cells to extract intracellular components.

Centrifugation Centrifugation is a technique used for the separation of particles from a solution according to their size, shape, density, viscosity of the medium, and rotor speed.

Antimicrobial Antimicrobials are agents that kill microorganisms or stop their growth.

Cytotoxic Cytotoxicity is the quality of being toxic to cells and generally refers to are drugs used to destroy cancer cells.

Spectrophotometer The spectrophotometer is an optical instrument for measuring the intensity of light relative to wavelength. It is used frequently in several areas of biological research.

Abbreviations

SPR	Surface plasmon resonance
NP	Nanoparticles
AgNP	Silver nanoparticles
AuNP	Gold nanoparticles
ZnONPs	Zinc oxide nanoparticles
NNI	National Nanotechnology Initiative
TSB	Tryptic soy broth
UV-Vis	Ultraviolet and visible range
SDB	Sabouraud dextrose broth
BG11	Blue green 11 (for cultivation of blue green algae)
$AgNO_3$	Silver nitrate
$HAuCl_4$	Chloroauric acid
$ZnNO_3$	Zinc nitrate
NaOH	Sodium hydroxide
psi	Pound per square inch

Long answer questions

1. What are the top-down and bottom-up approaches of nanomaterial synthesis? Elaborate with examples.
2. Provide one generalized protocol each for synthesis of nanoparticles using bacteria, fungi, algae, and plants?
3. What are the ethical issues connected to synthesis and research with nanoparticles?
4. Describe few medical applications of nanoparticles with suitable examples.
5. What are different methods of physical and biochemical methods for characterization of nanoparticles? Describe using suitable examples.

Short answer questions

1. Give examples of a few nanoparticles found in nature?
2. What makes graphene a unique nanomaterial?
3. What are the different physical and biological properties displayed by silver nanoparticles?
4. Give a recent example of biogenic synthesis of graphene.
5. What are the advantages of biogenic synthesis of nanoparticles?

Answers to short answer questions

1. Nanomaterials have been existing in the nature even before the first man made nanomaterial. Various sources of natural nanoparticles in nature are as follows:
   a. Fires and volcanoes which produce nanodimensional carbon and other particles.
   b. Liquid sprays contain small amounts of dissolved substances, which produce nanoparticles as they evaporate, for example, sea spray.
   c. Sometimes natural compounds react in atmosphere to form nanoparticles. For example in Blue Ridge mountains terpenes and other hydrocarbon compounds released by some trees react with ozone to form a blue haze caused due to nanomaterials.
2. Graphene is a unique nanomaterial due the following reasons:
   a. Graphene is only one atom thick so it the world's first 2D nanomaterial.
   b. It is 200 times tougher than steel and conducts 13 times better conductor than copper.
   c. It is impervious as even helium cannot pass through it.
   d. It is a highly transparent material and allows 97%–98% light to pass through it.
3. Physical and biological activities displayed by silver nanoparticles are as follows:
   a. Silver nanoparticles show antimicrobial action against several microorganisms.
   b. They exhibit cytotoxic effect against several cancer cell lines.
   c. They display excellent catalytic properties.
   d. Some reports have shown silver nanoparticles to possess antiviral properties.
4. In a recently published article by Benjamin A.E. Lehner and others, in the journal *Chemistry Open*, a novel approach of graphene synthesis with the help of bacterium *Shewanella oneidensis* has been described. The graphene synthesized by this method shows a better electrical conductivity and other enhanced properties.
5. There are several advantages associated with biogenic synthesis of nanoparticles:
   a. Biogenic synthesis eliminates the use of toxic chemicals and solvents, which are harmful to environment.
   b. Nanoparticles synthesized through biological means often have properties different from those produced through chemical means.
   c. Nanoparticle synthesis by biological methods can mostly be done at ambient temperature, while those by chemical methods often have high temperature and energy requirements.

Yes/No type questions

1. Michael Faraday discovered the nanomaterial graphene in year 1856.
2. A nanometer means one millionth of a meter.
3. Bacteria cannot be used to synthesize nanoparticles.
4. Gold and silver nanoparticles are 2D nanomaterials.
5. Biogenic nanoparticle synthesis uses hazardous chemicals as reducing and capping agents.
6. Nanoparticles are engineered materials not found in nature.
7. Physical and biological properties of nanoparticles are same as bulk materials.
8. Graphene is one atom thick and is a two-dimensional nanomaterial.
9. Silver nanoparticles have antimicrobial and antiviral properties.
10. Gold nanoparticles are prospective photothermal agents in cancer treatment.

Answers to Yes/No type questions

1. No
2. No.
3. No.
4. No.
5. No.
6. No.
7. No.
8. Yes.
9. Yes.
10. Yes.

CHAPTER

35

Ethical issues in animal biotechnology

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Summary

This chapter provides a brief overview of relevant ethical theories and principles for examining the various ethical issues in animal biotechnology. These issues include intrinsic concerns such as religious objections, and extrinsic concerns such as those for the environment. Ethical issues and debates pertaining to the creation of chimeras, biopharming of animals, as well as those arising out of the recent application of genome editing techniques such as CRISPR-Cas9 on human embryos are also discussed.

What you can expect to know

Simply ethics is a set of moral principles that guide the decisions and actions of people and enable them to determine right and wrong behavior. Ethical theories are divided into metaethics, normative ethics, and applied ethics. Metaethics explores the foundations of morality; normative or prescriptive ethics lists ethical behavior; and applied ethics deals with specific issues that call for debate and discussion, using the concepts and principles contained in metaethics and normative ethics. Applied ethics include diverse issues such as animal rights, environmental ethics, euthanasia, cloning, xenotransplantation, and others. New applied ethics issues are likely to emerge from time to time with the introduction of new technology and practices.

The ancient Greeks like Aristotle and Plato based their ethics on virtue, happiness, and the soul. The deontological approaches to ethics describe discharge of duties irrespective of consequences as the hallmark of an ethical way of life. On the contrary, utilitarian ethics is a form of consequentialist ethics that considers the action that brings the greatest good to the greatest number as ethical.

The major principles of normative ethics, medical ethics, and ethics of science and technology, include beneficence, nonmaleficence, autonomy, justice, human dignity, equality, tolerance, informed consent and choice, animal rights and welfare, and environmental compatibility, among a host of others. These principles can be important tools for an ethicist to examine particular animal biotechnology innovations.

The growing interest in ethical analysis of animal biotechnology stems from the high manipulative ability of the gene-based technologies that place greater control with humans than ever before. These technologies are consequently subject to ethical analysis from religious, environmental, safety-based, animal rights-based, and welfare-based approaches.

Concerns over animal biotechnology can include both intrinsic and extrinsic concerns. Of the former, biotechnology has been accused of playing God by infringing upon the divine order of the nature of a species by transferring genes from one species to another and creating hitherto unknown forms of life. This is the most common intrinsic concern, although there is hardly any consensus among the adherents of the different religions, or even within the different sects or groups of a given religion on this concern. Another concern is the insertion of genes from prohibited animals into other animals and plants, which raises the question of their acceptability as food. Similarly, animal genes inserted into a plant could be thought of compromising the vegetable nature of the plant itself. As opposed to these religious objections, the secular intrinsic concerns allege that biotechnology is disturbing the natural species boundaries and is therefore unnatural, as it destabilizes the evolutionary order of life. However, the detractors of this concern point out that because of hybridization occurring in nature, species boundaries cannot be regarded as fixed and immutable.

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Surveys to record public perceptions about animal biotechnology in Europe and the United States revealed that most people were against animal cloning for food, while they approved the use of animal biotechnology for medical purposes, subject to the framing of strong regulatory guidelines.

Among the extrinsic concerns, animal welfare in terms of both physical and mental health is judged against the yardstick of the five freedoms laid down in the Brambell Report of 1964. While taking into account many curtailments of these five freedoms in traditional animal breeding, it can be seen that animal biotechnologies, such as gene knockout technology, produce a large number of surplus animals, which coupled with the current state of inefficiency and unpredictability of gene technologies, result in several health problems in livestock animals.

The concern for human health arises from two directions, namely use of genetically engineered animals or their products as food, and their use in medicine, along with their attendant ethical dilemmas. The ethical issues arising out of these concerns are evaluated in relation to a concise set of public health ethics.

The environmental concerns of animal biotechnology can be viewed in relation to ethical positions in human relationships with the environment, which could be categorized as anthropocentric (human-centered), biocentric (life-centered), and ecocentric (ecosystem-centered). Deep ecology is similar to ecocentrism, with an emphasis on self-realization. Anthropocentrism recognizes only instrumental or extrinsic value in the environment, while the others recognize intrinsic value in varying degrees. Viewed against this perspective, animal biotechnology poses certain risks to the environment. The major concern is the release of transgenic animals into the environment, where they can create various problems for their nontransgenic counterparts. Transgenic fish especially pose such hazards to a large extent. The precautionary principle should be applied to take decisions for safeguarding the environment from such hazards.

Ethical issues also arise when human cells such as pluripotent stem cells are placed in a nonhuman animal to create human-to-animal chimeras, which is accused of compromising human dignity and generates moral abomination by evoking the idea of bestiality. Chimeras also represent a metaphysical threat to the self-image of human beings. There is a utilitarian angle to the chimera debate in view of the medical benefits, but this ought to be regulated by not allowing chimeras with human cognitive abilities to be produced, especially in nonhuman primates.

Another issue that has been addressed in this chapter is that of biopharming, where specific human genes are engineered into cattle, goat, pig, sheep, or even rabbits, to produce milk containing a number of human proteins of therapeutic value. The ethical issues in animal biopharming revolve around risks to human health, gene transfer, and animal welfare, and can be analyzed by general utilitarian, public health, and animal welfare principles, as well as the by precautionary principle. The message of the chapter is that ethical analysis of any new technology can help remove concerns in everybody's mind and enable its safe and wise adoption for the benefit of all.

Finally, the readers are also made aware of the ethical issues and concerns pertaining to the use of genome editing techniques such as CRISPR-Cas9 on human embryos for therapeutic purposes as well as for germline modification.

History and methods

Introduction

According to the Internet Encyclopedia of Philosophy, the field of ethics (or moral philosophy) involves systematizing, defending, and recommending concepts of right and wrong behavior. The word ethics has its root in the Greek word ethos or ethicos, which means the moral ideas or attitudes that belong to a particular group or society; in other words, it can mean its custom, habit, character, or disposition. Put simply, ethics is a set of moral principles that guide the decisions and actions of people and enable them to determine right and wrong behavior. Ethical theories are divided into three broad areas: metaethics, normative ethics, and applied ethics. Metaethics explores the foundations of morality, probing the theoretical basis and scope of moral values. In other words, it tries to define morality itself. In contrast, normative or prescriptive ethics tells us what is ethical and what is not. Applied ethics deals with specific issues that call for debate and discussion. It uses the concepts and principles contained in metaethics and normative ethics as tools to deal with issues that include animal rights, environmental ethics, euthanasia, cloning, and xenotransplantation, among many others. Issues in applied ethics may arise from time to time and generate public debate.

For example, exposure to non-ionizing radiation from mobile towers (cellphone base stations) has been shown by some studies to be harmful to human health as well as other life forms, like plants and animals. However, several other studies also exist that show it to cause no perceptible damage. Thus, the scientific evidence for either its capacity to cause harm or its innocuous nature can be said to be far from unequivocal. In such a situation, would it be ethical to set up mobile phone towers in densely populated localities in urban

areas? What kind of policy decision taken by the city administration and other concerned authorities or the promotional policy adopted by mobile phone companies may be considered ethical? And what should be considered an ethical stand on the setting up of mobile towers in commercial or residential areas or near schools and hospitals? One may argue that since the scientific evidence for potential harm by mobile towers to human health or the environment is not convincing, technological progress, improved and facile communication, and economic development through trade, employment generation, and the like should not be hampered (i.e., setting up towers should be allowed to continue). However, others may cite the precautionary principle, which states that "where there are threats of serious or irreversible damage, lack of full scientific certainty shall not be used as a reason for postponing cost-effective measures to prevent environmental degradation" (COMEST, 2005); they may then plead for not allowing mobile towers to be set up inside residential or other vulnerable areas until they are proved to be harmless through conclusive scientific evidence.

With the increasing emergence of issues (such as the one above) in different fields of science and technology that are not easily resolved, applied ethics has assumed importance not only in policy-making and governance, but in our day-to-day lives as well. The ethical debates around many contentious issues such as end-of-life decisions, euthanasia, cloning, and others have questioned the very basis of normative theories and spilled over into the domain of metaethics. Animal biotechnology also opens several ethical issues that need to be discussed in order to have a complete understanding of the pros and cons of different techniques that are employed, or are likely to be employed, in the near future. While identifying and discussing ethical issues, it must be borne in mind that ethics is a prescriptive, and not a descriptive or a discipline. It is concerned with what ought to be and not what is. There is a tendency to group moral and ethical issues together. Despite the overlap commonly found between morals and ethics, these two fields of enquiry are distinct from each other. Most people have some moral views on various subjects, which may be similar or different (in varying degrees) from another individual's. For example, one may think it is totally wrong to cut down forests or go hunting, while another person may feel it is alright to clear forests for developmental projects, but not for urban expansion, and so on. Thus, moral views come naturally to people and may depend on their upbringing and experiences, their type of education or religious training, and a host of other factors. While ethics also deals with right and wrong, it provides arguments and justifications about the stand that it takes on a given issue. Some people may instinctively think that cloning or transfer of genes is wrong, and this is part of their moral views on these issues. Ethicists, on the other hand, may also be against cloning or gene transfer, but they will present reasons and justifications to their views. Thus, moral views are based more on intuitive feelings, while ethical stands rely more on logical or factual arguments and analyses (Straughan, 1999).

