Human Blood Groups

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Foreword by Ruth Sanger

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Foreword to 1st edition

It is a particular pleasure for me to welcome this new book on human blood groups, the more so since it emanates from the Medical Research Council's Blood Group Unit. For 25 years this Unit devoted its energies to the search for new red cell antigens and the application of those already known to various problems, particularly to human genetics. During these years Rob Race and I produced six editions of *Blood Groups in Man*.

Dr Geoff Daniels joined the Unit in 1973 on Dr Race's retirement; soon after, concurrently with the Unit's move from the Lister Institute to University College, the scope of the Unit's interest was broadened.

Having been divorced from blood groups and otherwise occupied in 12 years of retirement, I am delighted and astonished at the rapid advances made in recent years. The number of blood group loci have increased to 23 and all except one have found their chromosomal home. The biochemical backgrounds of most of the corresponding antigens are defined and hence several high and low incidence antigens gathered into systems. The molecular basis of many red cell antigens has provided an explanation for some confusing serological relationships which were observed many years before.

Dr Daniels is to be congratulated on his stamina in producing a comprehensive text and reference book on human blood groups, for which many scientists will be grateful.

Ruth Sanger
December 1994

Preface to the third edition

The primary purpose of this book, like the first two editions, is to describe human blood group antigens and their inheritance, the antibodies that define them, the structure and functions of the red cell membrane macromolecules that carry them, and the genes that encode them or control their biosynthesis. In addition, this book provides information on the clinical relevance of blood groups and on the importance of blood group antibodies in transfusion medicine in particular.

The second edition of *Human Blood Groups* was published in 2002; this new edition will appear 11 years later. There have been many new findings in the blood group world over those years. In order to prevent the book from becoming too cumbersome, my goal has been to produce a third edition roughly the same size as the first two. I have tried to do this without eliminating anything too important, although this has not been easy, with so much new material to include. Since 2002, about 69 new blood group antigens and seven new blood group systems have been identified, and all of the 38 genes representing those systems have been cloned and sequenced.

In the preface of the sixth edition of *Blood Groups in Man*, the predecessor of *Human Blood Groups*, Race and Sanger wrote, 'Here is the last edition of this book: the subject has grown to need more than our two pencils'. Well, here is the last edition of *Human Blood Groups*; the subject is rapidly growing too vast to be contained in a textbook. In the previous two editions I strove to include all fully validated blood group antigens and genetic changes associated with their expression or loss of expression. This has proved impossible and pointless in this edition so, although the genetic bases of all the important blood group polymorphisms are described, in many cases the reader is directed to web sites for a more complete list of mutations, particularly those responsible for null phenotypes. In the next few years, next-generation sequencing will become readily available and affordable, and the number of genetic variations associated with red cell change will increase exponentially.

I wish to thank again all the people who helped me produce the first two editions, in particular Patricia Tippett, Carole Green, David Anstee, and Joan Daniels. I would like to add my thanks to Dr Nicholas Burton at the University of Bristol who provided many of the protein models for this edition. Finally I would like to thank all the numerous colleagues from around the world who have provided so much of the information in this book, in published or unpublished form, over so many years.

Geoff Daniels

Some abbreviations used

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
AET	2-aminoethylisothiourunium bromide
AIHA	Autoimmune haemolytic anaemia
bp	Base-pair
CDA	Congenital dyserythropoietic anaemia
cDNA	Complimentary DNA
CFU-E	Colony-forming unit-erythroid
Da	Daltons
DAT	Direct antiglobulin test
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
Gal	Galactose
GalNAc	*N*-acetylgalactosamine
GlcNAc	*N*-acetylglucosamine
GDP	Guanosine diphosphate
GPI	Glycosylphosphatidylinositol
GSL	Glycosphingolipid
GTA	A-transferase
GTB	B-transferase
HCF	Hydatid cyst fluid
HDFN	Haemolytic disease of the fetus and newborn
HTR	Haemolytic transfusion reaction
IAT	Indirect antiglobulin test
ISBT	International Society of Blood Transfusion (may refer to ISBT terminology)
kb	Kilo-bases
kDa	Kilo-Daltons
MAIEA	Monoclonal antibody immobilisation of erythrocyte antigens
mRNA	Messenger ribonucleic acid
MW	Molecular weight
PCR	Polymerase chain reaction
RFLP	Restriction fragment-length polymorphism
RNA	Ribonucleic acid
SDS PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SNP	Single nucleotide polymorphism

1 Human Blood Groups: Introduction

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1.1 Introduction

What is the definition of a blood group? Taken literally, any variation or polymorphism detected in the blood could be considered a blood group. However, the term blood group is usually restricted to blood cell surface antigens and generally to red cell surface antigens. This book focuses on the inherited variations in human red cell membrane proteins, glycoproteins, and glycolipids. These variations are detected by alloantibodies, which occur either 'naturally', due to immunisation by ubiquitous antigens present in the environment, or as a result of alloimmunisation by human red cells, usually introduced by blood transfusion or pregnancy. Although it is possible to detect polymorphism in red cell surface proteins by other methods such as DNA sequence analysis, such variants cannot be called blood groups unless they are defined by an antibody.

Blood groups were discovered at the beginning of the twentieth century when Landsteiner [1,2] noticed that plasma from some individuals agglutinated the red cells from others. For the next 45 years, only those antibodies that directly agglutinate red cells could be studied. With the development of the antiglobulin test by Coombs, Mourant, and Race [3,4] in 1945, non-agglutinating antibodies could be detected and the science of blood group serology blossomed. There are now 339 authenticated blood group antigens, 297 of which fall into one of 33 blood group systems, genetically discrete groups of antigens controlled by a single gene or cluster of two or three closely linked homologous genes (Table 1.1).

Most blood group antigens are synthesised by the red cell, but the antigens of the Lewis and Chido/Rodgers systems are adsorbed onto the red cell membrane from the plasma. Some blood group antigens are detected only on red cells; others are found throughout the body and are often called histo-blood group antigens.

Biochemical analysis of blood group antigens has shown that they fall into two main types:
1 protein determinants, which represent the primary products of blood group systems; and
2 carbohydrate determinants on glycoproteins and glycolipids, in which the products of the genes controlling antigen expression are glycosyltransferase enzymes.
Some antigens are defined by the amino acid sequence of a glycoprotein, but are dependent on the presence of carbohydrate for their recognition serologically. In this book the three-letter code for amino acids is mainly used, though the single-letter code is often employed in long sequences and in some figures. The code is provided in Table 1.2.

In recent years, molecular genetical techniques have been introduced into the study of human blood groups and now most of the genes governing blood group systems have been cloned and sequenced (Table 1.1). Many serological complexities of blood groups are now explained at the gene level by a variety of mechanisms, including point mutation, unequal crossing-over, gene conversion, and alternative RNA splicing.

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Table 1.1 Blood group systems.

No.	Name	Symbol*	No. of antigens	Associated membrane structures	CD no.	HGNC symbol(s)	Chromosome
001	ABO	ABO	4	Carbohydrate		*ABO*	9
002	MNS	MNS	46	Glycophorins, GPA, GPB	CD235 A & B	*GYPA, GYPB, GYPE*	4
003	P1PK	P1PK	3	Carbohydrate		*A4GALT*	22
004	Rh	RH	54	Rh family, RhD, RhCcEe	CD240 D & CE	*RHD, RHCE*	1
005	Lutheran	LU	20	IgSF	CD239	*BCAM*	19
006	Kell	KEL	35	Endopeptidase	CD238	*KEL*	7
007	Lewis	LE	6	Carbohydrate		*FUT3*	19
008	Duffy	FY	5	G protein-coupled SF, chemokine receptor	CD234	*DARC*	1
009	Kidd	JK	3	Urea transporter		*SLC14A1*	18
010	Diego	DI	22	Band 3, anion exchanger (AE1)	CD233	*SLC4A1*	17
011	Yt	YT	2	Acetylcholinesterase		*ACHE*	7
012	Xg	XG	2	Glycoproteins	CD99**	*XG, CD99*	X/Y
013	Scianna	SC	7	IgSF, erythroblast membrane-associated protein		*ERMAP*	1
014	Dombrock	DO	8	ADP-ribosyltransferase 4	CD297	*ART4*	12
015	Colton	CO	4	Aquaporin SF, aquaporin-1		*AQP1*	7
016	Landsteiner-Wiener	LW	3	IgSF, intercellular adhesion molecule-4	CD242	*ICAM4*	19
017	Chido/Rodgers	CH/RG	9	Complement components C4A, C4B		*C4A, C4B*	6
018	H	H	1	Carbohydrate, Type 2 H	CD173	*FUT1*	19
019	Kx	XK	1	Xk protein		*XK*	X
020	Gerbich	GE	11	Glycophorins, GPC, GPD	CD236	*GYPC*	2
021	Cromer	CROM	18	CCP SF, decay-accelerating factor	CD55	*CD55*	1
022	Knops	KN	9	CCP SF, complement regulator-1	CD35	*CR1*	1
023	Indian	IN	4	Link module SF of proteoglycans	CD44	*CD44*	11
024	Ok	OK	3	IgSF, basigin	CD147	*BSG*	19
025	Raph	RAPH	1	Tetraspanin SF	CD151	*CD151*	11
026	John Milton Hagen	JMH	6	Semaphorin SF	CD108	*SEMA7A*	15
027	I	I	1	Carbohydrate		*GCNT2*	6
028	Globoside	GLOB	1	Carbohydrate, globoside		*B3GALT3*	3
029	Gill	GIL	1	Aquaporin SF, aquaporin-3		*AQP3*	9
030	RHAG	RHAG	4	Rh family, Rh-associated glycoprotein	CD241	*RHAG*	6
031	Forssman	FORS	1	Carbohydrate, Forssman glycolipid		*GBGT1*	9
032	Junior	JR	1	ATP-binding cassette transporter ABCG2		*ABCG2*	4
033	Lan	LAN	1	ATP-binding cassette transporter ABCB6		*ABCB6*	2

HGNC, Human Genome Organisation Gene Nomenclature Committee; SF, superfamily; IgSF, immunoglobulin superfamily; CCP, complement control protein.
*ISBT gene name when in italics.
**Does not include Xg glycoprotein.

Table 1.2 The 20 common amino acids: one- and three-letter codes.

A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine

Discovery of the ABO blood groups first made blood transfusion feasible and disclosure of the Rh antigens led to the understanding, and subsequent prevention, of haemolytic disease of the fetus and newborn (HDFN). Although ABO and Rh are the most important systems in transfusion medicine, many other blood group antibodies are capable of causing a haemolytic transfusion reaction (HTR) or HDFN. Red cell groups have been important tools in forensic science, although this role was diminished with the introduction of HLA testing and has recently been displaced by DNA 'fingerprinting'. For many years blood groups were the best human genetic markers and played a major part in the mapping of the human genome.

Blood groups still have much to teach us. Because red cells are readily available and haemagglutination tests relatively easy to perform, the structure and genetics of the red cell membrane proteins and lipids are understood in great detail. With the unravelling of the complexities of blood group systems by molecular genetical techniques, much has been learnt about the mechanisms responsible for the diversification of protein structures and the nature of the human immune response to proteins of different shapes resulting from variations in amino acid sequence.

1.2 Blood group terminology

The problem of providing a logical and universally agreed nomenclature has dogged blood group serologists almost since the discovery of the ABO system. Before going any further, it is important to understand how blood groups are named and how they are categorised into systems, collections, and series.

1.2.1 An internationally agreed nomenclature

The International Society of Blood Transfusion (ISBT) Working Party on Red Cell Immunogenetics and Blood Group Terminology was set up in 1980 to establish a uniform nomenclature that is 'both eye and machine readable'. Part of the brief of the Working Party was to produce a nomenclature 'in keeping with the genetic basis of blood groups' and so a terminology based primarily around the blood group systems was devised. First the systems and the antigens they contained were numbered, then the high and low frequency antigens received numbers, and then, in 1988, collections were introduced. Numbers are never recycled: when a number is no longer appropriate it becomes obsolete.

Blood group antigens are categorised into 33 systems, seven collections, and two series. The Working Party produced a monograph in 2004 to describe the terminology [5], which was most recently updated in 2011 [6]. Details can also be found on the ISBT web site [7].

1.2.2 Antigen, phenotype, gene and genotype symbols

Every authenticated blood group antigen is given a six-digit identification number. The first three digits represent the system (001 to 033), collection (205 to 213), or series (700 for low frequency, 901 for high frequency); the second three digits identify the antigen. For example, the Lutheran system is system 005 and Lu^a, the first antigen in that system, has the number 005001. Each system also has an alphabetical symbol: that for Lutheran is LU. So Lu^a is also LU001 or, because redundant sinistral zeros may be discarded, LU1. For phenotypes, the system symbol is followed by a colon and then by a list of antigens present, each separated by a comma. If an antigen is known to be absent, its number is preceded by a minus sign. For example, Lu(a–b+) becomes LU:–1,2.

Devising a modern terminology for blood group alleles is more complex. One antigen, the absence of an antigen, or the weakness or absence of all antigens of a system

may be encoded by several or many alleles. Over the last few years the Working Party has been developing a new terminology for bloods group alleles. Unfortunately at the time of publication of this book, it was still incomplete, controversial, and in draft form. Consequently, it has only partially been used in this book. Basically, alleles have the system symbol followed by an asterisk followed in turn by a number or series of numbers, separated by full stops, representing the encoded antigen and the allele number. Alternatively, in some cases a letter can be used instead of a number. For example, Lu^a allele can be *LU*01* or *LU*A*. Genotypes have the symbol followed by an asterisk followed by the two alleles separated by a stroke. For example, Lu^a/Lu^b becomes *LU*01/02* or *LU*A/B*. The letters N and M represent null and mod. For example, one of the inactive Lu^b alleles responsible for a null phenotype is *LU*02N.01*, the *02* representing the Lu^b allele, even though no Lu^b antigen is expressed. Genes, alleles, and genotypes are italicised. For lists of blood group alleles in the ISBT and other terminologies see the ISBT and dbRBC web sites [7,8].

Symbols for all human genes are provided by the Human Genome Organisation (HUGO) Gene Nomenclature Committee (HGNC) [9]. These often differ from the ISBT symbols, as the HGNC symbols reflect the function of the gene product (Table 1.1). When referring to alleles defining blood group antigens, the ISBT gene symbol is preferred because the HGNC symbols often change with changes in the perceived functions of the gene product.

1.2.3 Blood group systems

A blood group system consists of one or more antigens, governed by a single gene or by a complex of two or more very closely linked homologous genes with virtually no recombination occurring between them. Each system is genetically discrete from every other blood group system. All of the genes representing blood group systems have been identified and sequenced.

In some systems the gene directly encodes the blood group determinant, whereas in others, where the antigen is carbohydrate in nature, the gene encodes a transferase enzyme that catalyses biosynthesis of the antigen. A, B, and H antigens, for example, may all be located on the same macromolecule, yet H-glycosyltransferase is produced by a gene on chromosome 19 while the A- and B-transferases, which require H antigen as an acceptor substrate, are products of a gene on chromosome 9. Hence H belongs to a separate blood group system from A and B (Chapter 2). Regulator genes may affect expression of antigens from more than one system: *In(Lu)* down-regulates expression of antigens from both Lutheran and P systems (Chapter 6); mutations in *RHAG* are responsible for Rh_{null} phenotype, but may also cause absence of U (MNS5) and Fy5 antigens (Chapter 5). So absence of an antigen from cells of a null-phenotype is never sufficient evidence for allocation to a system. Four systems consist of more than one gene locus: MNS has three loci; Rh, Xg, and Chido/Rodgers have two each.

Table 1.3 Blood group collections.

No.	Name	Symbol	No. of antigens	Chapter
205	Cost	COST	2	20
207	Ii	I	1	25
208	Er	ER	3	28
209		GLOB	2	4
210	(Le^c & Le^d)		2	2
212	Vel	VEL	2	30
213	MNCHO	MNCHO	6	3

1.2.4 Collections

Collections were introduced into the terminology in 1988 to bring together genetically, biochemically, or serologically related sets of antigens that could not, at that time, achieve system status, usually because the gene identity was not known. Thirteen collections have been created, six of which have subsequently been declared obsolete (Table 1.3): the Gerbich (201), Cromer (202), and Indian (203) collections have now become systems; Auberger (204), Gregory (206), and Wright (211) have been incorporated into the Lutheran, Dombrock, and Diego systems, respectively.

1.2.5 Low frequency antigens, the 700 series

Red cell antigens that do not fit into any system or collection and have an incidence of less than 1% in most populations tested are given a 700 number (see Table 29.1). The 700 series currently consists of 18 antigens. Thirty-six 700 numbers are now obsolete as the corresponding antigens have found homes in systems or can no longer be defined owing to lack of reagents.

1.2.6 High frequency antigens, the 901 series

Originally antigens with a frequency greater than 99% were placed in a holding file called the 900 series, equivalent to the 700 series for low frequency antigens. With the establishment of the collections, so many of these 900 numbers became obsolete that the whole series was abandoned and the remaining high frequency antigens were relocated in a new series, the 901 series, which now contains six antigens (see Table 30.1). The 901 series antigen Jr^a and Lan became systems 32 and 33 in 2012 when their genes were identified (Chapter 27).

1.2.7 Blood group terminology used in this book

The ISBT terminology provides a uniform nomenclature for blood groups that can be continuously updated and is suitable for storage of information on computer databases. The Terminology Working Party does not expect, or even desire, that the numerical terminology be used in all circumstances, although it is important that it should be understood so that the genetically based classification is understood. In this book, the alternative, 'popular' nomenclature, recommended by the Working Party [5], will generally be used. This does not reflect a lack of confidence in the numerical terminology, but is simply because most readers will not be well acquainted with blood group numbers and will find the contents of the book easier to digest if familiar names are used. The numerical terminology will be provided throughout the book in tables and often, in parentheses, in the text.

The order of the chapters of this book is based on the order of the blood group systems, collections, and series. There are, however, a few exceptions, the most notable of which are the ABO, H, and Lewis systems, which appear together in one mega-chapter (Chapter 2), because they are so closely related, biochemically.

1.3 Chromosomal location of blood group genes

Blood groups have played an important role as human gene markers. In 1951, when the Lutheran locus was shown to be genetically linked to the locus controlling ABH secretion, blood groups were involved in the first recognised human autosomal linkage and, consequently, the first demonstration of recombination resulting from crossing-over in humans [10,11]. When, in 1968, the Duffy blood group locus was shown to be linked to an inherited visible deformity of chromosome 1, it became the first human gene locus assigned to an autosome [12]. Since all blood group system genes have now been sequenced, all have been assigned to a chromosome (Table 1.1, Figure 1.1).

1.4 DNA analysis for blood group testing

Since the discovery of blood groups in 1900, most blood group testing has been carried out by serological means. With the application of gene cloning and sequencing of blood group genes at the end of the twentieth century, however, it became possible to predict blood group phenotypes from the DNA sequence. The molecular bases for almost all of the clinically significant blood group polymorphisms have been determined, so it is possible to carry out blood grouping by DNA analysis with a high degree of accuracy.

There are three main reasons for using molecular methods, rather than serological methods, for red cell blood grouping:

1 when we need to know a blood group phenotype, but do not have a suitable red cell sample;

2 when molecular testing will provide more or better information than serological testing; and

3 when molecular testing is more efficient or more cost effective than serological testing.

1.4.1 Clinical applications of molecular blood grouping

A very important application is determination of fetal blood group in order to assess the risk of HDFN. This is a non-invasive procedure carried out on cell-free fetal DNA in the maternal plasma, which represents 3–6% of the cell-free DNA in the plasma of a pregnant woman [13]. This technology is most commonly applied to RhD typing (Section 5.7), but also to Rh C, c, and E, and K of the Kell system.

Molecular methods are routinely used for extended blood group typing (beyond ABO and RhD) on multiply transfused patients, where serological methods are unsatisfactory because of the presence of transfused red cells. These patients are usually transfusion dependent and

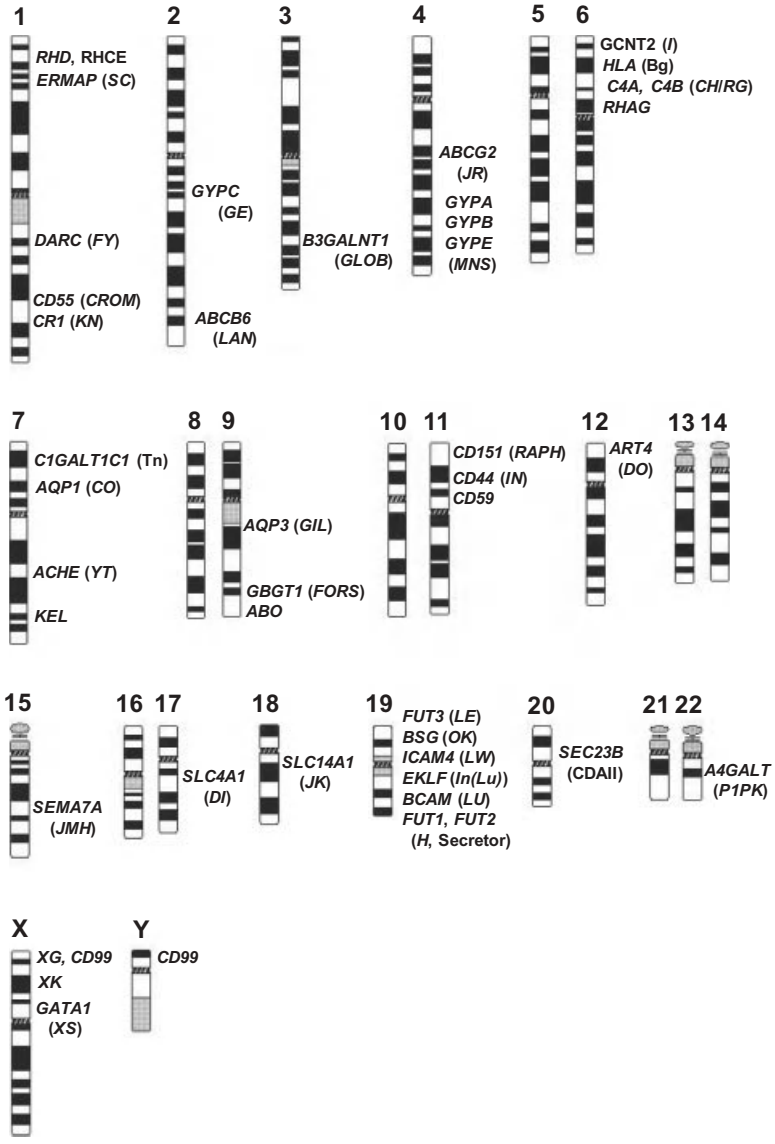


Figure 1.1 Human male chromosomes, showing location of blood group and related genes.

knowledge of their blood groups means that matched blood can be provided in an attempt to save them from making multiple antibodies and, if the patient is already immunised, to facilitate antibody identification. Molecular methods can be used for determining blood group phenotypes on red cells that are DAT-positive (i.e. coated with immunoglobulin), which makes serological testing difficult. This is particularly useful in helping to identify underlying alloantibodies in patients with autoimmune haemolytic anaemia (AIHA).

There are numerous variants of D. Some result in loss of D epitopes and some in reduced expression of D; most probably involve both (Section 5.6). Individuals with some of these variant D antigens can make a form of alloanti-D that detects those epitopes lacking from their own red cells. In many cases D variants cannot be

distinguished by serological methods, so molecular methods are often used for their identification. This assists in the selection of the most appropriate red cells for transfusion in order to avoid immunisation whilst conserving D-negative blood. There are some rare D antigens, such as DEL, that are not detected by routine serological methods. Consequently, blood donors with these phenotypes would be labelled as D-negative, although evidence exists that transfusion of DEL red cells can immunise a D-negative recipient to make anti-D. As DEL and other very weak forms of D are associated with the presence of a mutated *RHD* gene, they can be detected by molecular methods. In some transfusion services all D-negative donors are tested for the presence of *RHD*, although this is still not generally considered necessary (Section 5.6.9).