A brief overview of ethical thoughts and principles

Virtue ethics

The ancient Greek moral theories attached great importance to virtue, happiness (eudaimonia), and the soul. Two well-known works on ethics by the Greek philosopher Aristotle (e.g., *The Nicomachean Ethics* and *The Eudemian Ethics*) contain examinations of, and discussions on, the subject of eudaimonia, which connotes happiness or flourishing, and virtue or excellence. These are the most important among the character traits that a person must have in order to lead an ethical life. Virtue was an important parameter in ancient Indian texts like the Mahabharata or the Ramayana where, for example, a warrior or a king was expected to have certain virtues in order to be considered an ideal example of his class. He was expected to sacrifice his possessions, his loved ones, and even his life, to uphold these virtues. However, as issues (controversial or otherwise) in science and technology or other areas of applied ethics were almost non-existent, there was no ethical debate or discussion in this area. Nevertheless, parts of an ancient Indian text, the Kautilya's Arthasastra, which was written or compiled between the 3rd-century BCE and the 2nd-century CE, prescribed ethically proper ways for carrying out agricultural activities and forestry. It also gave detailed instructions for how to treat elephants, which were the living war machines of that period, as well as guidelines for the welfare of other domestic and wild animals.

Deontological (duty-based) ethics

Another major theory of normative ethics that provides valuable guidelines for adopting ethical positions on specific issues in applied ethics includes the deontological approach to ethics. The deontological theories are based on the concept of duty or obligation and are often called nonconsequentialist, as they are not dependent on the consequences of a given action. For example, our duties or obligations to children, to the old and infirm, to our community, or to not commit felonies or murder are considered ethical actions regardless of their

consequences. The duty-based approach to ethics was first developed by the 17th-century German philosopher Samuel Pufendorf, who classified duties under three major categories (i.e., duties to God, oneself, and others). The renowned philosopher Immanuel Kant based his deontological approach on the concept of categorical imperative, which recommends an action irrespective of its contribution to personal happiness. Thus, we are to treat another person as an end and not as a means to an end. In other words, we should not use other people as mere instruments to achieve our own happiness. Another well-known, duty-based approach is the rights theory postulated by the 17th-century British thinker John Locke, who stated that every human being enjoys certain rights given by God that include life, liberty, health, and possessions. Any action that hampers or alienates these rights is, therefore, unethical. The British philosopher W.D. Ross developed the most recent duty-based approach, where he identified some universal duties such as fidelity, reparation, gratitude, justice, beneficence, self-improvement, and nonmaleficence.

Consequentialist ethics

As the name indicates, a consequentialist ethical theory judges the rightness or wrongness of an action by the nature of its consequences. Utilitarianism is an important consequentialist ethical theory. In its classical form, postulated by the British philosopher Jeremy Bentham (1748–1832) and his follower John Stuart Mill (1806–1873), utilitarianism is concerned with the maximization of good and aims to bring about "the greatest amount of good for the greatest number." While maximizing the good, classical utilitarianism treats everybody's good as the same, without any partiality, and the reason that prompts somebody to undertake an action is the same for everybody else. John Sidgwick (1838–1900) and G.E. Moore (1873–1958) were among the other thinkers who contributed richly to utilitarian ethical thinking. Consequently, utilitarianism today has many variations. It is also perhaps the most commonly used tool in the hands of policy-makers when deciding whether to build a dam across a river by submerging villages or tracts of agricultural or forest land, acquiring private land for building a highway, approving a new drug, or banning a pesticide.

Principles

Many general principles of normative ethics, medical ethics, and the ethical guidelines for science and technology are also applicable to issues in animal biotechnology. Some such principles are defined as follows (Weed and McKeown, 2001):

1. *Rationality*: Rationality is an important prerequisite for ethics, as all morally appropriate actions should be defendable by logical reasoning.
2. *Beneficence*: Beneficence means doing good. From an ethical perspective, it has an underlying utilitarian basis that is concerned with producing the greatest possible proportion of good over evil for the greatest number of people.
3. *Nonmaleficence*: It means not doing harm.
4. *Least harm*: This principle deals with a situation where neither choice is beneficial. In such a case, one ought to choose the option that is less harmful in extent or magnitude, or which causes harm to the least number of people.

   Beneficence, nonmaleficence, and least harm can be seen to be part of a continuum where doing good is the best option, but when that is not possible, doing any harm at all must be avoided. In the worst scenario of not being able to totally prevent harm, the least harmful option ought to be chosen.
5. *Autonomy*: This involves respect for persons who should enjoy freedom from coercion. It automatically opposes the principle of paternalism, which tends to impose upon others' will. In other words, the principle of autonomy allows people to make their own decisions.
6. *Informed consent*: A cardinal principle in medical and research ethics, informed consent involves provision of complete information, including risk involved, chances of failure, etc., about a course of treatment or a trial offered to patients or volunteers. They can then either opt for the suggested treatment/trial or decide against it.
7. *Informed choice*: This is one step beyond informed consent, where a range of options are offered to a patient/volunteer with explanations about the pros and cons of each option. The patients/volunteers can select the preferred one.

   We can see here that the path of ethical progression moves from paternalism, where consent is taken for granted, to informed consent, to informed choice, with greater autonomy of the subject at each successive stage.
8. *Justice*: Justice means treating everybody with fairness, impartiality, and consistency, with equitable distribution of risks and benefits of research, health care, or other goods and services. It should be borne in mind that the word justice in an ethical vocabulary is distinct from the retributive justice of criminal law.
9. *Confidentiality*: This implies respect for privacy of information provided by patients or research subjects.

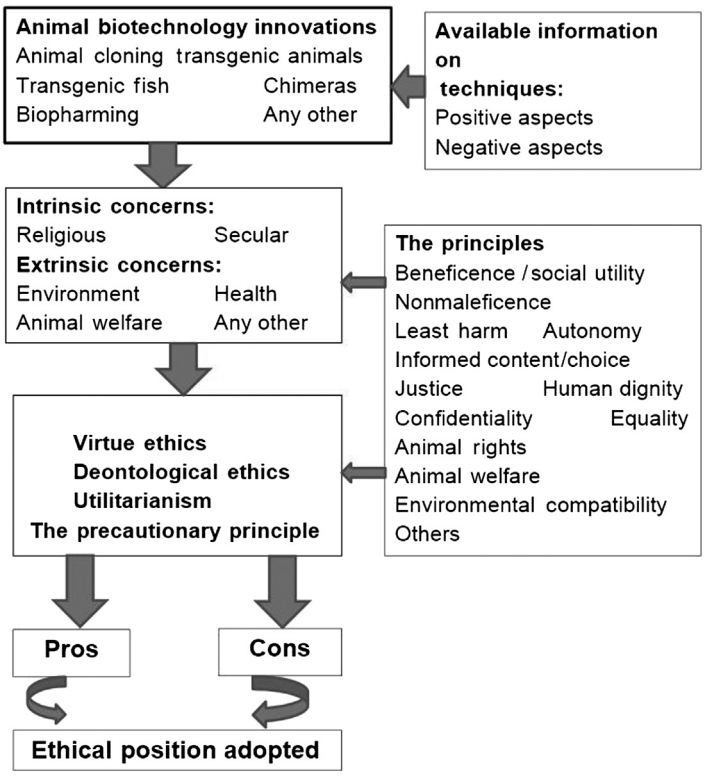
10. *Equality*: This is the duty to treat every human subject as a moral equal.
11. *Tolerance*: This calls for understanding and acceptance of diverse viewpoints that may be expressed by different subjects or stakeholders.
12. *Human dignity*: This warrants treating every human being with dignity and keeping intact their dignity as human beings.

    It may be said that the five aforesaid ethical principles are closely related to some amount of overlap so that observance or violation of one is likely to affect the others to varying degrees.
13. *Animal rights*: The Merriam-Webster Dictionary defines animal rights as "rights (as to fair and humane treatment) regarded as belonging fundamentally to all animals."
14. *Animal welfare*: This pertains to the general health, happiness, and safety of an animal. Modern views of animal welfare tend to be concerned not only with the health of the animal's body, but also with its feelings (Hewson, 2003).
15. *Environmentally benign nature*: This implies that an essential prerequisite for an action to be ethically appropriate is that it not be harmful to the environment.
16. *Intrinsic value*: A value in itself, regardless of its utility for others. For instance, people generally believe that other human beings have a value in themselves as conscious intelligent beings, regardless of their utility for others. Thus, human beings do not lose their value even if they are not able to work or be useful to other human beings.
17. *Extrinsic value*: Derives from the objective properties of something or somebody that has use for others by virtue of its functions. For example, a pen or pencil has value only until it is considered fit to write with, after which it loses its value.
18. *The Precautionary Principle*: According to the definition adopted in the Rio Declaration (1992), the precautionary principle (PP) states that "where there are threats of serious or irreversible damage, lack of full scientific certainty shall not be used as a reason for postponing cost-effective measures to prevent environmental degradation" (COMEST, 2005).

The aforesaid principles can serve as powerful tools in the hands of ethicists to examine the different animal biotechnological innovations.

Methodology

The methodological approach followed in this chapter to examine several important innovations in animal biotechnology with ethical analysis follows an analytical step-by-step model (Flow Chart 35.1). The available supportive data on positive and negative aspects of the



FLOW CHART 35.1 Methodology adopted in the ethical analysis of animal biotechnology innovations.

innovation are extensively used. This information is first subjected to the relevant intrinsic (both religious and secular) and extrinsic (in terms of environment, health, animal welfare, and any other relevant concerns) factors. This is followed by an analysis of the innovations from different normative ethics approaches and the precautionary principle, wherever applicable. The whole process is examined against the backdrop of ethical principles such as beneficence, nonmaleficence, autonomy, justice, and others, depending upon their applicability in a given situation. At the end of this exercise, the pros and cons of the technology get sorted out, and a final ethical decision can be made.

Application of ethics in animal biotechnology

There has been a surge in studies on the ethical concerns of biotechnology, including animal biotechnology. Why have ethical questions become an integral part of public concern about the phenomenal breakthroughs in biotechnology research in recent years? Perhaps, it is because of the increasing ability of humans to chart and manipulate the genome of plants and animals and utilize this knowledge to innovate in several subject fields, such as medicine, agriculture, veterinary science, and others (i.e., humans are able to tinker with the very basis of life). A large number of people are not comfortable with such manipulations and believe that they are tantamount to playing God, which raises questions about the ethical propriety of such research. Such beliefs that humans should not interfere with the fabric of life are usually linked to religious faith, and certain worldviews and convictions.

Other concerns include animal rights and animal welfare; possible harmful effects on human consumers, recipients or volunteers, along with infringement of their autonomy; and potential impact on the environment. Thus, of the different ethical principles discussed in the "Principles" section, those like beneficence, informed consent and choice, animal rights and welfare, environmentally benign nature, and the precautionary principle (among others) are especially relevant for biotechnology, in general, and animal biotechnology in particular.

Ethical questions are also coming more and more to the foreground because of the revolutionary changes in animal breeding in the last few decades. The pre-20th-century animal breeders did not have the knowledge of the underlying genetic mechanisms that controlled the expression of a given morphological character and based their experiments on experience and observation. This approach changed in the twentieth century when knowledge of Mendelian genetics was applied to achieve greater control over breeding. Beginning in the 1960s, animal breeding experienced progressively greater control over associated processes with the application of modern biotechnological techniques such as artificial insemination (AI), which became more effective and widespread with the development of various techniques and concepts: deep-freezing semen, superovulation of females, embryo transfer, and cloning. With greater control and deeper manipulation, ethical, regulatory, and safety-related concerns were also expressed from more quarters and at increasing frequency.

Ethical concerns in animal biotechnology

Before going into the ethical concerns in animal biotechnology, it is to be understood that ethics cannot provide conclusive proof about the rightness or wrongness of a technology. There could be arguments in favor of or against a given technology and its applications. However, ethical debates and discussions can provide a basis for individuals to reach their own decisions instead of being provided with a paternalistic prescription. Such an approach in turn can pave the way for taking appropriate policy decisions.

Intrinsic concerns

Before discussing the ethical concerns of specific biotechnology applications, it is appropriate to introduce the ethical concerns about biotechnology in general. Some researchers have called these concerns intrinsic if they are inherent in the technologies themselves, and extrinsic if they revolve around the consequences of the technology (Straughan, 1999; Kaiser, 2005). If a given practice or technique is intrinsically wrong, then there is no further grounds for the continuation of that practice or technique. For example, slavery is intrinsically unethical, and there is no excuse to perpetuate it. The major intrinsic arguments against biotechnology are summarized in the following sections.

Religious intrinsic critique of biotechnology

One of the most common ethical allegations against modern biotechnology is that it amounts to playing God. Large numbers of people belonging to certain denominations of Christianity, as well as other religious faiths, believe that the divine and human realms are distinct from each other. The natural world is made up of myriad kinds of plants and animals that exhibit similarities and differences among each other

in varying degrees. This arrangement is part of a divine order. The ability of modern biotechnology to transfer genes from one species to another and make replicas having characteristics selected by humans violates this divine order. The characteristics of a given species are the gifts of God, and therefore, any human fabrication of the very basis of life is tantamount to playing God. This critique of biotechnology in general, and genetic engineering in particular, delves into the domain of metaethics as it defines and delimits the rights of humans to alter the living world, which is the creation of the Almighty God. Such acts, therefore, cannot receive the ethical sanction/approval.

However, there is lack of consensus among the believers in different religions, including Christianity, on such a negative view of biotechnology. There is support for the dominion over nature by humans in Christianity itself, and many believe that God has provided this opportunity to humans (the chosen ones among all the species) to work as co-creators (Straughan, 1999). Genetic changes, albeit less drastic and taking place over a long time period, have also been achieved via selective breeding throughout ages. One example is the numerous breeds of dogs that have widely different morphological characters and behavioral attributes produced through selective breeding. This raises the question of whether the timespan in which genetic changes in a species can be produced (and which in the case of genetic engineering happens to be short) is an important factor for considering biotechnology as intrinsically wrong and equivalent to playing God. It is debatable whether such an argument can be considered valid. Nevertheless, gene transfer techniques bestow much more precision and control in the hand of humans over the life of animals than was available earlier. The question therefore arises whether there should be a limit to the modifications that humans should be allowed to make in the genetic composition of animals. At the same time, numerous modifications and re-designing of nature have taken place and are continuing around us. Exotic plants and animals have been introduced into strange environments, many of which have turned into invasive species that in turn resulted in the extinction or severe decimation of native life forms. Although these changes did not take place through direct genetic manipulations, they could also be perceived as playing God, since the morphology of a species or the composition of plant or animal communities of a given area is also the work of God. Perceptions and beliefs are important issues to consider when deciding where to draw the line on tinkering with the genetic makeup of various organisms.