Molecular tests can be used for screening for donors when serological reagents are of poor quality or in short supply. For example, anti-Do^a and -Do^b have the potential to be haemolytic, yet satisfactory reagents are not available for finding donors for a patient with one of these antibodies (Chapter 14). Some Rh variants, such as hr^B-negative and hr^S-negative, are relatively common in people of African origin but are difficult to detect serologically (Section 5.9.5). Molecular tests are often employed to assist in finding suitable blood for patients with sickle cell disease, to reduce alloimmunisation and the risks of delayed HTRs [14,15].

Molecular methods are extremely useful in the blood group reference laboratory for helping to solve serological difficult problems.

In most countries, all blood donors are tested for ABO and D, but often a proportion of the donors are also tested for additional blood group antigens, especially C, c, E, e, and K, but sometimes also C^w, M, S, s, Fy^a, Fy^b, Jk^a, and Jk^b. This testing is usually performed by automated serological methods, but it is likely that in the future these serological methods will be replaced by molecular methods [16–18]. Molecular typing for this purpose has already been introduced in some services [19,20]. Molecular methods are more accurate than serological methods, they are more suited to high-throughput methods, and they are either cheaper or are likely to become so in the near future. This provides justification for a switch of technologies.

1.4.2 Current and future technologies

Laboratories performing blood group testing on cell-free fetal DNA in the maternal plasma generally use real-time quantitative PCR with Taqman technology, but an alternative technology that is becoming available involves the application of matrix-assisted laser desorption/ionisation time-of-flight (MALDI TOF) mass spectrometry [21].

For other applications of molecular blood grouping, many laboratories use methods traditionally applied to single nucleotide polymorphism (SNP) testing, involving PCR with the application of restriction enzymes or PCR with allele-specific primers, followed by gel electrophoresis. Other technologies that are becoming more commonly used involve the application of allele-specific extension of primers tagged with single fluorescent nucleotides, pyrosequencing, DNA microarray technology, on chips or coloured beads coated with oligonucleotides, and MALDI TOF [18,22]. The future of molecular blood grouping and of molecular diagnostics probably lies with next generation (massively parallel) sequencing, which will be truly high-throughput [23,24]. Next generation sequencing is an extremely powerful technology that provides the capacity to sequence many regions of the genome in numerous different individuals in one run, including fetal DNA from maternal plasma [25].

1.5 Structures and functions of blood group antigens

For the half-century following Landsteiner's discovery, human blood groups were understood predominantly as patterns of inherited serological reactions. From the 1950s some structural information was obtained through biochemical analyses, firstly of the carbohydrate antigens and then of the proteins. In 1986, *GYPA*, the gene encoding the MN antigens, was cloned and this led into the molecular genetic era of blood groups. A great deal is now known about the structures of many blood group antigens, yet remarkably little is known about their functions and most of what we do know has been deduced from their structures. Functional aspects of blood group antigens are included in the appropriate chapters of this book; provided here is a synopsis of the relationship between their structures and putative functions. The subject is reviewed in [26] and computer modelling of blood group proteins, which gives detailed information about protein structure, is reviewed in [27].

1.5.1 Membrane transporters

Membrane transporters facilitate the transfer of biologically important molecules in and out of the cell. In the

red cell they are polytopic, crossing the membrane several times, with cytoplasmic N- and C-termini, and are *N*-glycosylated on one of the external loops. Band 3, the Diego blood group antigen (Chapter 10) is an anion exchanger, the Kidd glycoprotein (Chapter 9) is a urea transporter, the Colton glycoprotein is a water channel (Chapter 15), the Gill glycoprotein is a water and glycerol channel (Chapter 26), and the Lan and Junior glycoproteins are ATP-fuelled transporters of porphyrin and uric acid (Chapter 27). Band 3 is at the core of a membrane macrocomplex, which contains the Rh proteins and the Rh-associated glycoprotein, which probably function as a CO_2 channel (Chapters 5 and 10).

1.5.2 Receptors and adhesion molecules

The Duffy glycoprotein is polytopic, but has an extracellular N-terminus. It is a member of the G protein-coupled superfamily of receptors and functions as a receptor for chemokines (Chapter 8).

The glycoproteins carrying the antigens of the Lutheran (Chapter 6), LW (Chapter 16), Scianna (Chapter 13), and Ok (Chapter 22) systems are members of the immunoglobulin superfamily (IgSF). The IgSF is a large family of receptors and adhesion molecules with extracellular domains containing different numbers of repeating domains with sequence homology to immunoglobulin domains. The functions of these structures on red cells are not known, but there is evidence to suggest that the primary functional activities of the Lutheran and LW glycoproteins occur during erythropoiesis, with LW probably playing a role in stabilising the erythropoietic islands.

The Indian antigen (CD44), a member of the link module superfamily, functions as an adhesion molecule in many tissues, but its erythroid function is unknown (Chapter 21). The glycoproteins of the Xg (Chapter 12) and JMH (Chapter 24) systems also have structures that suggest they could function as receptors and adhesion molecules. The Raph antigen, a tetraspanin, may associate with integrin in red cell progenitors to generate complexes that bind the extracellular matrix (Chapter 23).

1.5.3 Complement regulatory glycoproteins

Red cells have at least three glycoproteins that function to protect the cell from destruction by autologous complement. The Cromer glycoprotein, decay-accelerating factor (Chapter 19), and the Knops glycoprotein, complement receptor-1 (CR1) (Chapter 20), belong to the complement control protein superfamily; CD59 is not polymorphic and does not have blood group activity (Chapter 19). The major function of red cell CR1 is to bind and process C3b/C4b coated immune complexes and to transport them to the liver and spleen for removal from the circulation.

1.5.4 Enzymes

Two blood group glycoproteins have enzymatic activity. The Yt glycoprotein is acetylcholinesterase, a vital enzyme in neurotransmission (Chapter 11), and the Kell glycoprotein is an endopeptidase that can cleave a biologically inactive peptide to produce the active vasoconstrictor, endothelin (Chapter 7). The red cell function for both of these enzymes is unknown. The Dombrock glycoprotein belongs to a family of ADP-ribosyltransferases, but there is no evidence that it is an active enzyme (Chapter 14).

1.5.5 Structural components

The shape and integrity of the red cell is maintained by the cytoskeleton, a network of glycoproteins beneath the plasma membrane. At least two blood group glycoproteins anchor the membrane to its skeleton: band 3, the Diego antigen (Chapter 10), and glycophorin C and its isoform glycophorin D, the Gerbich blood group antigens (Chapter 18). Mutations in the genes encoding these proteins can result in abnormally shaped red cells. In addition, there is evidence that glycoproteins of the Lutheran (Chapter 6), Kx (Chapter 7), and RHAG (Chapter 5) systems interact with the cytoskeleton and their absence is associated with some degree of abnormal red cell morphology.

1.5.6 Components of the glycocalyx

Glycophorin A, the MN antigen (Chapter 3), band 3 are the two most abundant glycoproteins of the red cell surface. The *N*-glycans of band 3, together with those of the glucose transporter, provide the majority of red cell ABH antigens, which are also expressed on other glycoproteins and on glycolipids (Chapter 2). The extracellular domains of glycophorin A and other glycophorin molecules are heavily *O*-glycosylated. Carbohydrate at the red cell surface constitutes the glycocalyx, or cell coat, an extracellular matrix of carbohydrate that protects the cell from mechanical damage and microbial attack.

1.5.7 What is the biological significance of blood group polymorphism?

Very little is known about the biological significance of the polymorphisms that make blood groups alloantigenic. In any polymorphism one of the alleles is likely to

have, or at least to have had in the past, a selective advantage in order to achieve a significant frequency in a large population, though genetic drift and founder effects may also have played a part [28]. Glycoproteins and glycolipids carrying blood group activity are often exploited by pathogenic micro-organisms as receptors for attachment to the cells and subsequent invasion; surviving malaria possibly being the most significant force affecting blood group expression. In some cases, however, selection may have nothing to do with red cells; the target for the parasite could be other cells that carry the protein. It is likely that most blood group polymorphism is a relic of the selective balances that can result from mutations making cell surface structures less suitable as pathogen receptors and resultant adaptation of the parasite in response to these selective pressures. It is important to remember that whilst blood group polymorphism undoubtedly arose from the effects of selective pressures, these factors may have disappeared long ago, so that little hope remains of ever identifying them. To quote Darwin (*The Origin of Species*, 1859), 'The chief part of the organisation of any living creature is due to inheritance; and consequently, though each being assuredly is well fitted for its place in nature, many structures have now no very close and direct relations to present habits of life'.

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2 ABO, H, and Lewis Systems

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Part 1: History and introduction

Described in this chapter are three blood group systems, ABO, H, and Lewis (Table 2.1), although Lewis is really an 'adopted' blood group system because the antigens are not intrinsic to the red cells, but introduced into the membrane from the plasma. These three systems are genetically discrete, but are discussed in the same chapter because they are phenotypically and biochemically closely related. A complex interaction of genes at several loci controls the expression of ABO, H, Lewis, and other related antigens on red cells and in secretions.

The science of immunohaematology came into existence in 1900 when Landsteiner [1] reported that, 'The serum of healthy humans not only has an agglutinating effect on animal blood corpuscles, but also on human blood corpuscles from different individuals'. The following year Landsteiner [2] showed that by mixing together sera and red cells from different people three groups, A, B, and C (later called O), could be recognised. In group A, the serum agglutinated group B, but not A or C cells; in group B, the serum agglutinated A, but not B or C cells; and in group C (O), the cells were not agglutinated by any serum, and the serum appeared to contain a mixture of two agglutinins capable of agglutinating A and B cells. Decastello and Stürli [3] added a fourth group (AB), in which the cells are agglutinated by sera of all other groups and the serum contains neither agglutinin. Healthy adults always have A or B agglutinins in their serum if they lack the corresponding agglutinogen from their red cells (Table 2.2).

Epstein and Ottenberg [4] suggested that blood groups may be inherited and in 1910 von Dungern and Hirschfeld [5] confirmed that the inheritance of the A and B antigens obeyed Mendel's laws, with the presence of A or B being dominant over their absence. Bernstein [6,7] showed that only three alleles at one locus were necessary to explain ABO inheritance (Table 2.2).

Some group A people produce an antibody that agglutinates the red cells of most other A individuals. Thus A was subdivided into A_1 and A_2, and the three allele theory of Bernstein was extended to four alleles: A^1, A^2, *B* and *O* [8] (Section 2.4). Many rare subgroups of A and B have now been identified (Sections 2.7 and 2.8).

The structure and biosynthesis of the ABO, H, and Lewis antigens is well understood, thanks mainly to

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Table 2.1 Numerical notation for the ABO, Lewis, and H systems, and for Le^c and Le^d.

ABO (system 001)		Lewis (system 007)		H (system 018)		Collection 210	
ABO1	A	LE1	Le^a	H1	H	210001	Le^c
ABO2	B	LE2	Le^b			210002	Le^d
ABO3	A,B	LE3	Le^{ab}				
ABO4	A1	LE4	Le^{bH}				
		LE5	Ale^b				
		LE6	BLe^b				

Obsolete: ABO5, previously H.

Table 2.2 The ABO system at its simplest level.

ABO group	Antigens on red cells	Antibodies in serum	Genotype
O	None	Anti-A,B	*O/O*
A	A	Anti-B	*A/A* or *A/O*
B	B	Anti-A	*B/B* or *B/O*
AB	A and B	None	*A/B*

the pioneering work in the 1950s of Morgan and Watkins [9,10] and of Kabat [11]. A and B red cell antigens are carbohydrate determinants of glycoproteins and glycolipids and are distinguished by the nature of an immunodominant terminal monosaccharide: *N*-acetylgalactosamine (GalNAc) in group A and galactose (Gal) in group B. The *A* and *B* genes encode glycosyltransferases that catalyse the transfer of the appropriate immunodominant sugar from a nucleotide donor to an acceptor substrate, the H antigen. The *O* allele produces no active transferase (Sections 2.2 and 2.3). The sequences of the *A* and *B* alleles demonstrate that A- and B-glycosyltransferases (GTA and GTB) differ by four amino acid residues; the most common *O* allele contains a nucleotide deletion and encodes a truncated protein.

There are a multitude of *ABO* alleles, many of which affect phenotype, and at least two different terminologies. In this chapter the original terminology (e.g. A^1, A^2, O^1) will be used, with the dbRBC terminology often provided in parentheses.

H antigen is synthesised by a fucosyltransferase produced by *FUT1*, a gene independent of *ABO*. Very rare individuals lacking *FUT1* have no H antigen on their red cells and, consequently, are unable to produce A or B antigens, even when the enzyme products of the *A* or *B* genes are present (Section 2.12).

H antigen is present in body secretions of about 80% of Caucasians. The presence of H in secretions is governed by *FUT2*, another fucosyltransferase that is closely linked to *FUT1*. Individuals who secrete H also secrete A or B antigens if they have the appropriate *ABO* alleles. Non-secretors of H secrete neither A nor B, even when those antigens are expressed on their red cells (Section 2.6).

The first two examples of anti-Lewis, later to be called anti-Le^a, were described by Mourant [12] in 1946. These antibodies agglutinated the red cells of about 25% of English people. Andresen [13] found an antibody, later to become anti-Le^b, that defined a determinant only present on Le(a–) cells of adults. Six percent of group O adults lacked both antigens. Although Le^a and Le^b are not synthesised by red cells, but are acquired from the plasma, they are considered blood group antigens because they were first recognised on red cells. The terminology Le^a and Le^b is misleading as these antigens are not the products of alleles.

The Lewis gene (*FUT3*) encodes a fucosyltransferase that catalyses the addition of a fucose residue to H antigen in secretions to produce Le^b antigen or, if no H is present (non-secretors), to the precursor of H to produce Le^a. Consequently, as these structures are acquired from the plasma by the red cell membrane, red cells of most H secretors are Le(a–b+) and those of most H non-secretors are Le(a+b–). The Lewis-transferase can also convert A to ALe^b and B to BLe^b. About 6% of white people and 25% of black people are homozygous for a silent gene at the

FUT3 locus and, as they do not produce the Lewis enzyme, have Le(a–b–) red cells and lack Lewis substances in their secretions (Sections 2.3 and 2.15). In East Asia the red cell phenotype Le(a+b+) is common, caused by a weak secretor allele (Section 2.6.3).

The antigens Le^c and Le^d represent precursors of the Lewis antigens and are present in increased quantity in the plasma of Le(a–b–) individuals. Le^c is detected on the red cells of Le(a–b–) non-secretors of H and Le^d is detected on the red cells of Le(a–b–) secretors of H. Le^x and Le^y antigens, isomers of Le^a and Le^b, are not present in substantial quantities on red cells (Section 2.18.2).

ABH and Lewis antigens are often referred to as histo-blood group antigens [14] because they are ubiquitous structures occurring on the surface of endothelial cells and most epithelial cells. The precise nature of the histo-blood group antigens expressed varies between tissues within the same individual because of the intricacy of the gene interactions involved (Section 2.19).

ABO is on chromosome 9; *FUT1*, *FUT2*, and *FUT3* are on chromosome 19 (Sections 2.3.1, 2.3.2.4, and 2.3.5).

Part 2: Biochemistry, inheritance, and biosynthesis of the ABH and Lewis antigens

2.2 Structure of ABH, Lewis, and related antigens

ABH and Lewis antigens are carbohydrate structures. These oligosaccharide chains are generally conjugated with polypeptides to form glycoproteins or with ceramide to form glycosphingolipids. Oligosaccharides are synthesised in a stepwise fashion, the addition of each monosaccharide being catalysed by a specific glycosyltransferase. The oligosaccharide moieties responsible for expression of ABH, Lewis, and related antigens are shown in Table 2.3 and abbreviations for monosaccharides are given in Table 2.4. The biosynthesis of these structures is described in Section 2.3 and represented diagrammatically in Figure 2.1. There is a vast literature on the biochemistry of these blood group antigens and only some of the relevant references can be given in this chapter. The following reviews are recommended: [10,14–27].

2.2.1 Glycoconjugates expressing ABH and Lewis antigens

Two major classes of carbohydrate chains on glycoproteins express ABH antigens:

1 *N*-glycans, highly branched structures attached to the amide nitrogen of asparagine through GlcNAc; and
2 *O*-glycans, simple or complex structures attached to the hydroxyl oxygen of serine or threonine through GalNAc.

Glycosphingolipids consist of carbohydrate chains attached to ceramide. They are classified as lacto-series, globo-series, or ganglio-series according to the nature of the carbohydrate chain. Glycosphingolipid-borne ABH and Lewis antigens are present predominantly on glycolipids of the lacto-series, although ABH antigens have also been detected on globo-series and ganglio-series glycolipids. The carbohydrate chains of most ABH-bearing glycoproteins and of lacto-series glycolipids are based on a poly-*N*-acetyllactosamine structure; that is, they are extended by repeating Galβ1→4GlcNAcβ1→3 disaccharides (see Table 2.5 for examples).

On red cells, most ABH antigens are on the single, highly branched, poly-*N*-acetyllactosaminyl *N*-glycans of the anion exchange protein, band 3, and the glucose transport protein, band 4.5 [28]. There are about 1 million monomers of band 3 protein and half a million monomers of band 4.5 protein per red cell [29]. The other major red cell glycoprotein, glycophorin A, carries very low levels of ABH activity on both *O*- and *N*-glycans (Sections 3.2.1 and 3.2.2) and ABH determinants have also been detected on the Rh-associated glycoprotein [30]. Lewis antigens on red cells are not expressed on glycoproteins; they are not intrinsic to red cells, but are acquired from the plasma.

Glycolipids play a minor role in red cell ABH expression compared with glycoproteins. Red cell glycosphingolipids of the poly-*N*-acetyllactosaminyl type that express ABH antigens may have relatively simple linear or branched carbohydrate chains [15] (Table 2.5) or may be highly complex, branched structures called polyglycosylceramides, with up to 60 carbohydrate residues per molecule [31].

All the early work establishing the structures of the ABH and Lewis determinants was carried out on body secretions, especially the pathological fluid from human ovarian cysts, an abundant source of soluble A, B, and H substances [32]. ABH and Lewis antigens in secretions are glycoproteins; oligosaccharide chains attached to mucin by *O*-glycosidic linkage to serine or threonine (for reviews see [9,10]). These macromolecules have molecular weights varying from 2×10^5 to several millions. In milk and urine, free oligosaccharides with ABH and Lewis activity are also found [33,34]. ABH and Lewis determinants are present in plasma on glycosphingolipids, some

Table 2.3 Structures of A, B, H, Lewis, and related antigens (for abbreviations see Table 2.4).

Type 1		*Type 2*	
Precursor (Le^c)	Galβ1→3GlcNAcβ1→R †	**Precursor**	Galβ1→4GlcNAcβ1→R
H (Le^d)	Galβ1→3GlcNAcβ1→R † 2 ↑ Fucα1	**H** (CD173)	Galβ1→4GlcNAcβ1→R * 2 ↑ Fucα1
A	GalNAcα1→3Galβ1→3GlcNAcβ1→R † 2 ↑ Fucα1	**A**	GalNAcα1→3Galβ1→4GlcNAcβ1→R * 2 ↑ Fucα1
B	Galα1→3Galβ1→3GlcNAcβ1→R † 2 ↑ Fucα1	**B**	Galα1→3Galβ1→4GlcNAcβ1→R * 2 ↑ Fucα1
Le^a	Galβ1→3GlcNAcβ1→R † 4 ↑ Fucα1	**Le^x**	Galβ1→4GlcNAcβ1→R 3 ↑ Fucα1
Le^b	Galβ1→3GlcNAcβ1→R † 2 4 ↑ ↑ Fucα1 Fucα1	**Le^y**	Galβ1→4GlcNAcβ1→R 2 3 ↑ ↑ Fucα1 Fucα1
ALe^b	GalNAcα1→3Galβ1→3GlcNAcβ1→R † 2 4 ↑ ↑ Fucα1 Fucα1	**ALe^y**	GalNAcα1→3Galβ1→4GlcNAcβ1→R 2 3 ↑ ↑ Fucα1 Fucα1
BLe^b	Galα1→3Galβ1→3GlcNAcβ1→R † 2 4 ↑ ↑ Fucα1 Fucα1	**BLe^y**	Galα1→3Galβ1→4GlcNAcβ1→R 2 3 ↑ ↑ Fucα1 Fucα1
sialyl-Le^a	Galβ1→3GlcNAcβ1→R 3 4 ↑ ↑ NeuAcα2 Fucα1	**sialyl-Le^x**	Galβ1→4GlcNAcβ1→R 3 3 ↑ ↑ NeuAcα2 Fucα1

of which may become incorporated into the red cell membrane (Section 2.15.4).

2.2.2 Carbohydrate determinants

Expression of H, A, and B antigens is dependent on the presence of specific monosaccharides attached to various precursor disaccharides at the non-reducing end of a carbohydrate chain. There are at least five precursor disaccharides, also called peripheral core structures (reviewed in [14,18,21,23]):

Type 1	Galβ1→3GlcNAcβ1→R
Type 2	Galβ1→4GlcNAcβ1→R
Type 3	Galβ1→3GalNAcα1→R
Type 4	Galβ1→3GalNAcβ1→R
Type 6	Galβ1→4Glcβ1→R.

(Type 5 has only been chemically synthesised.)

H-active structures have Fuc α-linked to C-2 of the terminal Gal [35,36]; A- and B-active structures have GalNAc and Gal, respectively, attached in α-linkage to

Table 2.3 (*Continued*)

Type 3: O-linked mucin type

Precursor (T antigen) Galβ1→3GalNAcα1→O-Ser/Thr

H
Galβ1→3GalNAcα1→O-Ser/Thr
2
↑
Fucα1

A
GalNAcα1→3Galβ1→3GalNAcα1→O-Ser/Thr
2
↑
Fucα1

B
Galα1→3Galβ1→3GalNAcα1→O-Ser/Thr
2
↑
Fucα1

Type 3: repetitive type

H
Galβ1→3GalNAcα1→3Galβ1→4GlcNAcβ1→R *
2 2
↑ ↑
Fucα1 Fucα1

A
GalNAcα1→3Galβ1→3GalNAcα1→3Galβ1→4GlcNAcβ1→R *
2 2
↑ ↑
Fucα1 Fucα1

Type 4: globo-series

Globo-H
Galβ1→3GalNAcβ1→R
2
↑
Fucα1

Globo-A
GalNAcα1→3Galβ1→3GalNAcβ1→R
2
↑
Fucα1

*Intrinsic to red cells and detected in significant quantity on red cells of individuals of appropriate genotype.
†Adsorbed onto red cells from plasma in individuals of appropriate genotype.

Table 2.4 Some abbreviations for monosaccharides and the structures they are linked to.

Gal	D-galactose	Cer	Ceramide
GalNAc	*N*-acetyl-D-galactosamine	Asp	Asparagine
GlcNAc	*N*-acetyl-D-glucosamine	Ser	Serine
Fuc	L-Fucose	Thr	Threonine
NeuAc	Sialic acid (*N*-acetylneuraminic acid)		
Man	Mannose		
Glc	Glucose	R	Remainder of molecule

C-3 of this α1→2 fucosylated Gal residue (Table 2.3). Although Fuc does not represent the whole H determinant, it is the H immunodominant sugar because its loss results in loss of H activity. Likewise GalNAc and Gal are the A and B immunodominant sugars, respectively.