Another issue of a religious nature that gene transfer creates is that many religions, such as Hinduism, Sikhism, Judaism, and Islam, prohibit the consumption of certain animals, some even recommending a purely vegetarian diet. If the gene from any such prohibited animal is inserted into a plant or other animal that humans are normally allowed to consume, how does that change the status of the altered organism? How do genetically modified (GM) food items fit into the guidelines of various religious doctrines? Or if genetic material from a fish or some other animal is inserted into a fruit or vegetable, would the latter still be considered the vegetarian food? No ready answers to these controversies are available, as none of the earlier methods of selective breeding of plants and animals crossed the genetic barrier between species.

Religious critique of human stem cell research

Human embryonic stem cells (hESCs) are cells derived from the early embryo that are pluripotent and can differentiate into any cell type of the adult body. Stem cell research or therapeutic cloning, therefore, holds great promise for developing effective medical treatments for many hitherto incurable diseases such as Parkinson's and Alzheimer's diseases, diabetes, spinal cord injury, and others. However, religions such as Catholicism object to the utilization of human embryos, as it is considered a human being with full moral standing from the moment of union of the male and female gametes. Consequently, the violation of its right to life at the hands of stem cell research is not approved by the Catholic religion. Larijani and Zahedi (2004) have explained that according to Islamic Jurisprudence, ensoulment of embryos occurs after 120 days of fertilization. Therefore, embryos that have existed for less than that period of time can be used for essential research, such as curing disease. Judaism, as well as Hinduism and Buddhism, puts great importance on healing and compassion and consequently does not raise any serious objections to hESC research.

Religious critique of human reproductive cloning

Human reproductive cloning is the production of a human fetus from a single cell by asexual reproduction. When compared to embryonic stem cell research and therapeutic cloning, human reproductive cloning has attracted more serious criticism from a number of religions, as it is seen as directly challenging the authority of God and is tantamount to playing God. Most Churches unequivocally object to human cloning. Hinduism advises caution and careful analysis before embarking on such research, while Buddhism and Judaism are relatively more tolerant to human cloning. On the contrary, the wasteful use of embryos, unnaturalness of reproduction, social harms, and contradiction of the diversity of creation are some of the many reasons for Islam not being supportive of human reproductive cloning (Larijani and Zahedi, 2004).

Religious critique of in vitro fertilization

In vitro fertilization (IVF) is an assisted reproductive technology (ART) that comprises a major technique for resolving infertility problems. In IVF, sperm is used to fertilize the egg outside the body. The fertilized egg is then transferred to the uterus of the patient in order to have a successful pregnancy and childbirth. The Catholic Church has expressed its moral opposition to IVF and considered it immoral. Islam accepts IVF, provided that it is carried out between a legally married couple, but it does not approve of third-party donations of sperm, eggs, or uterus.

Secular intrinsic objections to biotechnology

Besides the intrinsic objections to biotechnology from a religious point of view, there are secular arguments against it as well. Biotechnology could be considered intrinsically unethical because it attempts to cross the natural dividing lines or barriers that naturally exist between species. Biotechnology, according to these critiques, is intrinsically wrong because it violates the natural integrity of species by deliberately bringing about genetic modifications.

The detractors of this view argue that the natural boundaries between species are not as fixed and immutable as they are made out to be. A certain amount of natural hybridization takes place in natural systems and is a part of the evolutionary process of speciation. For example, gene flow between species has been shown to occur fairly frequently in insects such as heliconiinae butterflies, cicadas, sea anemones, parasites, fish, dolphins, and killer whales. An estimated 10% of animal species are known to hybridize in nature. Thus, the species boundary could be visualized as a continuum (Mallet et al., 2007).

Nevertheless, this evidence of the natural transgression of species boundaries does not entirely nullify the intrinsic secular arguments against biotechnology since the drastic genetic modifications in biotechnological research, such as transfer of genetic material from plants or microbes to animals and vice versa, find no parallel in nature and could be treated as a gross and anthropogenic breach of species boundaries.

Public perceptions about genetic modifications in biotechnology

How do common people who are the potential recipients of biotechnological innovations respond to genetic engineering? Some surveys to gauge public response have been carried out in Europe and the United States; it is not known whether such surveys have been carried out in developing countries. Several Eurobarometer surveys on biotechnology have been conducted in EU countries since 1989 (Lassen et al., 2006). The responses indicate that there is no outright rejection of biotechnology by Europeans, and there is even a wide acceptance of those technologies that have medical applications. For instance, in the 2010 survey, stem cell research and embryonic stem cell research received the support of 68% and 63% of the respondents, respectively. It is also noteworthy that xenotransplantation, which involves the introduction of human genes into animals to produce organs for transplantation into humans, did not receive majority approval for a long time. However, in the 2010 survey, 58% of the respondents approved this technology. On the contrary, animal cloning for food products was among the least popular technology, receiving support only from 18% of the respondents. "Unnaturalness" was one of the major objections to cloned animal food and GM food crops. Thus, the concern expressed is intrinsic in nature. Furthermore, even in the case of technologies receiving majority support, such as human enhancement, xenotransplantation, gene therapy, and embryonic and non-embryonic stem cell research, a considerable percentage of the public expressed their approval, subject to the administration of strict regulatory laws (Gaskell et al., 2010). In the United States, a 2003 survey conducted by Rutgers University revealed that the majority of interviewees disapproved of the idea of animal-based genetically modified food and considered the creation of hybrid animals through genetic manipulation morally wrong (Van Eenennaam, 2006; CAST, 2010).

People are the ultimate end-users of the technology. Therefore, their perceptions, even if more intuition-based than founded in logic or scientific information, have to be respected and taken into account before introducing new technologies and related products on a large scale. The ethical principles of informed consent and informed choice have to be followed, and paternalism avoided at all cost. Biotechnology and genetic engineering may not be unethical per se, but their imposition on an uninformed or poorly informed public is definitely unethical. The surveys conducted in Europe and the United States point out the need to inform and educate the people regarding the pertinent facts about gene technology so that they are able to make informed decisions.

Extrinsic concerns

Animal welfare

How is animal welfare best defined? Traditionally, the term animal welfare pertained to the animal's morphological and physiological status, its level of nutrition, and the quality and adequacy of shelter provided to it, etc. In other words, the criteria for welfare had to

do with the physical health and the body of the animal. However, a more modern concept of welfare would ideally take into account the mental health of the animal as well. For example, a pet dog which is getting adequate food and housing and is in a good state of bodily health could still be facing cruel treatment from one of its masters, which results in its remaining in a perpetual state of anxiety. In such a case, its welfare has been violated. Thus, the definition of animal welfare is that it comprises the state of the animal's body and mind, and the extent to which its nature (genetic traits manifested in breed and temperament) is satisfied (Hewson, 2003).

While evaluating the animal welfare concerns of animal biotechnology, it is necessary to examine the nature and extent of infringement, if any, that biotechnology can inflict on animal welfare. In this context, it is necessary to list the five freedoms advocated in the Brambell Report that are the most well-known yardsticks of animal welfare. Roger Brambell drafted this document on the welfare of farm animals in 1965 and submitted it to the UK government. The guidelines subsequently developed by the Farm Animal Welfare Advisory Committee were later elaborated as the five freedoms that comprise the following:

1. Freedom from hunger and thirst by ready access to fresh water and a diet to maintain full health and vigor.
2. Freedom from discomfort by providing an appropriate environment, including shelter and a comfortable resting area.
3. Freedom from pain, injury, or disease by prevention or rapid diagnosis and treatment.
4. Freedom to express normal behavior by providing sufficient space, proper facilities, and company of the animal's own kind.
5. Freedom from fear and distress by ensuring conditions and treatment that avoid mental suffering.

It may be noted that these five freedoms take care of the animal's physical needs as well as its feelings, and provide a broad guideline for animal welfare. However, each of these five points needs to be delineated for particular farm animals. For example, the food composition and quantity for broiler chickens and dogs, and their space requirements, would be different. Again, it would also vary among different dog breeds. These five criteria could serve as a basic yardstick to find out whether animal biotechnology meets or violates the minimum basic requirements of animal welfare. It is perhaps worth mentioning here that the current practices of animal rearing that are not mediated by biotechnology also pose challenges to these five freedoms. For instance, British and French bull dogs, pugs, boxers, and several others experience breathing problems, high blood pressure, and low oxygen levels in blood due to their short muzzle, accompanied by a disproportionately large amount of muzzle soft tissue, which is the characteristic of these breeds. Similarly, some dog breeds have difficulty giving birth without surgical intervention, while others have difficulty walking and so on. Broiler poultry also have leg problems, while the double-muscled Belgian Blue cattle experience extreme calving difficulties, among a host of other problems. Questions may therefore be raised as to the ethical propriety of creating these breeds just for human entertainment and business, and subjecting them to a life afflicted by suffering and incapacitation.

While looking for ethical issues related to biotechnology (i.e., animal welfare), one has to examine the technology of genetic engineering that involves the manipulation of animal genomes using recombinant DNA (rDNA) techniques. Gene knockout techniques are used in animal experimentation where a gene with unknown functions is removed to determine its role in physiological processes. Animal cloning is another technique that is going to be increasingly used in animal biotechnology. The welfare aspects of transgenic and cloned animals, therefore, need to be evaluated.

The ethical basis for the treatment of animals by humans can be summarized in three or four approaches. The Greek philosopher-naturalist, Aristotle (384–322 BCE), and the philosopher, St. Thomas Aquinas (1225–1274), were of the view that since only humans possessed the ability to use reason to guide their actions, only they could be accorded moral standing. The French mathematician–philosopher René Descartes (1596–1650) held the view that animals were mere machines possessing only instrumental or extrinsic value and were exclusively meant for service to (and use by) humans. This exploitative view is consequently called the Cartesian view. Descartes believed that animal behavior could be explained on the basis of mechanistic principles, as an animal was akin to an "automaton" (a machine or mechanical device) produced by humans and not to a conscious being. Needless to say, such an ethic would allow experimentation without expressing any concern for animals and is no longer acceptable. A second approach is a kind of ethical utilitarianism put forward by the renowned ethicist Peter Singer, which states that the pain and suffering experienced by an animal during a given experiment or use should be weighed against the benefits accrued from that act or experimentation before deciding on a course of action. The "rights" approach of Tom Regan rejects this kind of a trade-off ethic. Regan argued that any being that was the "experiencing subject of a life" possessed inherent

or intrinsic value irrespective of its having sufficient intelligence, autonomy, or reason. It would be ridiculous to consider a human being in this manner; therefore, following this principle we can also recognize intrinsic value in animals, so they deserve to be accorded moral standing. It would therefore be unethical to use animals simply as a means to an end. In this sense, the animal rights argument is very much a part, or an extension of, the human rights movement. Another pioneering researcher in animal ethics, Bernard Rollin, pointed out that despite their good intentions of using animal experiments for curing disease or protecting people from toxicity, scientists were nevertheless responsible for a lot of animal suffering. The increasing use of animals in research necessitates the creation of a new ethic. Such an ethic would prevent cruelty and ensure for animals a life that respects their natural behavior and preferences to the best extent possible, and would remove or reduce pain without abolishing animal use in agriculture and research (Rollin, 2011).

The above discussion shows that the implications of animal biotechnology in animal welfare should be assessed for each specific type of technological applications against the backdrop of the five freedoms and the various ethical approaches, like deontology, utilitarianism, and the rights approach. Some examples are provided here to illustrate this point of view.

As said earlier, gene knockout technology involves the removal of a particular gene in order to understand its role in physiological processes. One problem associated with this technology is the production of surplus animals that do not have the desired genotype. A small percentage of the embryos in genetic engineering experiments survive, and of those that survive, a mere 1%–30% carries the intended genetic alteration. With the substantial increase in such experimentation worldwide, the number of surplus animals also increases greatly, creating problems of animal welfare in terms of pain and suffering experienced by them. The present uncertainty and inefficiency of genetic engineering experiments also add to animal welfare problems. For example, unpredictable anomalies such as lameness, susceptibility to stress, and low fertility characterized the early transgenic livestock. Cloning has also remained inefficient, and cloned animals suffer several abnormalities. These include developmental abnormalities like prolonged gestation, large birth weight, reduced placenta, and abnormalities in hepatic, cardiovascular, renal, neural, and muscular tissues, resulting in pain and suffering for the newborn as well as for the surrogate mother. However, with continuing refinement of genetic engineering techniques, unpredictability is expected to be reduced, leading to the improvement of animal welfare (Ormandy et al., 2011). Pigs with a gene for human somatotropin or bovine somatotropin (bST) exhibited weight gain and improved ability to convert feed to flesh, but at the same time, they also experienced a number of physical problems such as lameness, lethargy, and even gastric ulcers. Similarly, cloning from blastomeres and somatic cells may result in large calves and lambs, with possibilities of more serious effects when cloning is done from somatic cells. Cloning is still an inefficient process, resulting in a high percentage of surviving eggs showing abnormalities in placental morphology, fetal development, the immune system, brain, and digestive system (reviewed in US National Research Council, 2004). This evidence, although far from adequate, points out the need to have continuous monitoring of animal welfare in transgenic and cloned animals. However, the situation is expected to improve with the refinement of existing technologies.

Human health

Nature of risk

The concern for human health arises from two directions, namely the use of genetically engineered animals or their products as food, and their use in medicine (along with their attendant ethical dilemmas). As the public surveys in Europe and the United States show, there is extremely low public acceptability of GM animal food, while relatively high approval of genetic medicine. Large percentages of the interviewees perceived GM/cloned animal food as low on benefits, unsafe, inequitable, and worrying. However, both the European Food Safety Authority (EFSA) and the US Food and Drug Administration (FDA) observed that the reasons for not approving GM animal food mostly lacked a scientific basis. However, there are concerns that the animal organs used in xenotransplantation may transmit viruses into the human body.

Public health ethics and animal biotechnology

Schröder-Bäck (2007) formulated a set of concise principles of public health ethics that can be useful for evaluating the health risks and benefits of animal biotechnology. Based on these principles, some key questions could be asked to examine the ethical propriety of animal biotechnology (i.e., human health). The following are basic principles and questions:

1. *Social Utility*: This is equivalent to beneficence in medical ethics. Therefore, one could ask whether, or how much, a given animal biotechnology would be indispensable to society, or is it merely intended to advance the interests of a small group of people, such as scientists and corporations. In other words, a utilitarian analysis can be useful in adopting an ethical position regarding a particular technology.

2. Respect for Human Dignity: The utilitarian bias of social justice is somewhat counterbalanced by having respect for human dignity, which prevents the sacrifice or instrumentalization of the interests of individual human beings. Therefore, this principle could protect specific groups from possible victimization because of the adoption or rejection of a particular technology. For example, people suffering from kidney, liver, or cardiac malfunction, and their friends and relatives, would welcome advances in xenotransplantation, irrespective of the ethical implications. Thus, social utility and respect for human dignity in a way they complement each other and enable us to examine both of these principles in a balanced manner.
3. *Social Justice*: This comprises another facet for balancing social utility. According to this principle, not only the utility, but also the distributive justice, is taken care of. Thus, a particular animal biotechnology ought to be assessed with regard to its ability to look after the interests of all spectrums of people, including disadvantaged people and people in economically developed, developing, and under-developed countries or regions. This could take the form of access to a given technology by all groups of people.
4. *Efficiency*: This is also an important parameter to determine the appropriateness of any technology. An efficient technology would allow it to reach more people in a country or region with limited resources at its disposal.
5. *Proportionality*: The fifth (and last) principle demands insurance that the health benefits of animal biotechnology outweigh the possible infringement on autonomy, privacy, and all other negative impacts.

Environmental concerns

Ethical positions

The ethical positions that humans can adopt in their relationships with the environment could be categorized as anthropocentric (human-centered), biocentric (life-centered), and ecocentric (ecosystem-centered). Deep ecology is similar to ecocentric positions, but with a strong emphasis on self-realization ("Self" with a capital "S") (Sessions, 1995). An anthropocentric environmental ethic grants moral standing exclusively to human beings. It considers nonhuman natural entities and nature as a whole to be only a means for human ends. Thus, it recognizes intrinsic value only in human beings, with nature accorded extrinsic value only. A biocentric ethic recognizes intrinsic value in animals, especially the sentient animals, while an ecocentric ethic assigns intrinsic value to nature as a whole, including all life forms, and even entire ecosystems, and ultimately, the biosphere. Environmental risks posed by all technology (including biotechnology) need to be evaluated against these ethical positions. Even with a moderately anthropocentric stance, one cannot ignore the potential and existing threats of technology and development to natural systems, including their nonhuman denizens.

Concerns posed by transgenic technology

The major environmental concern with transgenic animals is that they may be accidentally or deliberately released into the environment, causing environmental problems. However, in such cases, the impact of the transgenic animals would be like that caused by the introduction of invasive alien species into the environment. Transgenic fish provide an example of such concerns. If a transgenic fish species has high juvenile and adult viability, high fecundity, and high fertility, then it could play the role of an invasive species, spreading its genes into the aquatic ecosystem. Thus, the impact of transgenic fish release will depend on the net fitness of the species compared to that of the wild stock; if it is higher than that of the latter, then the transgene will spread through the wild population (Kaiser, 2005). Transgenic fish with greatly enhanced growth are coveted both in aquaculture and in sport fishery. Transgenic coho salmon (*Oncorhynchus kisutch*), rainbow trout (*O. mykiss*), cutthroat trout (*O. clarki*), and chinook salmon (*O. tshawytsha*) have juveniles 10–15 times larger than their non-transgenic counterparts. Both juvenile and adult GM tilapia are known to be three times the size of non-GM members of the same species. These growth-enhanced fish can reach sexual maturity earlier than their wild relatives, resulting in a lasting genetic effect in the population. In a model developed with the fish Japanese medaka (*Oryzias latipes*), it was shown that transgenes could spread rapidly in the population, provided certain conditions are fulfilled (Muir and Howard, 2001). The transgenic Atlantic salmon, which is to be marketed soon, grows twice as fast as the wild individuals. It contains the DNA sequence of the code for the growth hormone of Chinook salmon and regulatory sequences derived from Chinook salmon and ocean pout. This creates the risk of these transgenics escaping into natural ecosystems and eventually decimating or even wiping out wild populations. However, the company producing the transgenic salmon has offered assurances of full-proof culture systems far from the sea, with adequate preventive measures. Furthermore, most of the fish are reported to be sterile triploids that pose low risk to the wild populations (Marris, 2010).

Precautionary principle

The regulatory issues emerging from human health and environmental concerns of biotechnology can be discussed under the guidelines of the UN precautionary principle.

This can be especially useful for animal biotechnology or any other emerging technologies that are characterized by a lack of full scientific certainty. However, this uncertainty cannot be an excuse for not adopting cost-effective measures to minimize health risks or prevent environmental degradation. The EU decision to impose a ban on cloned animals for food reflects not only a respect for public opinion, but also adherence to the precautionary principle, notwithstanding the fact that evidence for adverse impacts of GM animal food is far from conclusive.

Some challenging ethical issues in animal biotechnology

Chimeras

In this section, the ethical implications of the production of chimeras, which are combinations of cells of different embryonic origin, including those of humans and nonhuman animals, are discussed. The term chimera denotes organisms comprised of cells from two or more individuals of the same (intraspecific) or different (interspecific) species. Chimeras can have cellular combinations at the preimplantation blastocyst stage of development, as well as other entities created by introducing cells at later stages, including in adult recipients (Lensch et al., 2007; Greely et al., 2007). Human-to-animal chimeras are those where human cells such as pluripotent stem cells are placed in a nonhuman animal. Such chimeras could be fetal or adult depending on whether the human cells were placed in an embryo or a postnatal animal. Similarly, there could be animal-to-human chimeras that come more under the heading of xenotransplantation.

Four ethical arguments can be raised against chimeras: moral taboo, species integrity, unnaturalness, and human dignity. The questions of moral taboo and playing God, as well as crossing species boundaries, have already been discussed in an earlier section. Hybrids occur in nature, and artificial hybrids of both plants and animals have been accepted by society without any ethical reservations. So, the real question raised by the human-to-animal chimeras is whether the species boundary is unique and consequently inviolable or not. The other question is that if the human stem cells are planted in a nonhuman embryo and not allowed to develop into adults, what ethical position should be adopted? Should the termination of human-to-animal chimeric embryos receive the same ethical attention as human abortion? Again, in another possible scenario, what ethical stance should be taken if a chimeric mouse having human sperm mates with another chimeric mouse having human eggs? It is also felt that the creation of interspecies creatures from human material evokes the idea of bestiality, an act of moral abomination, just as sexual intimacy between humans and animals is considered abominable and a moral taboo. Chimeras also represent a metaphysical threat to the self-image that human beings have (Robert and Baylis, 2003).

Here, it could also be argued that species, besides being genetic and ecological constructs, could also be viewed as mental or cultural constructs so far as human beings are concerned. We have mental images of a cat or a monkey, and for a human being. If human-to-animal or animal-to-human chimeras violate those images, it could lead to moral–ethical problems. On the contrary, a human being with a pig's heart, liver, kidney, or neural tissue, or a cat with grafts of human tissue but otherwise human-like or cat-like characteristics (respectively) may not disrupt that mental construct of the species. Such constructs of species have been called commonsense species categories (Karpowicz, 2003). The author adopts a utilitarian argument by pointing out the fact that stem cell research could pave the way for the successful treatment of hitherto untreatable diseases such as those caused by the dysfunction of retinal or neural tissues. Mouse chimeras with areas of human neural or retinal tissue could find immense use in therapies and should be ethically defendable. Regarding the question of granting greater moral status to such chimeras, Karpowicz further argues that the mouse brain is small and has a much shorter development time than that of humans. In order to have a neuronal complexity equivalent to a human brain, a chimeric mouse would need to have a huge brain several hundred times larger than its own. Only such a chimera, which is extremely unlikely to be produced, would create moral abomination or demand greater moral status. In this context, a question arises as to whether the transplantation of human stem cells into the brain of nonhuman primate embryos should be allowed, as it would definitely increase the risk of a future moral confusion. Johnston and Eliot (2003) are of the opinion that the ethical parameter that is most offended by chimeras is human dignity in terms of cruelty to such a creature, treating it as a means to an end and not an end in itself, which is morally unacceptable. Greely et al. (2007), in their review of the findings of a host of other workers, concluded that chimeras compromised human dignity if the human cells transplanted into another animal were allowed to proliferate to develop into a human-like

brain rendering human-like capabilities in these animals. They also reported that research involved in transplanting human neural stem cells into the brains of nonhuman primates should be regulated to minimize the risk of the resulting chimera having human-like cognitive capacities.

Thus, the creation of human-to-animal chimeras poses certain interesting ethical questions that need to be addressed before such research could move forward to produce creatures that may have more and more human-like characteristics in their brains. Recently, Wu et al. (2017) have shown that naïve human induced pluripotent stem cells (iPSCs in the naïve state are those cells having unbiased development potential) successfully engrafted in the preimplantation blastocysts of both pig and cattle. Thus, naïve human iPSCs could be explored to generate interspecies chimeras not only in mouse, but also in ungulates like pig, cattle, and sheep.

The Nobel Prize in physiology or medicine 2012

The Nobel Prize in physiology or medicine in 2012 was jointly awarded to Sir John B. Gurdon and Shinya Yamanaka for the discovery that mature, fully differentiated and specialized cells had the potential to become pluripotent and give rise to all types of cells. John B. Gurdon in 1962 conducted an experiment where he removed the nucleus of a fertilized egg cell of a frog and inserted a nucleus from a mature intestinal cell. The cell with the transplanted nucleus grew into a tadpole, proving that a mature, specialized cell still had all the information needed to form all types of cells (The Nobel Prize, 2012). The same technique of nuclear transfer was used to produce the cloned sheep Dolly in 1996. Shinya Yamanaka in 2006 showed that pluripotent stem cells could be induced by reprogramming mouse fibroblasts with the introduction of four transcription factors: Oct3/4, Sox2, c-Myc, and Klf4. These cells—termed as induced pluripotent stem cells (iPSCs)—exhibit the property of embryonic stem cells (Takahashi and Yamanaka, 2006). Yamanaka's finding paves the way for human iPSC therapy whereby several diseases could potentially be cured by transplantation of specific healthy cells such as dopaminergic neurons for Parkinson's disease, oligodendrocytes for spinal cord injury, and retinal pigmented epithelial cells for retinal degenerative diseases. Further, human iPSCs could be transplanted in sheep, cattle, or pig to generate organs such as kidney, liver, heart, lung, and others, which in turn could be transplanted in humans requiring organ transplantation. This could alleviate the problem of organ shortage for transplantation and address the vexed issue of organ trade.

Animal biopharming

Biopharming is a popular term that describes the manufacture of complex therapeutic proteins such as blood clotting factors, fibrinogen, and alpha-1-antitrypsin in the milk of transgenic animals such as sheep, goat, cattle, and others. Alpha-1-antitrypsin is used in the treatment of cystic fibrosis in children, thus lending great utilitarian value to such innovative technologies. While recombinant proteins like insulin and human growth hormone have been manufactured for a long time in genetically engineered bacteria and yeast, more complex proteins can only be manufactured in mammalian cells. For example, the gene coding for alpha-1-antitrypsin can also be inserted into plants and microbes, but they lack the ability to add a carbohydrate moiety that is needed to make it biologically active. The biochemical similarity of animals to humans lends them an advantage over plants and microbes. Another advantage of animal biopharming is that lactating goats, sheep, or cattle can be made to secrete the therapeutic protein in their milk, from which it can be purified. Antithrombin, an anticoagulant protein, was the first therapeutic protein produced in the milk of transgenic goats that has subsequently been approved for administration to persons with a congenital defect that prevents them from producing sufficient amounts of this protein. Spider silk has also been produced in the milk of goats in a similar fashion. A large number of other therapeutic proteins are now in the process of being produced in the milk of transgenic animals. These products include albumin, human growth hormone, collagen, fibrinogen, lactoferrin, humanized polyclonal antibodies, and others, and they are being produced in the milk of cows, goats, sheep, pigs, and rabbits (Goven et al., 2008).

Ethical issues in animal biopharming revolve around risks to human health, gene transfer, and animal welfare, and can be analyzed by general utilitarian, public health, and animal welfare principles, as well as by the precautionary principle. The risk factors have been reviewed in detail by Goven et al. (2008). The information provided in the following sections is largely taken from their work and subjected to ethical analysis.

Risks to human health

The major potential health risks include contamination of milk with infectious agents such as prions in cattle. Prions are associated with transmissible spongioform encephalopathies (TSEs), including bovine sponigioform encephalopathy (BSE), variant Creutzfeldt–Jakob disease (vCJD), and scrapie. Although prion aggregation, activation, and transmission are complex phenomena, this is a major health hazard that

can occur from biopharming. Other possible health risks include allergenic and immunogenic responses, and autoimmune reactions. These may be linked to the unpredictability of the microinjection process of producing transgenic animals. These risks, therefore, need detailed scrutiny not only from scientific experimentation and assessment, but also from the point of view of public health ethics, medical ethics, and the precautionary principle. Striking a balance between social utility/beneficence and human dignity, and social justice and efficiency, is an essential exercise that should be undertaken for a realistic analysis of health risks. While biopharming holds great promise for providing health benefits to people, it should be noted that the only biopharm product that has received marketing authorization from the European Medicines Agency (EMEA) is ATryn®, which is a form of recombinant antithrombin produced in transgenic goats. Several others are in the preclinical and research stages.