Le^a and Le^b antigens are expressed when Fuc is attached to the GlcNAc residue of the Type 1 precursor and Type 1 H, respectively [37–40]. Le^x and Le^y are the Type 2 isomers of Le^a and Le^b [36,39,41,42]. Fuc is linked α1→4 to the GlcNAc residue of a Type 1 chain in Le^a and Le^b and α1→3 to the GlcNAc of a Type 2 chain in Le^x and Le^y. Le^x and Le^y are not present in significant quantities on red cells [43]. The monofucosylated Le^a and Le^x

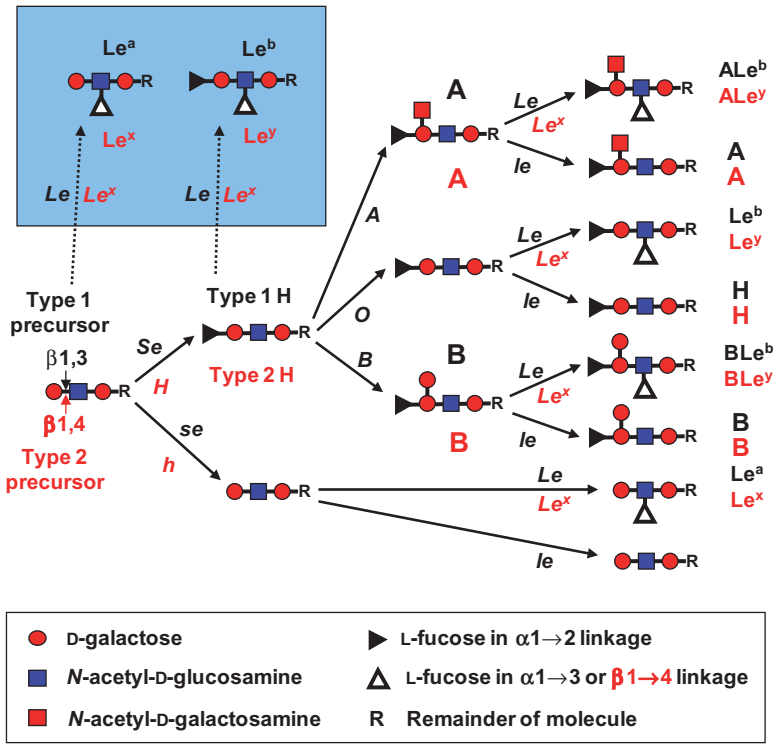


Figure 2.1 Diagram representing the biosynthetic pathways of ABH, Lewis, Le^x, and Le^y antigens derived from Type 1 and Type 2 core chains. Genes controlling steps in the pathway are shown in *italics* and the gene products are listed in Table 2.6. Type 1 and Type 2 precursors differ in the nature of the linkage between the non-reducing terminal Gal and GlcNAc: β1→3 in Type 1 and β1→4 in Type 2. Type 1 and Type 2 structures and the genes acting on them are shown in black and red, respectively. Dashed lines show how Le^a (Le^x) and Le^b (Le^y), produced from the precursor and H structures respectively, are not substrates for the H, Se, or ABO transferases and remain unconverted.

structures may be sialylated at the C-3 of Gal [44–46] (Table 2.3).

Type 1 ABH and Lewis structures are present in secretions, plasma, and endodermally derived tissues [21]. They are not synthesised by red cells, but are incorporated into the red cell membrane from the plasma [47]. Lewis antigens (Le^a and Le^b) are only present on Type 1 structures. Elongated carbohydrate chains with Type 1 peripheral structures are generally extended by repeating poly-*N*-acetyllactosamine disaccharides with the Type 2 (β1→4) linkage [48] (Table 2.5). Extended Type 1 structures with Le^a and Le^b activity have been detected in plasma, particularly in persons with Le(a+b+) red cells [49,50].

Antigens on Type 2 chains represent the major ABH-active oligosaccharides on red cells and are also detected in secretions and various ectodermally or mesodermally derived tissues [15,21]. Type 2 structures in secretions are probably more often difucosylated (Le^y, ALe^y, BLe^y) than monofucosylated (H, A, B) [51,52].

There are two forms of Type 3 ABH antigens, the *O*-linked mucin type and the repetitive A-associated type. In the *O*-linked mucin type the precursor exists as a disaccharide linked directly, by *O*-glycosidic bond, to a serine or threonine residue of mucin [53]. This precursor represents the T cryptantigen (see Section 3.17.2), but is not usually expressed because it is masked by substitution with sialic acid residues or other sugars. Type 3 ABH antigens of the *O*-linked mucin type are not found on red cells [54]. Repetitive Type 3 chains are present on red cell glycolipids and secreted mucins from group A individuals. They are restricted to group A because they are biosynthesised by the addition of Gal in β1→3 linkage to the terminal GalNAc of an A-active Type 2 chain followed by

Table 2.5 Examples of H-active glycoconjugates with Type 2 precursor chains (for abbreviations see Table 2.4).

Glycosphingolipid (simple linear)

Fucα1→2Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→Cer

Glycosphingolipid (branched)

Fucα1→2Galβ1→4GlcNAcβ1→3(Galβ1→4GlcNAcβ1→3)$_n$Galβ1→4Glcβ1→Cer
6
↑
Fucα1→2Galβ1→4GlcNAcβ1

N-*linked glycoprotein*

Fucα1→2Galβ1→4GlcNAcβ1
↓
6
Fucα1→2Galβ1→4GlcNAcβ1→3(Galβ1→4GlcNAcβ)$_n$1→2Man
Man-GlcNAc-GlcNAc-Asn
Fucα1→2Galβ1→4GlcNAcβ1→3(Galβ1→4GlcNAcβ)$_n$1→2Man
6
↑
Fucα1→2Galβ1→4GlcNAcβ1

O-*linked glycoprotein (complex mucin type)*

Fucα1→2Galβ1→4GlcNAcβ1
↓
6
Fucα1→2Galβ1→4GlcNAcβ1→3(Galβ1→4GlcNAcβ1→3)$_n$GalNAcα1→Ser/Thr
6
↑
Fucα1→2Galβ1→4GlcNAcβ1

n, 0–5 or more.

the fucosylation of that Gal to form Type 3 H [43,54–56] (Figure 2.2). Repetitive Type 3 chains are only present on group A cells because they are produced by the addition of Gal to the terminal GalNAc of a Type 2 A chain.

Type 4 ABH structures are only located on glycolipids. Type 4 precursor chain of the globo-series results from the addition of terminal Gal to globoside [57] (P antigen, see Chapter 4). Type 4 globo-H and globo-A have been detected in small quantities on red cells [57,58], but are more abundant in kidney [59]; Type 4 globo-B has only been found, in minute quantities, in kidney [60]. Kidney from a group A person with the p phenotype, which prevents extension of the globo-series structures, lacked Type 4 A [61] (see Chapter 4).

Type 6 chains have been found as free oligosaccharides in milk and urine [33,34].

The internal carbohydrate chains express I and i antigens. In fetal cells linear chains predominate and i antigen is expressed, whereas in adult glycoproteins and glycolipids there is branching of the inner core chains and I antigen is expressed (see Chapter 25).

2.3 Biosynthesis, inheritance, and molecular genetics

The carbohydrate antigens of the ABO, H, and Lewis blood group systems are not the primary products of the genes governing their expression. Carbohydrate chains are built up by the sequential addition of monosaccharides, each extension of the chain being catalysed by a specific glycosyltransferase. These enzymes catalyse the

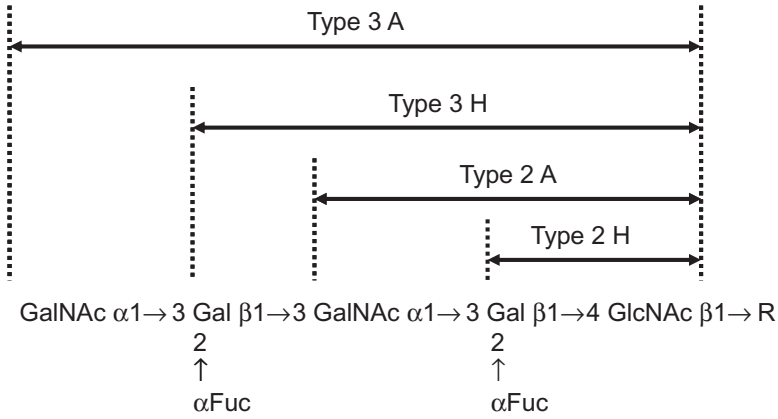


Figure 2.2 Diagram showing how a repetitive Type 3 A chain is built up from a Type 2 H chain. From right to left, Type 2 H is converted to Type 2 A in group A people. Type 2 A may be converted to Type 3 H. Type 3 H is then further converted to Type 3 A.

Table 2.6 Some ABH-related blood group genes and the glycosyltransferases they produce.

Locus	Allele	Transferase	
FUT1 (*H*)	*H*	α1,2-L-fucosyltransferase	EC 2.4.1.69
	h	None	
FUT2 (*SE*)	*Se*	α1,2-L-fucosyltransferase	EC 2.4.1.69
	se	None	
ABO	*A*	α1,3-*N*-acetyl-D-galactosaminyltransferase	EC 2.4.1.40
	B	α1,3-D-galactosyltransferase	EC 2.4.1.37
	O	None	
FUT3 (*LE*)	*Le*	α1,3/4-L-fucosyltransferase	EC 2.4.1.65
	le	None	

transfer of a monosaccharide from its nucleotide donor and its attachment, in a specific glycosidic linkage, to its acceptor substrate. Glycosyltransferases represent the primary products of the *ABO*, *FUT1* (H), *FUT2* (secretor), and *FUT3* (Lewis) genes (Table 2.6).

At least 100 glycosyltransferases are required for synthesis of the known human carbohydrates. The genes producing most of them have been identified and sequenced, including those for the ABO, H, and Lewis blood groups, and for secretion of H. The gene products are trans-membrane proteins of the Golgi apparatus. They share a common domain structure comprising a short N-terminal cytoplasmic tail, a 16–20 amino acid membrane-spanning domain, and an extended stem region followed by a large C-terminal catalytic domain. Soluble glycosyltransferases present in secretions may result from the release of membrane-bound enzymes by endogenous proteases or they may lack the membrane-spanning domain as a result of mRNA translation-initiation at an alternative site (reviewed in [62,63]).

The regulatory mechanisms required to assure that carbohydrate chains with the appropriate sequences are produced are complex. They involve the presence or absence of certain enzymes according to the genes expressed in various tissues and at different stages of development, and according to the genotype of the individual. Competition between different transferases for the same donor or acceptor substrate is also important in determining the carbohydrate chain produced (reviewed in [16]).

2.3.1 H antigen

H antigen is produced when an α1,2-L-fucosyltransferase catalyses the transfer of Fuc from a guanosine diphosphate (GDP)-L-fucose donor to the C-2 position of the terminal Gal of one of the precursor structures shown in Section 2.2.2 (Table 2.3, Figure 2.1). Two α1,2-L-fucosyltransferases, produced by *FUT1* (*H*) and *FUT2* (*SE*), catalyse the biosynthesis of H-active structures in different tissues. H-transferase, the product of *FUT1*, is active in tissues of endodermal and mesodermal origin, and synthesises red cell H antigen; secretor-transferase, the product of *FUT2*, is active in tissues of ectodermal origin, and is responsible for soluble H antigen in secretions (reviewed in [63]). *FUT1* has a higher affinity for Type 2 acceptor substrate than Type 1, whereas *FUT2* shows a preference for Type 1 acceptor substrate [64–67]. *FUT1* consists of four exons and *FUT2* of two exons, but in both genes only one exon (exon 4 in *FUT1*, exon 2 in *FUT2*) encodes the protein product [68,69].

FUT1 and *FUT2* share about 70% sequence identity and are 35 kb apart at chromosome 19q13.33 [70,71]. A pseudogene, *SEC1*, located within about 50 kb of *FUT2*, shares over 80% sequence identity with *FUT2*, but contains translation termination codons. *FUT1*, *FUT2*, and *SEC1* probably arose by gene duplication and are part of a linkage group that also includes the genes for the Lutheran (*BCAM*) and LW (*ICAM4*) blood groups (Section 6.2.4).

2.3.1.1 Red cells

A gene-transfer method was used to isolate *FUT1* [72–74]. Human genomic DNA was transfected into cultured mouse cells, which have all of the apparatus necessary to produce H-active carbohydrate chains apart from the *H*-gene-specified α1,2-fucosyltransferase. Transfected cells expressing H antigen were isolated with H-specific monoclonal antibodies and the human DNA in those cells used to produce secondary transfectants in mouse cells. Again cells producing H antigen were isolated immunologically. With an *Eco*RI restriction fragment common to all secondary transfectants expressing H as a probe, a mammalian cDNA library was screened; the *H* gene was isolated, cloned, sequenced, and expressed in cultured monkey (COS-1) cells [72,73]. The expressed enzyme was an α1,2-L-fucosyltransferase with an apparent K_m very similar to that of H-transferase and different from the putative *Se* gene product (see Section 2.3.1.2).

Stable transfection of Chinese hamster ovary (CHO) cells with human *FUT1* cDNA revealed that H-transferase does not indiscriminately act on all glycans, but favours glycoproteins containing polylactosamine sequences [75]. This explains why ABH expression is restricted to relatively few red cell surface glycoproteins.

Most people have H antigen on their red cells. Rare alleles at the *FUT1* locus produce little or no active transferase and individuals homozygous for these alleles have little or no H on their red cells (see Section 2.12).

2.3.1.2 Secretions

Almost everybody expresses H antigen on their red cells, but only about 80% of Caucasians have H antigen in their body secretions. These people are called ABH secretors because, if they have an *A* and/or *B* gene, they also secrete A and/or B antigens. The remaining 20% are called ABH non-secretors as they do not secrete H, A, or B, regardless of *ABO* genotype. In people of European and African origin, ABH secretor status appears to be controlled by a pair of alleles, *Se* and *se*, at the secretor locus (*FUT2*). *Se*, the gene responsible for H secretion, is dominant over *se* [76] (see Section 2.6).

The very different conformations of Type 1 (Galβ1→3GlcNAc) and Type 2 (Galβ1→4GlcNAc) disaccharides in two-dimensional models led Lemieux [77] to suggest the probable existence of two distinct fucosyltransferases, one specific for a Type 1 chain and the other for a Type 2 chain. It was well established that red cells produce only Type 2 H structures, whereas secretions of ABH secretors contain both Type 1 H and Type 2 H. Oriol and his colleagues [78,79] proposed that the *H* gene codes for an α1,2-fucosyltransferase specific for Type 2 substrate and is present in haemopoietic tissues, and that the *Se* gene codes for an α1,2-fucosyltransferase that utilises both Type 1 and Type 2 substrates and is present in secretory glands. Identification of two human α1,2-fucosyltransferases with slightly different properties and subsequent cloning of two α1,2-fucosyltransferase genes has confirmed the concept of two structural genes.

Le Pendu *et al.* [64] compared α1,2-fucosyltransferase from the serum of non-secretors with that from the serum of rare ABH secretors who lack H from their red cells (para-Bombay phenotype, see Section 2.12.3). The former transferase mostly originates from haemopoietic tissues and is the product of *FUT1*; the latter is believed to be the *FUT2* product [64,80]. Fucosyltransferases from these two sources differed from each other in various physicochemical characteristics such as K_m for GDP-fucose and sensitivity to heat inactivation. The transferase present in the serum of the non-secretors (*FUT1* product) favoured Type 2 acceptors, whereas that in

serum from the secretors with H deficient red cells (*FUT2* product) showed a definite preference for Type 1 substrate. Other, similar studies produced comparable results [65,66] and two α1,2-fucosyltransferases with different K_m values and electrophoretic mobilities were purified from pooled human serum [81].

In 1995, Rouquier *et al.* [70] exploited the close homology between the two α1,2-fucosyltransferase genes to clone *FUT2* from a human chromosome 19 cosmid library by cross-hybridisation with *FUT1* cDNA. *FUT2* encodes a 332 amino acid polypeptide, with substantial sequence homology to the product of *FUT1*, plus an isoform with 11 extra residues at the N-terminus [71]. The expressed product had α1,2-fucosyltransferase activity with a pH optimum and K_m similar to that ascribed to the secretor-transferase.

The common non-secretor allele of *FUT2* in people of European and African origin (se^{428}), with frequencies of 43–52% and 22–47%, respectively, contains a 428G>A nonsense mutation converting the codon for Trp143 to a translation stop codon, so no active enzyme is produced [71,82,83] (Table 2.7). This allele often also encodes a Gly247Ser substitution, but that change alone does not affect α1,2-fucosyltransferase activity [67,71].

The se^{428} allele is rare in Eastern Asia, but another *FUT2* allele (Se^{w385}), common in Eastern Asia and the South Pacific, encodes Ile129Phe in the stem region of the α1,2-fucosyltransferase [67,83,85–89]. This enzyme has identical substrate specificities to the normal *FUT2* product, but has at least a five-fold reduction in enzyme activity [67,86,87]. Se^{w385} has a gene frequency of 44% in Eastern Asia [83,86] and 40% in Samoa [94], but is very rare in Europeans and Africans [67,82]. Homozygosity for Se^{w385} (or heterozygosity for Se^{w385} and a non-secretor allele) results in reduced levels of secreted H and the Le(a+b+) red cell phenotype (Section 2.6.3). Se^{w385} also contains 357C > T, a synonymous change. In the Uygur of Urumqi (west of China) and in Bangladeshis both se^{428} and Se^{w385} are present with similar frequencies, suggesting admixed populations [94,97].

Many other inactive (non-secretor) alleles containing nonsense mutations have been found, some of which are listed in (Table 2.7) [83,84,89]. An allele with a single base deletion (778delC) was found in two of 101 black South Africans (Xhosa) [82].

Three alleles with deletions of exon 2 of *FUT2*, the whole of the coding region of the gene, were generated by three distinct *Alu–Alu* recombinations: se^{del} (10 kb deletion); se^{del2} (9.3 kb); se^{del3} (4 kb). Indian people with the rare Bombay phenotype have no H antigen on their red cells or in their secretions (Section 2.12.1). This phenotype results from homozygosity for an inactivating missense mutation in *FUT1* (Leu242Arg) together the se^{del} allele of *FUT2* [92,93]. The se^{del} allele linked with an active *FUT1* is relatively common in Bangladesh (7.4%) and in the Tamils of Sri Lanka [94,95]. Another *FUT2* deletion, se^{del2}, has a frequency of 10.4% in Samoans [94]. The se^{del3} allele was found in one Chinese [98].

Two inactive fusion genes are hybrids of *FUT2* and the pseudogene *SEC1*. One, se^{fus}, with a frequency of 5.5–7.9% in Japanese [96], consists of the 5′ region of *SEC1* and the 3′ region of *FUT2* and is presumably a product

Table 2.7 Some *FUT2* alleles responsible for ABH non-secretor phenotypes (*se*) or partial-secretor phenotype (Se^w).

Allele		Mutation	Amino acid substitution	Population	References
se^{302}		302C>T	Ile101Pro	Bangladeshi, Sri Lankan	[84]
Se^{w385}	*FUT2*01W.02*	385A>T	Ile129Phe	E. Asian, Polynesian, Filipino	[67,85–89]
se^{428}	*FUT2*01N.02*	428G>A	Trp143Stop	European, African	[71,82]
se^{571}	*FUT2*01N.04*	571C>T	Arg191Stop	E. Asian, Polynesian, Filipino, European	[71,82,86,89–91]
se^{688}	*FUT2*01N.09*	688_690del GTC	del230Val	Filipino	[91]
se^{778}	*FUT2*01N.11*	778delC	259fs275Stop	African	[82]
se^{849}	*FUT2*01N.12*	849G>A	Trp283Stop	Eastern Asian, Filipino	[89–91]
se^{del}	*FUT2*0N.01*	del exon 2		Indian, Bangladeshi, Sri Lankan	[92–95]
se^{del2}	*FUT2*0N.02*	del exon 2		Polynesians	[94]
se^{fus}	*FUT2*0N.03*	*SEC1–FUT2* fusion		Japanese	[86,96]

del, deletion; fs, reading frameshift.

of unequal crossing-over [86]. The other, a *SEC1–FUT2–SEC1* hybrid that was probably generated by gene conversion with the *FUT2* sequence derived from a se^{428} allele, has only been found in one person [99].

A single, multiplex PCR technique followed by RFLP digestion has been devised to detect many of the known *FUT2* mutations [100].

2.3.1.3 Other tissues

Control of expression of H antigen in various human tissues follows a general trend, summarised as follows: H antigens on tissues of ectodermal and mesodermal origin (e.g. primary sensory neurons, skin, vascular endothelium, and bone marrow) are Type 2 structures and produced by *FUT1*-specified α1,2-fucosyltransferase; those on tissues of endodermal origin (digestive and respiratory mucosae, salivary glands) are Type 1 and Type 2 structures and produced by the *FUT2*-specified enzyme [21]. There are, however, a number of exceptions to these rules (Section 2.19.3). Plasma α1,2-fucosyltransferase is predominantly haemopoietic in origin [101] and may originate from circulating red cells and platelets [102].

2.3.2 ABO antigens

2.3.2.1 ABO biosynthesis

H antigen, whether synthesised by the product of *FUT1* or *FUT2*, is the acceptor substrate of both *A* and *B* gene-specified glycosyltransferases (GTA and GTB) (Figure 2.1). GTA is an α1,3-*N*-acetyl-D-galactosaminyltransferase that transfers GalNAc from a uridine diphosphate (UDP)-GalNAc donor to the fucosylated Gal residue of the H antigen. GTB is an α1,3-D-galactosyltransferase that transfers Gal from UDP-Gal to the fucosylated Gal of H (Figure 2.3). *A* and *B* are alleles at the *ABO* locus; a third allele, *O*, does not produce an active enzyme and in *O* homozygotes H antigen remains unmodified. If no H structure is available, owing to the absence of H-transferase, A and B antigens cannot be produced despite the presence of GTA or GTB. This situation occurs in the secretions of ABH non-secretors and on red cells of the rare H-deficient (Bombay) phenotypes. The different species of GTA associated with A_1 and A_2 phenotypes are described in Section 2.4.1.

Anti-H reagents agglutinate group O cells far more readily than most A and B cells because H antigen activity is masked by GalNAc and Gal in A- and B-active structures.

A-, B-, and H-transferase activity has been demonstrated *in vitro*. GTA prepared from human gastric mucosa and other sources converts O or B cells to A or AB in the presence of UDP-GalNAc; likewise GTB from similar sources converts O cells to B cells in the presence of UDP-Gal [103–106]. Bombay phenotype cells, which lack the H-active substrate, could not be converted to B with GTB [104].

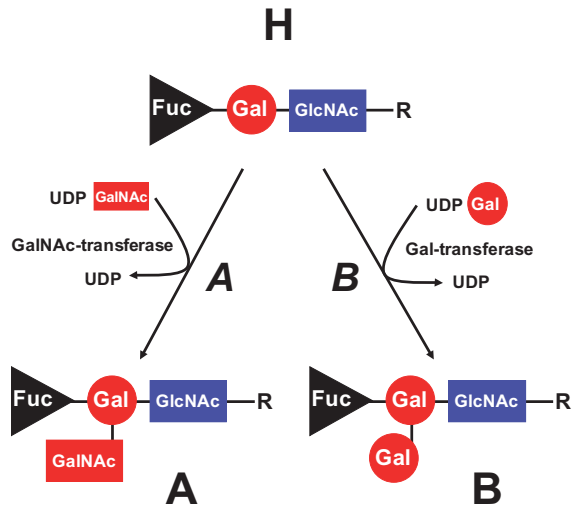


Figure 2.3 Pathways for biosynthesis of A and B antigens from their precursor, H.