Food chain contamination

All animals used in biopharming have to lactate in order to produce milk and therefore have to produce offspring. This results in the production of a large number of surplus animals. It is probable that these animals will be sold on the market and thus enter the human food chain. Similarly, the excess milk that is not required or suitable for pharmaceutical production will also enter the human food chain. This problem calls for strict regulatory mechanisms to prevent the entry of any such products legally or illegally into the market. Countries with inadequate policy frameworks and/or poor governance are therefore at higher risk of these problems. Another hazard could be in the form of these animals being marketed as processed meat or milk/milk products through clandestine channels, a possibility that cannot be entirely ruled out.

Escape of biopharm animals

Small animals pose a higher risk of such escape, and these may mix and mate with their relatives outside. The risks are similar to those posed by the possible escape of transgenic fish or other transgenic animals. The risks are relatively less in large, fully domesticated animals like cattle. From an ethical standpoint, careful weighing of potential benefits against risks may help to arrive at appropriate decisions. Besides utilitarian principles, this problem also raises the question of the value of animals. Should the animals be accorded only extrinsic or instrumental value, where the highest or sometimes sole priority is attached to their use-value for humans? On the contrary, should they be regarded as partners worthy of receiving intrinsic value because of their central role in producing life-saving molecules that alleviate human suffering? These questions call for intensive ethical discussion. Again, a rigorous application of the precautionary principle is needed in these cases to do justice to all concerned.

Horizontal gene transfer

Horizontal gene transfer may take place through the presence of the programmer gene in blood, feces, and other waste materials and could be spread by other organisms such as bacteria and blood-sucking insects. These possibilities, however remote, constitute another set of risks in biopharming. Again, the precautionary principle and the principles of beneficence, nonmaleficence, and least-harm can be the major instruments in resolving this issue.

Welfare issues of biopharm animals

This is one of the most important ethical concerns associated with biopharming. Various abnormalities of cloned animals cause high morbidity and mortality. Conditions such as large-calf syndrome, hydrops, or hydroallantois and musculature abnormalities occur at higher rates among cloned animals. The ethical implications of these welfare issues have already been discussed and are applicable to biopharm animals as well.

Genome editing

Genome editing is a very powerful tool that can be used to alter and redesign somatic cells, germ cells, and embryos alike. At present, genome editing employs several technologies such as zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and the most recently developed clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9 system. All of these technologies utilize the DNA repair mechanisms of the cell, which could be broadly of two types. In nonhomologous end joining (NHEJ), any break in DNA strand is simply joined without any means to ensure precision and thereby prone to targeted mutations at the repair site. On the other hand, homology-dependent repair (HDR) involves copying from a strand which is largely homologous with the sections around the repair site. In genome editing, the experimenter takes advantage of this mechanism by introducing a DNA strand having the desired sequences that are then copied at the repair site. This enables the experimenter to replace any 'defective' sequence at a given site with a 'normal' sequence. Of the three major genome-editing technologies mentioned earlier, CRISPR/Cas9 technique is the most versatile and promising, and consequently besides being hailed as the most revolutionary biotechnology of the recent times, this has also attracted considerable ethical

controversy. The potential applications of these technologies, especially CRISPR/Cas9, are immense, ranging from the creation and production of pigs resistant to a wide array of viral diseases, cattle that can experience a 'painless' dehorning and have more muscle mass, sterile female mosquitoes and transgenic animals, snails that no longer transmit the parasitic disease schistosomiasis, greatly improved and novel pets like 'micropigs', and many more such marvels. Scientists are even optimistic of applying these technologies to 'resurrect' extinct species such as the passenger pigeon and the woolly mammoth (Carroll and Charo, 2015; Reardon, 2015). Recently, a group of Chinese researchers led by J. Huang edited the genomes of human embryos in an attempt to modify the endogenous β-globin (HBB) gene responsible for β-thalassemia using the CRISPR/Cas9 technique. However, the rate of repair of the HBB gene was low, and there was the formation of mosaicism in the edited embryos, which is the presence of populations of genetically distinct somatic cells in the same organism. Further, several untoward mutations were also produced in 'off-target' areas. Thus, the fidelity and specificity of the CRISPR/Cas9 platform was questionable and posed serious limitations to the medical use of this technique in treating diseases (Liang et al., 2015; Cyranoski and Reardon, 2015). Although the researchers used 'non-viable' human embryos, the study raised extensive ethical debate in many quarters. At this point, it needs to be mentioned that genome editing could also involve making genetic changes in somatic cells, This type of genome editing, therefore, cannot be inherited and hence pose no threat to future generations. Hence, many scientists are of the opinion that a distinction must be made between genome editing in somatic and germ cells. A group of leading scientists in the field of genome editing (Lanphier et al., 2015) are, therefore, not only concerned that genetic manipulations in human embryos at the present stage of research are fraught with great risks and may be used for unethical non-therapeutic purposes, but also worried that the resultant public outcry against such research may also raise obstacles for the therapeutically valuable experiments on somatic cells that are relatively safe. Genome editing technologies in somatic cells have great potential in curing diseases such as HIV/AIDS, hemophilia, sickle-cell anemia, cystic fibrosis, some types of cancer, and others. On the contrary, human germline modification has a range of implications that need extensive discussion and dialogue on safety issues and ethical concerns. First, at its present state of development, germline editing was found to produce a large number of 'off-target' detrimental modifications of the genome. Second, it could be used to produce 'designer babies or superbabies' and could be opted by those privileged few who would then engage in preferential modification of fetuses to produce changes that they cherish. This would bring about a 'genetic divide' within the population with consequent feeling of inferiority and misery among those not endowed genetically (Pollack, 2015; quoted in Yang, 2015). This could also further accentuate disparity between developed and developing nations with the latter not able to afford such technologies. Thus, the world would be divided into an affluent and 'genetically improved' and a less affluent and 'genetically natural' population. Such a divide is undoubtedly unethical and unjust and would further increase the already existing inequity between regions. Another danger is the possible irresponsible use of such technologies under authoritarian regimes that could bring back the horror of 'eugenics' policies to bring about racial, linguistic, or religious discriminations. Risks are present in the environmental and biodiversity fronts as well. CRISPR and other genome-editing technologies could also be used to modify mosquitoes and other insects and invertebrates, and these changes could be transmitted to future generations. Accidental release of such genome-edited organisms into the natural environment would have consequences of which we have no or very little knowledge at the present moment. Such research, therefore, should ideally be subjected to the tenets of the UN Precautionary Principle and regulated.

Thus, it is evident that germline editing has several important medical, ethical, legal, and broadly scientific implications. In January 2015, a group of scientists, ethicists, and other interested stakeholders met at Napa, California, USA, to have threadbare discussions to ensure safe, responsible, and ethically acceptable application of genome-editing technology such as CRISPR. The group advocated for extensive open discussions on the benefits and risks of human genome editing among scientists, social scientists, medical professionals, legal experts, and the general public. They also felt that at the present state of knowledge, legal approval should not be accorded to human germline modification. However, they recommended formation of forums where scientists and bioethicists could disseminate information on this revolutionary technology, encourage transparent research to evaluate the efficacy of such research, and recommend appropriate policies (Baltimore et al., 2015). The Hinxton Group, which is an international consortium on stem cells, ethics, and law, issued a statement where they agreed that genome editing is a valuable tool in the field of medical science, although several scientific questions must be answered before human

reproductive applications. These questions, among a host of others, include those on unintended off-target effects and the type of embryos to be used in this kind of research. However, the group also cautioned against over-regulation which might hinder the progress and refining of this useful technology (The Hinxton Group, 2015).

Besides these secular arguments, religious perceptions of genome editing could vary from outright rejection of these technologies as 'playing God' to more tolerant 'partnering with God' or 'participating with God' (Carrol and Charo, 2015) or view these as 'God operating through human hands'. Thus, it may be difficult to arrive at a consensus among all religions on the ethical sanction to use these technologies. At the same time, it is very likely that despite their different positions on the ethical propriety of using these technologies, the safety aspects will definitely be given priority and all religions would advocate exercising prudence and taking all possible precautions while using these technologies.

The CRISPR-twin controversy

Recently, the scientific world was agog with the ethical and safety debates arising from the claim by He Jiankui of the Southern University of Science and Technology in Shenzhen, China, that he had used the gene-editing tool CRISPR-Cas9 to modify the DNA of twin girls born in November 2018. He used CRISPR-Cas9 to disable the *CCR5* gene, which allows HIV to enter human cells. Some humans possess a natural variation of this gene—called the delta-32 mutation—that confers resistance against some strains of HIV. It is now known that the elimination of *CCR5* gene in mice makes them smarter and also improves brain recovery after stroke in humans, besides other possible effects. Thus, many neuroscientists feel that the alteration could have affected the brains of the twins in an unknown manner and affect their cognitive function, which is not at all ethical (Regalado, 2019). Further, it is possible that the mutations generated by He Jiankui did not match the delta-32 mutation and, therefore, might not even offer the intended protection against HIV infection (Zimmer, 2018). He's institution has stated that it had no knowledge of the trial, which He conducted with his own resources (Williams, 2019). He Jiankui is also under investigation by the Chinese Government, and the experiment has been condemned by the scientific community in general. China now proposes to have strict regulations on research involving gene editing, gene transfer, and other similar technologies, which would require government approval before researchers can proceed with the experiments (Akst, 2019).

Constitution of ethics committees

The formation of ethics codes and committees can be traced back to the Nuremberg Trials in 1945 and 1946, held after World War II in order to try Nazi war crimes. Several crimes were committed in the guise of scientific research that involved inhuman and unethical treatment of prisoners and detainees in concentration camps. Such experiments involved immersing the subjects in ice-cold water or exposing them to freezing temperatures; muscle, bone, and nerve transplantations without anesthesia; infecting subjects with malarial parasites and other pathogens; head injury experiments; mustard gas exposure; and so on. The subjects (who included children) were forced to participate without any informed consent. The trials led to the drafting of the Nuremberg Code, which first addressed the question of volunteer consent for protecting human subjects in research. Subsequently, the World Medical Association (WMA) framed the Declaration of Helsinki, which still serves as a standard for physicians undertaking research on human subjects. This declaration was subsequently revised and updated several times. Important principles and guidelines were also provided in the Belmont Report (1979) published by The National Commission for the Protection of Human Subjects of Biomedical and Behavioral Research, USA. This report emphasized several ethical principles such as autonomy, respect for persons, beneficence, and justice. The Declaration of Helsinki gave precedence to the wellbeing of human research subjects over every other consideration. The Declaration stated that since medical research with human subjects involved risks to the subject, the details of such research must be clearly defined and described in a research protocol. This protocol should explain how it conforms to the principles of the Declaration and should provide information on funding, sponsors, institutional affiliations, conflicts of interest (if any), and so on. This protocol was to be submitted to a research ethics committee before starting the study. These committees were required to be free from any undue influence or interference from sponsors and other interested parties so as to be able to serve as an independent body to protect the rights and ensure the safety of participants in human trials. They were to take into consideration pertinent rules and regulations of the country where the research was to be carried out, along with applicable international norms and standards, but were not to withdraw or reduce any protection for research subjects contained in the Declaration. Once approved, no further changes could be made to the protocol without prior consideration and approval of the Committee. Such independent bodies are known as institutional review boards

(IRBs), independent ethics committees (IECs), or ethical review boards (ERBs). It is now customary for every university, institute, hospital, or other organization where research with human subjects is conducted, to have one of these bodies for ensuring the ethical propriety of research and for safeguarding the dignity, safety, and rights of human subjects. The goal is to obtain the prior informed consent of human subjects, assess risks, and protect individual privacies before any trial would become mandatory. An IRB or IEC must have a minimum of five voting members, at least one of whom must be a non-scientist, and one member should in no way be connected with the institution concerned.

Translational significance

Human therapeutic cloning and other techniques in animal biotechnology

Generally speaking, translational research (TR) means research aimed at converting the findings of basic science to their practical applications. More specifically, however, it means the clinical application of the outcome of basic research. TR can therefore be said to hold the key to the ultimate fate of scientific findings. Many path-breaking discoveries and inventions in every conceivable scientific discipline never find their deserving use in society because of the absence of appropriate TR. Although basic research is not aimed at solving practical problems, it can nevertheless provide clues or directions toward solutions to practical problems. Thus, it could provide the knowledge platform from which practical application-oriented studies could take off. In this sense, basic and translational research could be said to represent a sort of continuum. TR is often recognized to have two distinct dimensions: one involves applying the findings of basic research or preclinical studies to the development of clinical trials (first stage or T1), followed by the adoption of best practices in the community (second stage or T2) (Rubio et al., 2010). Thus, TR moves from basic research to patient-oriented research to culminate in population-based research, with society receiving the benefits and enduring the risks (if any) at this stage. However, it must be remembered that this flow is not strictly unidirectional, as there could be frequent to-and-fro movement and exchange of knowledge and experience between T1 and T2 (and even between TR as a whole) and basic research, as new issues and questions arise while applying the results at the community level. A large proportion of significant findings in basic science that hold great promise for developing cures for deadly diseases never reach the therapeutic development and application stage because of the absence of proper translational studies.

Citing the possible developments in human embryonic stem cell research as an example, the use of pluripotent embryonic stem cells (ESC) has created immense possibilities for developing therapeutic solutions for hitherto incurable diseases such as neurodegenerative diseases and others. It is to be understood that the main objective of raising ethical questions is to provide a moral–ethical framework to regulate and streamline these developments for the maximum welfare of humankind without compromising human dignity or harming other forms of life and the environment as a whole.