2.3.2.2 Molecular genetics

GTA was purified to homogeneity from human lung and gastric tissues, and partial amino acid sequences were obtained [107,108]. Degenerate synthetic oligodeoxynucleotides based on the GTA partial amino acid sequence were employed by Yamamoto *et al.* [109] in the isolation and cloning of cDNA representing the *A* allele. The cDNA library was constructed from RNA isolated from a human gastric carcinoma cell line that expressed high levels of A antigen. The 1062 basepair (bp) sequence predicted a 353 amino acid protein with the three-domain structure characteristic of a glycosyltransferase. After the initial publication [109], it became apparent that the original clone from a gastric carcinoma contained a unique 3 basepair deletion [110]. The numbering of nucleotides and encoded amino acids used in this chapter and in most publications reflects the usual sequence of the gene. Based on the cDNA clone encoding GTA, *B* and *O* cDNA was also cloned and sequenced [111,112].

The coding region of *ABO* is organised into seven exons, spanning 18 kb. Exons 6 and 7 constitute 77% of the coding sequence [110,113] (Figure 2.4).

A and *B* alleles differ by seven nucleotides in exons 6 and 7, four of which encode amino acid substitutions (Figures 2.5 and 2.6). The most common *O* sequence is identical to that of A^1 apart from a deletion of nucleotide 261 in exon 6 causing a shift in the reading frame and generation of a premature translation stop signal at the codon for amino acid residue 116. This allele encodes a truncated protein with no catalytic domain (Figure 2.6) and may produce a mRNA transcript of reduced stability [114]. Cloned *A* and *B* cDNA transfected into recipient cells expressing H antigen resulted in A and B phenotypes that could be detected immunologically. The common *A* sequence in Caucasians (A^1 or *A101*) is often referred to as the 'consensus sequence' and is used as a reference for the sequences of all other *ABO* alleles. About 80% of A^1 alleles (*A102*) in Japanese and 93% in Chinese Han differ from A^1 in Europeans (*A101*) by 467C>T encoding Pro-156Leu [115–117]. This has no apparent affect on the phenotype. The A^2 (*A201*) allele has a single nucleotide deletion in the codon before the translation stop codon of A^1, resulting in disruption of that stop codon and a GTA product with an extra 21 amino acids at the C-terminus [118] (see Section 2.4.1).



Figure 2.4 Genomic organisation of the *ABO* gene, showing the seven coding exons and the number of amino acids encoded by each exon.

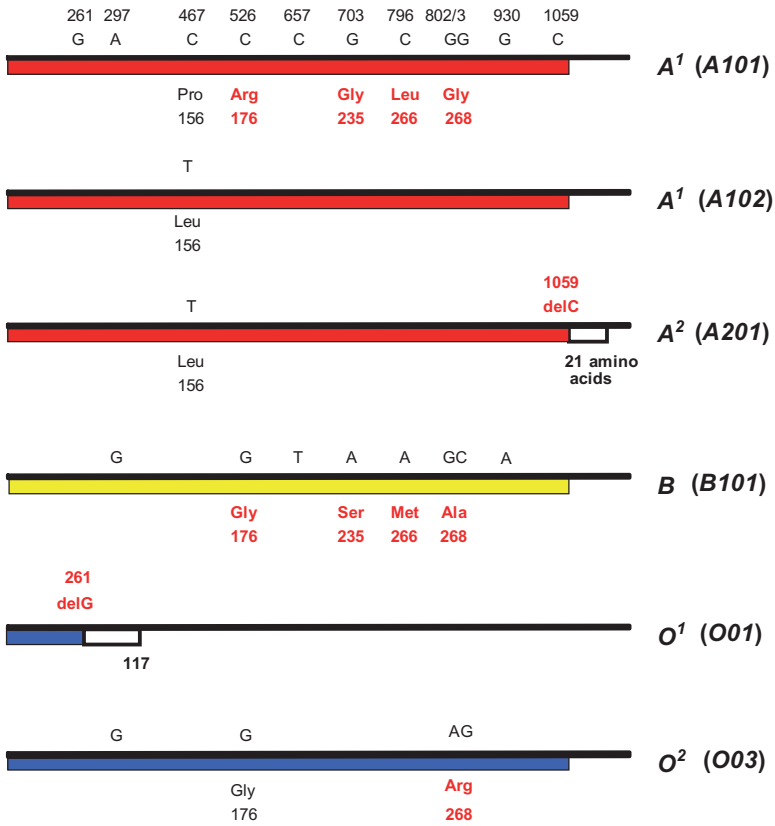


Figure 2.5 Diagram representing cDNA (black line) and protein products (coloured box) of six common *ABO* alleles, showing how they differ from the A^1 (*A101*) cDNA and its product. Seven nucleotide changes distinguish *A* and *B* alleles and result in four amino acid differences between GTA and GTB. A^1 (*A101*) and A^1 (*A102*) are the common A^1 alleles in Caucasians and East Asians, respectively. Single base deletions in A^2 and O^1 result in reading frameshifts and introduction and abolition of stop codons in O^1 and A^2, respectively. Amino acid substitution at position 268 is responsible for inactivation of the O^2 product.

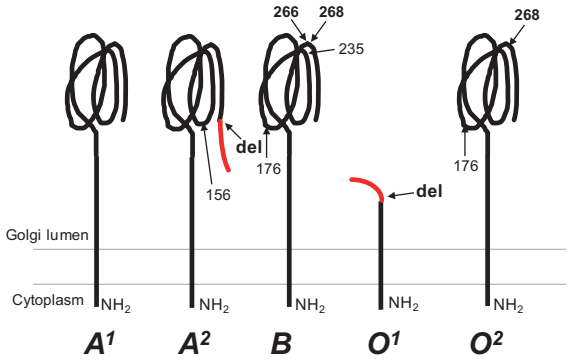


Figure 2.6 Diagrammatic representation of the products of five *ABO* alleles located in the membrane of the Golgi apparatus (modified from Clausen *et al.* [20], Copyright 1994, with permission from Elsevier), showing the positions of amino acids that differ from those of GTA_1 and the positions relating to the nucleotide deletions (del) in the A^2, O^1 alleles. Regions shown in red are the extra 21 amino acids in GTA_2 and the sequence encoded between the nucleotide deletion and the stop codon in the product of O^2.

The *O* allele described by Yamamoto *et al.* [111,112], with an A^1 sequence disrupted by a single base deletion, 261delG, is now named O^1 (*O01*). Another very common *O* allele, O^{1v} (O^1-variant, *O02*), has 261delG of O^1, preventing the production of any active transferase, but contains at least nine other nucleotide differences from O^1 and A^1 [119]. O^1 and O^{1v} are by far the most common *O* alleles and are present in all populations tested. The proportions of *O* alleles with 261delG that are O^{1v} are as follows: Swedes, 42% [119]; Australians, 42% [120]; Kuwaitis, 45% [121]; black Brazilians, 31% [122]; native Brazilians, 91% [122]; Japanese, 49–55% [115,116]; and Chinese, 40% [117,123]. Many rare variants of O^1, differing by a few point mutations, have been described [24]. A much less common *O* allele than O^1 and O^{1v}, O^2 (*O03*), lacks 261delG, but has nucleotide differences from A^1 exon 7 that encode Arg176Gly (identical to that of GTB) and Gly268Arg [124,125] (Figures 2.5 and 2.6). The substitution at position 268 introduces a charged arginine residue, completely blocking the donor GalNAc-binding site of GTA, whilst leaving the acceptor binding site unaffected [126]. *In vitro* expression of an A^1 cDNA construct with the Gly268Arg substitution introduced by site-directed mutagenesis resulted in no GTA activity or A antigen expression [127]. Between 2 and 6% of *O* alleles in white donors from Europe, Australia, and the United States are O^2 [120,125,128–131]; O^2 is not found in Japanese or Chinese [115,116,123]. Any *ABO* mutation that prevents production of an active transferase will be an *O* allele and numerous unique or very rare *O* alleles have been found, some having 261delG, others containing nonsense, frameshift, or enzyme-inactivating missense mutations [132–135]. In addition, hybrid genes containing 261delG will be *O* alleles. The debate on whether O^2 and other apparently inactivated *A* alleles produce any A antigen is discussed in Section 2.7.8.

Yamamoto and Hakomori [112] constructed *A-B* cDNA chimeras representing all 16 possible combinations of the four amino acid substitutions distinguishing *A* and *B* cDNA. Transfection experiments, in a group O human cell line, demonstrated that the third (266) and fourth (268) amino acid substitutions (Figure 2.5) are the most important in determining the specificity of the transferase. An enzyme with Met266 and Gly268 had dual GTA and GTB activity. *In vitro* mutagenesis experiments, in which cDNA constructs encoding every possible amino acid residue at position 268 were expressed, led to the conclusion that the side chain of the amino acid residue at position 268 is responsible for determining both activity and donor-substrate specificity of the transferase product [127] (and see Section 2.3.2.3).

The *ABO* gene contains a CpG island that extends from the immediate 5′ flanking region, through the first exon, and into the first intron. Methylation of this CpG island may play an important role in regulation of *ABO* expression in different tissues [136]. The most commonly used transcription site appears to be 12–38 bases upstream of the translation initiation codon in exon 1, but an alternative first exon (exon 1a) and transcription start site, utilised by both erythroid and epithelial lineages, is present at the 5′ end of the CpG island [137,138]. Exon 1a does not contain an ATG codon, but translation may be initiated from an alternative site in the transmembrane domain [138]. The promoter region binds the ubiquitous transcription factors Sp1 or Sp1-like [139] and transcription from the proximal promoter is partially dependent on an upstream N box [140]. In addition there is an erythroid-specific enhancer element within intron 1, 5.6–6.1 kb from the translation initiation site, containing two binding sites for the GATA-1 haemopoietic transcription factor [141]. Deletion of this intron 1 site results in the rare B_m phenotype, with almost no red cell B antigens expression, yet normal B antigen content in saliva (Section 2.8.3). Transcription regulation of *ABO* may also be dependent on a minisatellite, 3.8 kb upstream of the start of the translated sequence, that contains a CBF/NF-Y transcription factor-binding motif [142]. This

minisatellite usually consists of four copies of a 43 bp repeat sequence in A^2, B, O^1, and O^{1v} alleles, but only one copy in A^1 and O^2 alleles [143–145]. Transient transfection assays in a gastric cancer cell line suggested that the transcriptional activity of the *A* enhancer was substantially less than that of the *B* enhancer [144,146]. Strangely, transcripts from A^1 and A^2 alleles were not detected in peripheral blood, in contrast to readily detectable transcripts from B, O^1, O^{1v}, and O^2, whereas erythroid cells cultured from bone marrow expressed higher levels of A^1 and A^2 transcripts than those from B, O^1, O^{1v}, and O^2 [147]. Some weak B phenotypes appear to have been caused by sequence variations in the CBF/NF-Y regulatory region [148], although any affect of the number of repeats on transcription levels in another weak B phenotype is disputed [149] (Section 2.8.5).

2.3.2.3 ABO fusion genes

Many complexities of the *ABO* genes have been encountered. Some unusual *ABO* genes affect activity of the gene products and may result in subgroups of A and B (Sections 2.7 and 2.8). Numerous genes have been identified that appear to be hybrids, comprising partly of sequences characteristic of one *ABO* allele and partly of sequences characteristic of another. These fusion genes have probably arisen by meiotic crossing-over; in most the recombination has occurred within intron 6. Chester and Olsson [24] remark that the presence of *Chi* or *Chi*-like sequences near the 3′ end of intron 6, sequences associated with recombination hot-spots in *Escherichia coli*. Hybrid genes with exon 6 derived from O^1 or O^{1v} have 261delG and are inactive, regardless of the origin of exon 7. Hybrid genes with exon 6 derived from *A* or *B* are generally active, with the origin of exon 7 determining specificity. Exon 7 with A^1 or O^1 origin gives rise to A_1 activity; exon 7 with O^{1v} origin results in weakened A activity (A_2 or A_x).