Conclusions

The preceding discussion of a number of important ethical concerns of animal biotechnology clearly shows that an ethical analysis of the different innovations cannot provide any readymade answers to the contentious issues raised because of their highly complicated nature. However, ignoring pertinent ethical issues can bring about detrimental consequences not only for the different stakeholders, but also for the technology itself. In recent years, we have witnessed huge public outcries against several new technologies. It is a matter of argument whether such resistance has a strong scientific basis, but at the same time the paternalistic attitude often adopted by a segment of scientists and policy-makers has not helped the situation. This is true not only for animal biotechnology, but also for many other technologies and even developmental activities, including those in developing countries. A communication gap between the promoters and detractors of new technologies is a major cause of the resultant imbroglio. The theories and principles of ethics constitute valuable tools for creating an atmosphere of dialog among the different factions and to ultimately arrive at the safest and wisest possible use of a given technology in a given time and place for the welfare of humans, nonhuman organisms, and natural systems alike.

World Wide Web resources

http://www.unesco.org/

The United Nations Educational, Scientific, and Cultural Organization (UNESCO) website is one of the richest repositories of publications on bioethics and ethics of science and technology, including biotechnology. After logging on to the website, one can

go to Social and Human Sciences, where the Themes drop-down menu leads to Bioethics and then to the Publications section, which contains books, reports, and advice on almost all conceivable ethical issues in science and technology. Furthermore, the site also contains announcements on seminars, workshops, and other events on bioethics. Information on the objectives and mode of functioning of the International Bioethics Committee (IBC), the Intergovernmental Bioethics Committee (IGBC), and the Global Ethics Observatory (GEObs) is also useful for understanding ethical perspectives in science and technology.

http://www.nhmrc.gov.au

This is the website of the Australian government's National Health and Medical Research Council. This site provides resources on the ethics of animal research and on medical ethics. The areas covered are diverse, such as perspectives on health ethics and human research ethics committees, animal research ethics, human embryos and cloning, ethical conduct in human research, research integrity, and the like.

http://ec.europa.eu

The website of the European Commission provides information on ethical issues pertaining to agriculture, the environment, and other areas. After accessing the website, one can go to the desired language (English, French, etc.) and then to Policies, Legislations, and Public consultations. In the Science and Technology sub-section in the Policies section, one can click on Ethics in Science and then on several ethics links, including those of the European Commission, as well as a large number of National Ethics Committees within and outside Europe.

www.nap.edu

The website of The National Academies Press, National Academy of Sciences, contains information on animal ethics and other issues. Free registration is possible, whereupon one can access and download a large number of documents. In the Biotechnology section, publications on translational studies, biosecurity, biosafety, and other subjects are available. Publications on the care of laboratory animals, regulations on the use of animals in neuroscience research, the use of nonhuman primates like chimpanzees in biomedical and behavioral research, and a host of other topics can be accessed for free download and/or online reading.

www.scu.edu/ethics/

The website of the Markkula Center for Applied Ethics, Santa Clara University, California, is another important site for online resources on bioethics, including ethical issues in animal biotechnology. Discussions on the implications of important ethical principles in pharmacogenomics research and applications such as informed consent, confidentiality, justice, and equity can be found here. Ethical issues in translational aspects of pharmacogenomics are also presented in the context of the normative framework.

http://www.bbsrc.ac.uk

This is the website of the Biotechnology and Biological Sciences Research Council, UK. In the Science in Society section, an external link to Understanding Animal Research provides resources such as documents, images, and videos on the use of animals in research. In the Publications section, Topic-Based Publications contains writeups on food security, industrial biotechnology, stem cell science, and related issues.

http://plato.stanford.edu

The website of the Stanford Encyclopedia of Philosophy (Principal Editor: Edward N. Zalta) is a valuable repository of information on all aspects of philosophy and ethics, including applied ethics. For example, a search using the term bioethics can lead to information on issues like human/nonhuman chimeras, cloning, the ethics of stem cell research, and other issues. General ethics topics like metaethics, deontology, utilitarianism, etc., can also yield substantial information in these areas.

http://www.iep.utm.edu

The Internet Encyclopedia of Philosophy contains useful definitions and explanations for concepts in ethics and philosophy. The search option can be used to access resources on ethical concepts such as deontology, metaethics, utilitarianism, virtue ethics, and the like, as well as the coverage of important ethicists and philosophers.

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Glossary

Animal Welfare A modern concept of animal welfare takes into account not only an animal's morphological and physiological status, its level of nutrition, and the quality and adequacy of its shelter, but also its mental health.

Anthropocentrism It is the human-centered ethical position in relation to the environment. It puts foremost importance on human needs and priorities.

Antithrombin Antithrombin is an anticoagulant protein. It is the first therapeutic protein produced in the milk of transgenic goats.

Applied Ethics Applied ethics deals with specific issues using the concepts and principles contained in metaethics and normative ethics as tools. It deals with wide-ranging issues like animal rights and environmental ethics, to euthanasia, cloning, and xenotransplantation, among others.

Biocentrism The biocentric or life-centered approach to environmental ethics recognizes intrinsic value in animals, especially sentient animals.

Biopharming Biopharming is a popular term that describes the manufacture of complex therapeutic proteins such as blood clotting factors, fibrinogen, and alpha-1-antitrypsin in the milk of transgenic animals such as sheep, goat, cattle, and others.

Chimera Chimeras are a combination of cells of different embryonic origin, including those of humans and nonhuman animals.

Deep Ecology Deep ecology puts emphasis on intrinsic relationships between man and environment, and a deep respect for all living organisms and ecosystems. The Norwegian philosopher Arne Ness is the main proponent of deep ecology.

Deontological Ethics Deontological ethics are based on the concept of duty or obligation and are often called nonconsequentialist, as they are not dependent on the consequences of a given action.

Ecocentrism An ecocentric ethic recognizes intrinsic value in nature as a whole.

Eudaimonia An important component of Aristotlean ethics, eudaimonia connotes happiness or flourishing, and virtue or excellence.

Gene Knockout Techniques These are used in animal experimentation where a gene with unknown functions is removed to determine its role in physiological processes.

Human Embryonic Stem Cells (hESCs) These are cells derived from the early embryo that are pluripotent and can differentiate into any cell type of the adult body.

Independent Ethics Committee An independent ethics committee (IEC) is also known as an institutional review board (IRB) or ethical review board (ERB). It is a committee that is authorized to examine, monitor, review, and approve (if found appropriate) biomedical and behavioral research involving humans, to ensure patient or volunteer safety.

Metaethics Metaethics studies the foundations of morality and establishes the theoretical basis and scope of moral values. In other words, it tries to define morality itself.

Normative or Prescriptive Ethics Normative or prescriptive ethics determines what is ethical and what is not.

Prion Prions are infectious pathogens that are devoid of nucleic acids and consist of a refolded protein. They are known to cause several diseases, including bovine spongiform encephalopathy (BSE) in cattle and Creutzfeldt–Jakob disease (CJD) in humans.

Utilitarian Ethics Utilitarianism is concerned with maximization of good and aims to bring about "the greatest amount of good for the greatest number."

Abbreviations

ART	assisted reproductive technology
BSE	bovine sponigioform encephalopathy
bST	bovine somatotropin
CAST	Council for Agricultural Science and Technology
CJD	Creutzfeldt–Jakob Disease
COMEST	World Commission on the Ethics of Scientific Knowledge and Technology
CRISPR	clustered regularly interspaced short palindromic repeat
ERB	Ethical Review Board
hESC	human embryonic stem cell
IEC	independent ethics committee
IRB	Institutional Review Board
IVF	*In vitro* fertilization
TR	translational research

Long-answer questions

1. Give a brief overview of the different ethical thoughts and theories. Enlist the major principles that could be of use in resolving ethical issues in science and technology.
2. Discuss the major intrinsic ethical concerns in animal biotechnology. What are your views on the accusation that animal biotechnology is "playing God." Do you think that religious beliefs should be used to impose restrictions on biotechnology research?
3. Give an account of the major extrinsic concerns in animal biotechnology.
4. Discuss the ethical issues arising out of the creation of chimeras.
5. Describe the potential benefits and risks associated with animal biopharming.
6. What is genome editing. Briefly discuss the benefits and risks associated with genome editing.

Short-answer questions

1. Name the three broad areas of ethics.
2. Explain the term beneficence.
3. What is the most important guiding principle in animal welfare?
4. Name the five concise principles of public health ethics.
5. Distinguish among anthropocentric, biocentric, and ecocentric approaches in environmental ethics.
6. Distinguish between somatic cell genome editing and germline genome editing.

Answers to short-answer questions

1. The three broad areas of ethics are metaethics, normative ethics, and applied ethics.
2. It literally means "doing good." From an ethical perspective, it has an underlying utilitarian basis that is concerned with producing the greatest possible proportion of good over evil for the greatest number of people.
3. The five freedoms outlined in the Brambell Report (1964) provide the most useful guideline in animal welfare. These include freedom from hunger and thirst; from discomfort; from pain, injury, or disease; for expressing normal behavior; and from fear and distress.
4. These are social utility, respect for human dignity, social justice, efficiency, and proportionality.
5. These approaches are human-centered, life-centered, and ecosystem-centered, respectively. A purely anthropocentric ethic does not recognize intrinsic values in nonhuman organisms and ecosystems.
6. Somatic cell genome editing involves genetic changes in somatic cells only, and these are not heritable.

Yes/no-type questions

1. The word ethics has its root in the Greek word *ethos* or *ethicos*.
2. The classical work *Nicomachean Ethics* was written by Plato.
3. Religious concerns about biotechnology are of an intrinsic nature.
4. Human embryonic stem cells are not pluripotent.
5. The Brambell Report is concerned with animal welfare.
6. Anthropocentric ethic recognizes intrinsic value in animals.
7. An intraspecific chimera has a combination of cells from two different species.
8. The Nobel Prize in physiology or medicine in 2012 was awarded jointly to Sir John B. Gurdon and Shinya Yamanaka.
9. Milk produced through biopharming can contain prions.
10. The gene *CCR5* allows HIV to enter human cells.

Answers to yes/no-type questions

1. Yes
2. No
3. Yes
4. No
5. Yes
6. No
7. No
8. Yes
9. Yes
10. Yes

CHAPTER

36

Approaches to the humane euthanasia of research animals

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Summary

Researchers who work with animals must sometimes euthanize those animals for scientific or ethical reasons. Any method used for euthanasia must be safe to personnel and should produce rapid and painless death to animals, unless otherwise justified for scientific reasons. Review of proposed methods by an ethics committee or institutional animal care and use committee (IACUC) can help ensure that a method appropriate to the research objectives, which considers humane treatment of the animal, is used. Both physical methods and those that use drugs or chemicals have been developed which, when used properly, allow for humane euthanasia. In all cases it is essential that personnel be properly trained so that whatever method is used, euthanasia is conducted in a way that minimizes distress to the animal.

What you can expect to learn

In this chapter we will discuss the ethical and practical reasons that euthanasia is conducted on research animals. In addition the importance of training of personnel will be highlighted, and the methods most commonly used to perform euthanasia on research animals will be described. Because rodents are overwhelmingly the most common animal used in research, focus will be given to euthanasia with relevance to mice and rats.

Introduction and background

The goal of euthanasia is to provide a painless death for an animal. Indeed the term "euthanasia" is derived from Greek to mean "good death." Though the idea of animal euthanasia can be troubling for some, it is important to recognize that research animals are euthanized for several compelling reasons, including declining health and the conclusion of the research project in which the animals were used.

Beyond an expectation for safe (for personnel) and humane conduct of euthanasia, there are few stated regulatory requirements for euthanasia of research animals. In general it is widely recognized that the guidelines for euthanasia described by organizations such as the American Veterinary Medical Association (AVMA) and the European Commission can be applied to achieve this expectation when appropriately followed (AVMA, 2020; Close et al., 1996).

Increasing recognition of the pain experienced by animals with some forms of euthanasia has directed improvements and refinements in approach. The importance of technical proficiency of personnel performing euthanasia, the environment in which animals are euthanized, and the methods used should be emphasized as central to the principle that animals should be euthanized in a way that minimizes pain and distress to that which is unavoidable and meets the scientific needs of the research.

Proper training of personnel

A basic premise of humane euthanasia is that personnel performing euthanasia on animals are properly trained and qualified. Because improper procedure can lead to animal distress and pain, proficiency of personnel should be established and documented.

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Several important aspects of euthanasia should be understood by personnel, including the goals of humane euthanasia, recognition of animal pain and distress, the proper environment for euthanasia, details of the specific method to be used, and the potential safety and emotional impacts of euthanasia on personnel.

Hands-on training with respect to methods of euthanasia should be available, even expected, for personnel. Aspects of training should include principles for ensuring a humane death of animals and which offers focus on the equipment to be used; specific techniques; safety of personnel when performing euthanasia; methods to confirm death of the animals; and safe disposal of carcasses and tissues following euthanasia. It is important that personnel train closely with someone experienced in the specific method being used and species to be euthanized. For the physical methods described later in this chapter, personnel might first practice on carcasses that have already been euthanized for other reasons by a skilled person. In all cases the ability of personnel to safely and effectively perform euthanasia should be confirmed by an experienced person.

An understanding that a rapid painless death is a goal that should be shared by all personnel performing euthanasia. Importantly those involved should have a clear appreciation for the contribution of the animal to advancement of science and medicine and of the need for euthanasia as part of the underlying work. Because each animal in a study may offer important data in support of study objectives, personnel should be trained to euthanize animals in a way that facilitates collection of needed samples for data analysis; and in the case of animals euthanized due to spontaneous illness, for collection of samples that will allow for diagnostic interpretation. In all cases, individuals must understand that the specific methods that have been approved by the IACUC should be followed.

Personnel who regularly perform euthanasia as part of their work with research animals may experience emotional distress (Scotney et al., 2015). Individuals who have a clear understanding of the need for using animals, as well as the potential scientific and translational benefit of the work, and the reason that the animals must be euthanized will be better prepared to handle any distress associated with performing euthanasia. In some cases carefully planned adoption of research animals as pets has helped to refine animal care and use and to reduce the emotional distress related to euthanasia (Carbone et al., 2003). If adoption is a possibility, a mechanism should be in place to ensure that the animal being adopted is healthy and will not, as a pet, pose a threat to people.

The environment in which euthanasia is performed should be one that minimizes distress to the animal. For example the area should be quiet with relatively little other activity. Because animals in distress may release alarm pheromones that alert conspecifics to threatening situations (Brechbül et al., 2013), the area should be cleaned so that animals to be euthanized are not distressed by alarm pheromones released from animals previously euthanized. Similarly animals should not be exposed to other animals undergoing euthanasia, as such exposure via pheromones or vocalization is regarded as a potential source of distress (AVMA, 2020), although work to investigate this possibility failed to confirm such an effect in C57BL/6N mice (Boivin et al., 2016).

Methodology, equipment, and principles

Methods to carry out euthanasia can be classified in different ways. From the point of view of the agents and mechanism of action, they can be broadly divided into physical and chemical methods. In contrast the AVMA Guidelines (2020) classify euthanasia methods as *acceptable*, *acceptable with conditions*, or *unacceptable* based on the immediate effects to the animals (i.e., time to induce unconsciousness, irreversibility, potential for pain or distress, consistency). Acceptable methods are those that consistently result in a humane death; methods characterized as acceptable with conditions are those that require additional provisions in order to consistently produce a humane death of the animal; and unacceptable methods are those that are always considered to be inhumane or pose significant risk to personnel. Other factors taken into consideration for this categorization are the safety for personnel, availability of the agent, and possible legal limitations as well as species differences.

Consideration must be taken about the place to carry out euthanasia: preferably in a separate room so that if distress vocalizations or the secretion of pheromones are elicited, they cannot be heard or smelled by other animals in the area (AVMA, 2020). Examples of methods for rodents that are acceptable and acceptable with conditions are given in Tables 36.1 and 36.2.

A crucial point is to confirm the animal's death after carrying out any euthanasia procedure and before disposal of the carcass. The election of the signs to observe will depend on the species in question. For laboratory rodents the cessation of vital signs such as respiration and heartbeat, corneal opacity, or the onset of rigor mortis are typically used. For some of the methods described below a secondary method might be necessary to ensure death and to completely avoid the potential for recovery.

TABLE 36.1 Euthanasia methods classified as acceptable for laboratory rodents.

Acceptable		
Method	**Advantages**	**Disadvantages**
Barbiturate overdose	Rapid action Gentle induction Low cost Availability Might be used for pregnant females and neonates	Ideally administer IV, but very difficult in rodents (requires skill and training), hence IP administration permitted Might be a controlled substance in some countries because of abuse potential
Ketamine/Xylazine overdose	Rapid action Availability Might be used for pregnant females and neonates	Might be a controlled substance in some countries because of abuse potential Costly

TABLE 36.2 Euthanasia methods classified as acceptable with conditions for laboratory rodents.

Acceptable with conditions			
Method	**Condition**	**Advantages**	**Disadvantages**
Inhaled anesthetic agents (e.g., Isoflurane)	Requires special equipment (vaporizer) and appropriate dose for the species Keep socially compatible groups Empty and clean chamber after each use	Gentle induction Does not require restraint Also useful as induction before the use of CO_2 Safe for personnel with scavenging of waste gas Might be used for pregnant animals	Requires special equipment Costly
Inhaled CO_2	Requires controlled flow rate (30%–70% vol/min) Flowmeter and procedure must be calibrated and validated regularly Empty and clean chamber after each use	Can be performed in home cage Allows high amount of animals to be euthanized at the same time Safe for personnel Relatively low cost Automated equipment available Might be used for pregnant females and neonates (but consider much longer exposure periods for neonates)	Time consuming
Cervical dislocation	Personnel must show performance standard to luxate cervical vertebrae without crushing bones and spinal cord, thus achieving rapid unconsciousness	Does not require special equipment No chemical contamination of tissues Low cost Very fast technique for small animal groups	Requires highly skilled personnel Might be unpleasant for some staff members

Chemical methods

Injectable agents

Euthanasia carried out with the injection of an anesthetic overdose is an *acceptable* method in most species used for research according to the AVMA Guidelines (2020), hence it should be the first option to consider. However care should be taken that the agent used does not affect the intended use of tissue samples. Preferably the administration route should be intravenous (IV) as this is the fastest way to achieve the desired effect for most drugs. In small species such as mice and rats it is acceptable to use the intraperitoneal (IP) route given that an appropriate IV route is more technically difficult, and it is likely to take longer, thus going against one of the most important requisites for euthanasia: that unconsciousness should be achieved

rapidly in order to avoid pain and distress (Close et al., 1996). The injectable anesthetic agent should be selected according to the effectiveness for the species; in many cases drugs that have been shown to have a narrow margin of safety as anesthetics are the ones chosen as they are effective in producing a rapid death (e.g., barbiturates). Dissociative agents, such as ketamine, should always be used in combination with sedatives (e.g., xylazine) or benzodiazepines (e.g., diazepam), given that overdoses of ketamine alone can cause stimulation in some species or individuals (AVMA, 2020).

Inhalant agents

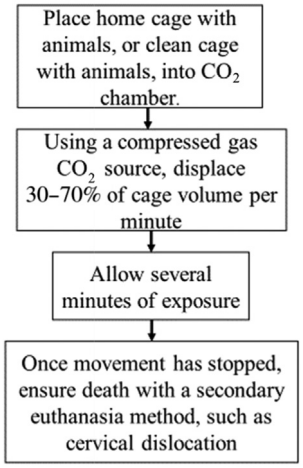
Volatile anesthetic agents (e.g., isoflurane) or CO_2 are typically used in this method. Both procedures require special equipment that render a known gas flow rate, and thus are classified as *acceptable with conditions*. In the case of isoflurane a concentration of 5% will first induce a surgical plane of anesthesia and by sustaining the same concentration over time, a depression of the central nervous system and the respiratory system typically occurs within 5 minutes (Marquardt et al., 2018; Roustan et al., 2012). An alternative method is to use a gauze or cotton soaked with isoflurane or other volatile inhalant anesthetic inside a closed chamber, taking care that the animal does not come in direct contact with the agent because of the risk of irritation and pain (AVMA, 2020). However this method is not recommended as it has been shown to be aversive to mice and rats (Moody and Weary, 2014).

Other inhalant anesthetics, such as ether and chloroform, have previously been used for euthanasia of small animals, as well as for anesthesia. However because of risks to personnel and because they are relatively slow acting and can be irritating to the animals, such compounds should not be used.

According to the AVMA Guidelines (2020), CO_2 inhalation should be carried out at a controlled flow rate that ensures gradual fill at a displacement rate from 30% to 70% of the chamber volume per minute. This requires the use of a flowmeter specific for CO_2. The volume of the chamber should be known, and the flowmeter should be adjusted accordingly. A prefilled chamber is *unacceptable*, as it causes great aversion to animals and pain due to membrane irritation (Conlee et al., 2005; Leach et al., 2002). A practical method is to use a purpose-made lid that fits over a cage that is connected to a CO_2 cylinder. For example, the flowmeter should be set at a rate of 1/2 of the cage volume, thus achieving a 50% filling of the cage per minute. The advantage of this method is that it is not necessary to remove animals from their cages and therefore it avoids a potential cause of stress. After the cessation of respiration, animals must be left for an appropriate time to ensure irreversibility or a secondary method, such as cervical dislocation, should be applied. There are commercially available systems that hold several cages and can be programmed to conduct euthanasia cycles automatically, according to species and cage size (Fig. 36.1). An

FIGURE 36.1 A carbon dioxide euthanasia chamber holding four cages of mice. When activated a light (right of figure) indicates gradual filling of the chamber with carbon dioxide and that the chamber is locked, preventing more animals to be added during the process. On the wall (back of the figure) is a flowmeter that closely regulates the rate at which carbon dioxide is added to the chamber. The system maintains an effective carbon dioxide concentration for a preset time and then automatically evacuates remaining carbon dioxide at the end of the cycle.

additional advantage of such systems is that they often have purpose-made valves that distribute the gas more homogeneously than a single entrance opening, thus ensuring the same concentration rate increase for all animals. Carbon dioxide should be provided via a compressed gas cylinder. Dry ice should not be used as a source of carbon dioxide for the euthanasia as its concentration and flow rate cannot be controlled. A preferred protocol for CO_2 euthanasia of rodents is summarized in the flowchart.



For both methods of euthanasia, death must be confirmed by observing the cessation of vital signs, the onset of rigor mortis, or by using a secondary method, such as cervical dislocation or induction of pneumothorax. Automated equipment that is programmed to allow for a dwelling time inside the chamber once it is saturated with CO_2 so that irreversibility is ensured without the need for a secondary method is available.

Physical methods

Cervical dislocation (Fig. 36.2) is perhaps one of the most commonly used physical methods for euthanasia of small rodents. It is a technique considered to be *conditionally acceptable*, because it requires the demonstration of proficiency by the operator to induce very rapid unconsciousness by achieving vertebral luxation without primarily crushing the bone and spinal cord. In this sense training and practice are critical and should be carried out first with dead animals to acquire manual dexterity. The next step should be the use of anesthetized animals, until proficiency and consistency are accomplished, before allowing the use of this method for conscious animals, otherwise death might not be achieved in a humane manner. Although some researchers find it a displeasing technique, and therefore it might not be their first choice, it is advisable to have this competence for critical situations

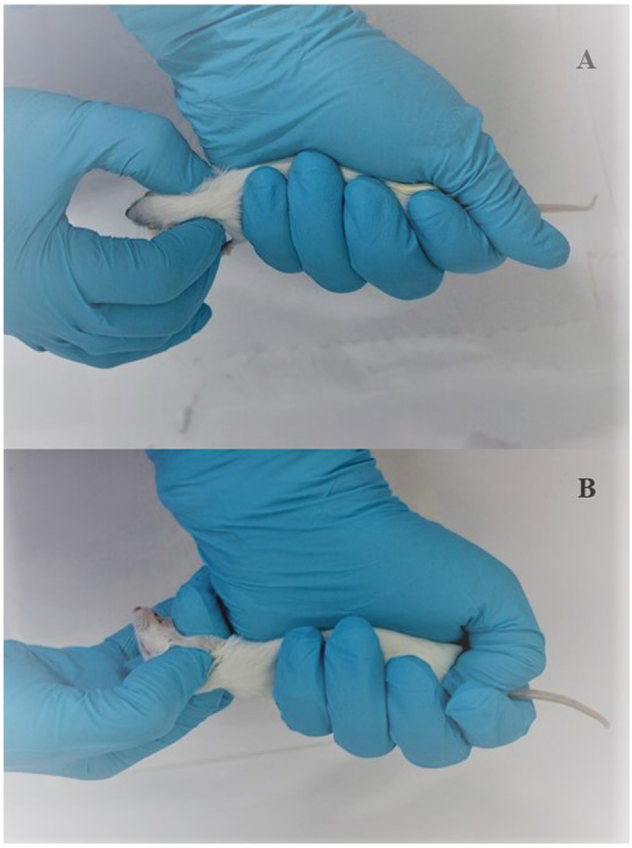


FIGURE 36.2 A method for cervical dislocation of an adult mouse. The body of the mouse is firmly grasped in one hand, while the head is controlled at the caudal aspect with the thumb and index finger (A). Initial hold, dorsal view (B). Holding the body firmly and steady, the head of the mouse is pulled forward and twisted simultaneously in one quick motion to dislocate the cervical vertebrae from the skull. Euthanasia by this method should be performed only by someone who has been properly trained.

when no other method is available for a rapid euthanasia. Cervical dislocation can be used for mice, rats, and other small mammals under 200 g of body weight, as well as for small birds and poultry (AVMA, 2020).

Decapitation is used in cases when it is necessary to preserve brain tissue that might be damaged by dislocation or might be altered by the use of anesthetic agents or CO_2. It is *conditionally acceptable* as it requires the use of special equipment (e.g., guillotine) that must be kept in good working condition to ensure euthanasia in one effective step. Personnel should be properly trained as this method implies a potentially higher occupational health risk. Equipment should have a maintenance schedule to ensure proper working order in every use.

Unacceptable methods

It is *unacceptable* to use injectable agents such as potassium chloride on unanesthetized animals, neuromuscular

blocking substances (solely or combined with anesthetic agents in the same syringe), opioids, α-chloralose, and urethane (unless used as an anesthetic before application of an acceptable adjunctive method).

Table 36.3 lists several chemical and physical methods that are considered as unacceptable for euthanasia.

Special considerations: fetuses and neonate animals

Methods for euthanasia of neonates that are acceptable with conditions are summarized in Table 36.4.

For mouse and rat fetuses and altricial neonates up to 7 days of age the use of hypothermia through gradual cooling is a common method for euthanasia and should be combined with an adjunctive method to ensure death following loss of movement. Care should be taken that the cooling is gradual and that they do not come in direct contact with ice or cold surfaces.

Physical methods, such as cervical dislocation and decapitation, are frequently used because they are easy to perform due to the softness of the tissues. For these small animals, decapitation is possible with surgical scissors.

Neonates have hemoglobin (fetal hemoglobin) that has greater oxygen binding capacity than that of adult animals; therefore the use of inhaled agents, although possible, is not recommended for euthanasia of neonates. For example it can take up to 60 minutes for neonates to die after CO_2 exposure (Pritchett et al., 2005).

Injectable agents might be used with the same considerations discussed for adult animals.

TABLE 36.3 Euthanasia methods classified as unacceptable for laboratory rodents.

Unacceptable as primary methods	
Method	**Comment**
Injectable agents	
Potassium chloride	Only under deep anesthesia as secondary method as they can produce convulsions and pain
Magnesium sulfate	
Neuromuscular blocking substances (NmB)	Only under deep anesthesia as secondary method as they can produce paralysis before unconsciousness
NmB + Barbiturates	
Opioids	Does not induce deep anesthesia
α-Chloralose	Does not induce deep anesthesia
Chloral hydrate	Carcinogenic for humans
Urethane	
Inhalable agents	
Nitrogen	Only under deep anesthesia as they produce aversion
Argon	Explosion risk
Ether	
Physical agents	
Concussion	Not consistent, might not induce rapid unconsciousness
Rapid freezing young/adult animals	Painful

TABLE 36.4 Euthanasia methods frequently used for fetuses and neonate laboratory rodents that are acceptable with certain conditions.

Fetuses and neonates: acceptable with conditions	
Method	**Condition**
Gradual cooling	<7 days (Neonates: only for altricial) No direct contact with ice or precooled surfaces
Rapid freezing in liquid N_2	<5 days (Neonates: only for altricial)
Decapitation	<7 days (Neonates: only for altricial) Scissors or sharp blades might be used Use another method whenever possible
Cervical dislocation	Personnel must show performance standard to luxate cervical vertebrae without crushing bones and spinal cord, thus achieving rapid unconsciousness

Ethical issues

In many cases, animals used in research must be euthanized at the end of the project as a part of the experimental design. In other instances animals are euthanized as a result of spontaneous, induced illness, or clinical decline. Among the animal research community there is consensus that euthanasia must be performed in a humane manner (Council for International Organization of Medical Sciences and The International Council for Laboratory Animal Science, 2012). While some pain or distress related to euthanasia is sometimes unavoidable due to the specific method required to meet the scientific objectives, these should always be minimized. In all cases, euthanasia should be reliable, timely, and rapid; that is, a suffering animal should be promptly euthanized using a method that ensures a certain and rapid death.

Research animals are sometimes euthanized due to illness that may be spontaneous or experimentally induced (Sivula and Suckow, 2018). For example research rodents may develop tumors spontaneously or induced to experimentally develop tumors. In either case specific endpoints based on clinical criteria should be established so that animals are euthanized at a time beyond which the animal would likely experience significant pain or distress (Foltz and Ullmann-Cullere, 1999;

Suckow and Doerning, 2014). Examples of endpoints that might be used are given in Table 36.5. For example because studies involving growing tumors may significantly impact animal health and well-being, recommendations and summaries for endpoints based on body condition and behavior of the animals have been described (Paster et al., 2009; Wallace, 2000). Relevant endpoints might include tumors that have become necrotic or ulcerated or have grown large enough that they impair the ability of the animal to easily access food and water; body condition scores, especially in cases where the tumors are internal (Paster et al., 2009); and specified tumor diameters (typically no greater than 1.5 cm in any dimension for a mouse and 2.8 cm for a rat; Workman et al., 2010) or volumes (typically greater than 530 mm^3 for mice and 4200 mm^3 for rats) where volume can be calculated using the following formula:

$$\text{Volume}\left(\text{mm}^3\right) = \frac{\left(1 \times w^2\right)}{2}$$

In this way a standard set of criteria for euthanasia is determined before the work is begun as an approach to set specific cut-offs that will limit the pain and distress of the animals. It has also been suggested that imaging technologies could be employed as a way to evaluate tumor size for ensuring the humane care of animals in cancer studies (Workman et al., 2010). Researchers and animal care staff should both understand in advance what limits will be placed on the degree of illness or debilitation and that will result in a decision to euthanize an animal. Such determinations should always be made with the input of a veterinarian who is experienced with the species of interest. When endpoint criteria are an essential part of determining the point at which animals will be euthanized, investigators should be expected to examine animals with sufficient frequency to detect changes in parameters that lead to euthanasia; and such examinations and findings should be documented.

TABLE 36.5 Clinical parameters commonly used as endpoint criteria.

Clinical parameter	Common endpoint
Level of activity, behavior	Lethargy, vocalization; failure to groom; listlessness, aggressiveness, or behavior that signals severe pain
Food and water consumption	Decrease in either; severe dehydration
Body weight	Loss of ≥20% over prestudy weight
Body temperature	Severe pyrexia or hypothermia; note that severe hypothermia is often a sign of shock and impending death
Infection	Severe infection, such as that involving a surgical wound, signs may include discharge from wounds, pyrexia, and listlessness
Wounds or tumors	Severe wounds involving large amount of tissue or at anatomic locations that impede normal activity; large tumors that are ulcerated or necrotic and are of significant size in terms of diameter or volume (e.g., for mice, a common tumor size limit is 2.0 cm diameter in any dimension); any tumor or wound that impedes ability of the animal to access food or water

In some cases extra animals may have been acquired or have been produced from a breeding colony. This is particularly true with breeding colonies of mice in which a litter may include individuals of the desired genotype along with individuals not having the genotype required by the research. Often individuals in the latter category are euthanized as they are not needed for experimental purposes. As well, because it can be difficult to predict the specific fecundity of a breeding colony, many more animals than are needed might be produced. These are all examples of circumstances in which otherwise healthy animals are potentially euthanized simply because they are not needed for experimentation.

To mitigate ethical conflicts related to euthanasia any institution conducting animal research should require an ethical review of proposed activities and methods, including those related to animal breeding as a way to discourage over-production of animals. Such a process should include review of procedures used for purposes of breeding research animals and should rigorously ensure that the number of surplus animals are kept to a minimum. In this way researchers, administrators, animal care workers, and the public will have confidence that all procedures involving animals, including euthanasia, have undergone scrutiny as a way to ensure that research with animals is conducted in a way that reduces both the number of animals and the potential pain of animals which is necessary to achieve the research objectives. In many locations such review is conducted by the Institutional Animal Care and Use Committee (IACUC; Brown and Shepherd, 2014), while in other locations review is undertaken by an Animal Ethics Committee, Animal Welfare Body, or Animal Care Committee (OIE, 2018).

Although the ethical review of proposed animal activities includes assessment of methods of euthanasia, there are some circumstances under which scientific requirements dictate the use of methods that might otherwise be discouraged. For example some areas of research include assays that are greatly influenced by the method of euthanasia (Overmeyer et al., 2015). A significant impact of the euthanasia method on brain amino acid concentrations has been demonstrated for rats (Miller et al., 1990); and methods such as decapitation of

unanesthetized animals are needed because of the rapid postmortem alterations that can occur with methods that result in a more delayed death. In all such cases it is important for ethical review of the proposed research to establish that such methods are scientifically justified and used only when necessary. Furthermore the institutional ethics review committee/IACUC should consider monitoring research activities involving animals, including euthanasia, to ensure that practices align with those described during application for approval.

Translational significance

The importance of euthanasia of research animals should be understood by everyone on the research team, including personnel who provide basic husbandry and care for the animals. It should be understood that to fully address the hypothesis of the work that sometimes tissues must be sampled for further evaluations or analyses, implants must be harvested for evaluation of function and biocompatibility, and in situ examinations made to determine the effects of experimental procedures and manipulations. Opportunities to communicate to the public the essential role that animals play in biomedical advancement should also be undertaken whenever available to promote general support for animal research. As described earlier it is essential that the method of euthanasia chosen is compatible with the objectives of the research. Importantly even the specific mode used for an euthanasia method can affect research parameters. For example immunologic and hematologic measures can be significantly affected depending on the concentration of carbon dioxide used to euthanize mice (Pecaut et al., 2000). Personnel should clearly understand, and be proficient with, the specific method of euthanasia to be used.

World Wide Web resources

https://www.avma.org/KB/Policies/Pages/Euthanasia-Guidelines.aspx

https://www.nc3rs.org.uk/news/laboratory-animal-euthanasia

https://oacu.oir.nih.gov/sites/default/files/uploads/arac-guidelines/b5-2017_rodent_euthanasia-adult_final_1-25-17.pdf

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Glossary

Endpoint The point at which an animal is euthanized based on clinical or experimental criteria. In the case of a humane endpoint the criteria are chosen so that euthanasia is performed at a point that will decrease the pain and distress an animal might experience.
Euthanasia The humane killing of an animal.
Institutional Animal Care and Use Committee An appointed committee that reviews and approves animal care and use practices to ensure the judicious use of animals and in a manner that minimizes pain and distress to that which is unavoidable for addressing the objectives of the research. Synonymous with the Animal Ethics Committee, the Animal Welfare Body, or Animal Care Committee.

Abbreviations

AVMA American Veterinary Medical Association
IACUC Institutional Animal Care and Use Committee
IP Intraperitoneal
IV Intravenous
NmB Neuromuscular blocking agent

Long answer questions

1. A research study lasting 18 months is conducted using mice. The experimental methods require that data can be collected from tissues harvested at the end of the study and that a minimum of six mice in each of four test groups are needed to achieve statistically relevant results. The researcher has included eight mice in each group to account for possible losses. However after 16 months many of the mice have begun to deteriorate due to a variety of clinical problems, including severe dermatitis, large tumors, and severe weight loss of undetermined origin. Some mice have died and now there are only six mice left in each test group. Is it appropriate to maintain the ill mice for an additional two months so that they may complete the study? How might the ethics review committee (IACUC) help to mitigate such potential situations? If the animals developed illness at 17 months, instead of at 16 months, would it be appropriate to delay euthanasia?
2. How should an Ethics Review Committee (IACUC) approach a proposal to euthanize animals that will likely lead to pain and distress of the animals?
3. What are some ways that personnel can reduce the distress that animals might experience as part of euthanasia?
4. An animal technician complains that he/she is always in charge of doing all euthanasia procedures in the facility/lab and that this is distressful, and he/she is not sure that this is not painful, or that this procedure should be carried out at all. What would be an appropriate way to proceed? Would changing the person in charge of this procedure solve the problem?
5. A new researcher at the institution intends to use ether inhalation for euthanasia of animals at the end of his/her research project. The reason purported is that in his/her previous institution this was an accepted method and he/she wants to be able to compare the data obtained between both projects. Is this an acceptable reason? If you are the person in charge of those animals, or indeed the researcher in question, what do you think would be the way forward?

Short answer questions

1. State the primary goal of euthanasia.
2. What are three desirable characteristics of a euthanasia method?
3. What is the largest size animal for which cervical dislocation may be used as a method of euthanasia?
4. What are typical maximum tumor diameters in rodents?
5. What aspects of euthanasia should be included for training of personnel?
6. Is it possible to use a euthanasia method that is not listed in this chapter?
7. What are the main factors to consider when choosing a euthanasia method within a given list of approved techniques?

Answers to short answer questions

1. The goal of euthanasia is to provide a painless death for an animal.

2. In all cases euthanasia should be reliable, timely, and rapid, that is, an animal suffering should be promptly euthanized using a method that ensures a certain and rapid death. Further the method should be safe for personnel.
3. Cervical dislocation can be used for mice, rats, and other small mammals under 200 g of body weight, as well as for small birds and poultry.
4. Typically tumor diameter should be no greater than 1.5 cm in any dimension for a mouse and 2.8 cm for a rat.
5. Aspects of training should include principles ensuring a humane death of animals and which offers focus on the equipment to be used; specific techniques; safety of personnel when performing euthanasia; methods to confirm death of the animals; and safe disposal of carcasses and tissues following euthanasia.
6. IACUCs usually have a list of approved methods for different kinds of animals used within their institution, considering species, age, size, physiological state, etc. If a researcher wants to use a method that is not in the list, he/she should communicate to the IACUC and justify the reason for using a different method. The IACUC will evaluate and decide according to factors such as national regulations, published evidence, and previous experience.
7. The potential effect of the technique on the data to be collected from the animals (e.g., quality of tissues), feasibility, both technical and personnel wise (does it need an apparatus? Is there on-site training available for personnel to learn how to perform the technique).

Yes/No type questions

1. The primary goal of euthanasia is to produce death by using methods that require pharmaceutical agents.
2. Humane endpoints for euthanasia should be flexible and open to interpretation by the research team.
3. Ethical review of proposed animal research activities should include consideration of animal breeding and production.
4. Euthanasia methods that involve pain to the animals may be acceptable if needed to meet the scientific needs of the study.
5. Inexperienced personnel may be allowed to perform animal euthanasia, because the methods are very easy to conduct.
6. Since rodents are social animals, it is important that they be euthanized in the presence of animals not being euthanized.
7. With carbon dioxide euthanasia of a mouse, one can correctly assume an animal to be dead once the animal has stopped moving.
8. When using an inhalant anesthetic such as isoflurane, it is important that the animal does not come into direct contact with the liquid form of the agent.
9. Dry ice should not be used as a source of carbon dioxide for euthanasia.
10. Euthanasia using carbon dioxide takes longer in neonates than in adult animals.

Answers to Yes/No type questions

1. No—The goal of euthanasia is to provide a painless death for the animal.
2. No—Specific endpoints based on clinical criteria and should be defined prior to beginning the research.
3. Yes—Review should ensure that practices are used to keep the number of surplus animals to a minimum.
4. Yes—Some areas of research include assays that are greatly influenced by the method of euthanasia. Methods that involve the animals experiencing pain may be acceptable if a scientific rationale is provided to explain the need for such euthanasia methods and why other non-painful methods cannot be used.
5. No—Because improper procedure can result in animal distress and pain, proficiency of personnel should be established and documented.
6. No—Since animals in distress may release alarm pheromones that alert conspecifics to threatening situations, euthanasia should be performed in an area separate from where other animals are maintained.
7. No—For proper euthanasia, secondary methods, such as cervical dislocation, should be used to ensure death.
8. Yes—One should take care that the animal does not come into direct contact with the liquid form of the anesthetic agent because of the risk of irritation.
9. Yes—Because the concentration and flow rate of carbon dioxide cannot be closely controlled from a dry ice source, gas from a compressed cylinder should be used.
10. Yes—Because of the greater oxygen binding capacity of fetal hemoglobin, neonatal animals are more able to withstand carbon dioxide narcosis.

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