Suzuki *et al.* [150] described a paternity case in which the mother was group B, the child group A, and the putative father group O; an apparent first order exclusion of paternity. Sequencing of the *ABO* genes showed that the child had an *ABO* gene in which exon 6 (and, presumably exons 1–5) had the sequence of a *B* allele and exon 7 the sequence of an O^1 allele. This hybrid gene had probably arisen in the germline of the mother as a result of crossing-over during meiosis. This B-O^1 gene would encode an enzyme with GTA activity because O^1 and A^1 have an identical sequence in exon 7, the region encoding the catalytic site; the absence of 261delG in exon 6 of *B* origin enables translation of this active enzyme. The child, therefore, had group A red cells, despite neither parent having an *A* gene. Such genetic events may be considered to be rare, yet similar recombinant alleles were estimated to occur with a frequency of about 1% in the Japanese population [150].

2.3.2.4 Linkage and evolution

ABO is closely linked to a gene for nail-patella syndrome (*LMX1B*), a dominantly inherited disorder characterised by dystrophic nails and deformed patellae and elbow joints, and the gene for adenylate kinase 1 (*AK1*). *ABO* location on the long arm of chromosome 9 was confirmed by *in situ* hybridisation [113] and the gene is now localised to 9q34.2.

The *ABO* genes have been well conserved during evolution [151,152,153]. *ABO* is part of the GT6 glycosyltransferases gene family, which is represented in all vertebrates [154]. Six GT6 genes other than *ABO* are present in humans, but all are pseudogenes and include *GBTG1*, the usually inactive Forssman-synthetase gene on chromosome 9q34.2 (but see Section 4.7), and the *ABO* pseudogene on chromosome 19 [111,155]. A minimum of 95% homology in nucleotide and deduced amino acid sequences was detected in the *ABO* genes of primates [151]. The critical substitutions differentiating the *A* and *B* genes occurred before the divergence of the lineages leading to humans, chimpanzees, gorillas, and orangutans [142]. The common human *O* mutation, 261delG, probably appeared once in human evolution, in the more ancient O^{1v} allele, with O^1 arising from recombination between O^{1v} and A^1 [132]. Kitano *et al.* [156] estimate that human *O* alleles appeared about 2 million years ago. From a phylogenetic network analysis involving the six most common *ABO* alleles, they propose that the original *A* allele became extinct in the human lineage and was resurrected less than 300 000 years ago as A^1 (*A101*) by recombination between *B* (exons 1–6) and O^1 (exon 7) [156].

2.3.2.5 Structure of the A- and B-transferases (GTA and GTB)

GTA and GTB, GalNAc-transferase and Gal-transferase, respectively, are called retaining enzymes because they do not alter the configuration of the nucleotide donor. They differ by four amino acids and are the two most homologous, naturally occurring glycosyltransferases that transfer different naturally occurring donors [157]. Models derived from the crystal structures of their catalytic

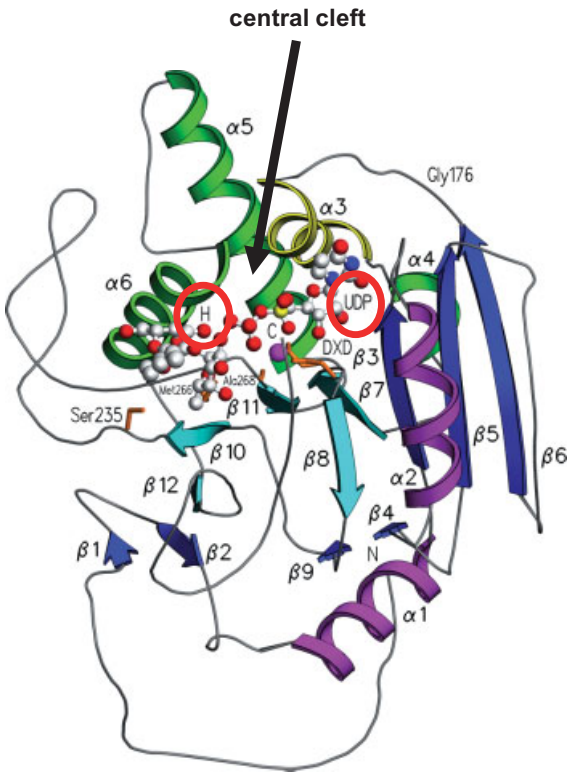


Figure 2.7 Structural model of GTB showing the two domains separated by a large central cleft, UDP-Gal and H antigen. Modified from Patenaude *et al.* [158] and provided by Dr Stephen Evans, University of Victoria, Canada.

domains, and of the enzymes in complex with the H-antigen disaccharide and UDP, reveal two domains separated by a cleft ~13 Å wide and containing the active site and all four amino acids that differ in GTA and GTB [158] (Figure 2.7). Of the four critical amino acids only residues 266 and 268 are positioned to contact donor and acceptor substrates and only Leu/Met266, which is most important for selection between donor sugars, is positioned to contact the characteristic acetamido/hydroxyl groups and so distinguishes UDP-GalNAc from UDP-Gal. The larger acetamido group in UDP-GalNAc is accommodated by the smaller Leu266 in GTA and the smaller hydroxyl group on UDP-Gal is accommodated by the larger Met266 in GTB. A fold near the active site, which is disordered in the unliganded state, undergoes conformational change and becomes ordered to cover the active site on binding of substrate [157,158].

2.3.2.6 Predicting ABO phenotype from DNA testing

Numerous methods have been developed for determining *ABO* genotype from genomic DNA and predicting ABO phenotype. Most tests involve two reactions: one determining the presence or absence of 261delG in exon 6; the other detecting the sequence for *A* (and possibly for A^1 and A^2), *B*, and O^2 in exon 7. Owing to the distance between the exon 6 and 7 critical sequences, haplotypes are rarely distinguished. Consequently, phenotypes are predicted on the basis that 261delG in exon 6 is usually associated with the *A* sequence in exon 7. To take an example, the presence and absence of 261delG in exon 6 together with an *A* and *B* sequence in exon 7 would be interpreted as group B phenotype, as the presence of 261delG would be assumed to be linked to the *A* sequence, representing an *O* allele, and the absence of 261delG would be assumed to be linked to the *B* sequence, representing a *B* allele. In most cases this interpretation would be correct, but errors could occur with A or B variants, non-deletion *O* alleles other than O^2, and hybrid alleles where 261delG is linked to a *B* sequence. Although errors arising from the presence of such confounding alleles would be relatively rare, any level of inaccuracy is unacceptable in ABO typing for transfusion purposes.

One method involving multiple PCR amplifications with allele-specific primers, some of which span from the 261-deletion site in exon 6 to various positions in exon 7 avoids most potential errors, including those arising from hybrid alleles [159]. A strategy that could be automated and could be accurate, involves sequence-based typing on physically separated haplotypes [160].

2.3.3 Lewis antigens

In 1948 Grubb [161] made the observation that people with Le(a+) red cells were mostly non-secretors of ABH. Subsequently the following general rule has been established for red cell Lewis phenotypes in adults:

Le(a+b–) red cells come from ABH non-secretors;
Le(a–b+) red cells come from ABH secretors;
Le(a–b–) red cells come from ABH secretors or non-secretors;
Le(a+b+) red cells come from ABH weak secretors.

Clearly there is an interaction between *FUT3*, the gene responsible for Le^a and Le^b on red cells, and the Secretor gene (*FUT2*). The Lewis and Secretor loci were shown by family studies to be genetically independent [162], although they are both on chromosome 19.

The Lewis-related antigens, Le^c and Le^d, are described in Section 2.18.2.

2.3.3.1 Lewis biosynthesis

The Lewis (*Le*) gene product is an α1,4-L-fucosyltransferase [163,164], which catalyses the transfer of L-fucose (Fuc) from GDP-Fuc to the GlcNAc of Type 1 acceptor substrates; to Type 1 precursor to form Le^a; to Type 1 H to form Le^b; to Type 1 A to form ALe^b; and to Type 1 B to form BLe^b. A pattern of interactions between *FUT3*, *FUT2*, and *ABO* determine whether Le^a or Le^b, or both, or neither, are present in secretions, plasma, and on red cells (Figure 2.1).

At the simplest level, two alleles at the *FUT3* locus can be considered: *Le*, which encodes an α1,4-fucosyltransferase, and *le*, which is apparently silent. People homozygous for *le* secrete neither Le^a nor Le^b and have the Le(a–b–) red cell phenotype, regardless of their ABH and secretor phenotypes.

In ABH non-secretors (*se/se*), no α1,2-fucosyltransferase is present in secretions to convert Type 1 precursor to Type 1 H. Consequently, the Type 1 precursor is available as an acceptor substrate for the Le-transferase, resulting in production of the monofucosylated Le^a antigen; so the secretions contain Le^a and the red cells are Le(a+b–). People with an *Se* allele produce Type 1 H, which can then be converted by the Le-transferase to the difucosylated Le^b antigen. If they also have an *A* or *B* gene, much of the Type 1 H will be converted to A or B structures and so the Le-transferase will produce ALe^b or BLe^b. Although Le-transferase can utilise either Type 1 precursor or Type 1 H acceptor substrates to produce Le^a and Le^b respectively, Le^a is a very poor substrate for the *Se* gene specified α1,2-fucosyltransferase. Consequently, there is competition between these two enzymes for substrate [165,166]. If any Le^a is produced from Type 1 'precursor' by the Le-transferase it cannot be converted further to Le^b by Se-transferase, so secretions of a person with *Le* and *Se* genes contain Le^a and Le^b, although very little Le^a is detected in the plasma or on the red cells. Similarly, Le^b is not an acceptor substrate for the A and B transferases, and secretions of an individual with *Le*, *Se* and *A* genes contain Le^a, Le^b, and ALe^b (Figure 2.1). The product of the weak secretor gene (Se^w), common in Eastern Asia and Pacific regions, competes with the Le-transferase less effectively than that of an *Se* allele, resulting in substantially greater production of Le^a than present in secretors. People homozygous for Se^w, or heterozygous Se^w/se, have Le^a and Le^b in their plasma and secretions and Le(a+b+) red cells [67,85,88,167,168].

Le-transferase has the exceptional ability to catalyse two distinct glycosidic linkages. In addition to α1,4-fucosyltransferase activity, it has some α1,3-fucosyltransferase activity and is often referred to as an α1,3/4-L-fucosyltransferase [169–172], although it is almost 100 times more efficient on Type 1 H than Type 2 H acceptors [173].

2.3.3.2 Molecular genetics

Kukowska-Latallo *et al.* [172] employed a gene transfer technique (like that described in Section 2.3.1.1 for isolation of *FUT1*) to clone and sequence cDNA encoding *Le* gene-specified α1,3/4-fucosyltransferase. The gene contains an intronless coding region that encodes a 361-amino acid protein with the three-domain structure typical of glycosyltransferases. There is a high level of sequence identity with some of the α1,2- and α1,3-fucosyltransferase genes.

The genetic basis for the Le(a–b–) red cell phenotype is heterogeneous, but is always associated with one or more missense mutations within the region of *FUT3* encoding the catalytic domain of the Lewis-transferase (Table 2.8). No Lewis nonsense mutation has been found. Transfection experiments with cDNA or chimeric *FUT3* constructs showed that Trp68Arg, Gly170Ser, and Ile356Lys caused complete or almost complete inactivation of α1,3/4-fucosyltransferase activity [175–177]. The enzyme is not inactivated by Thr105Met, which is associated with Trp68Arg [175]. The mutation encoding Leu20Arg is common in Lewis-negative alleles (Table 2.8). This substitution occurs within the transmembrane domain of the enzyme and does not affect catalytic activity [176,178,179], but may affect anchoring of the enzyme in the Golgi membrane [176]. Leu20Arg in the absence of any other Lewis mutation is relatively common in Indonesians and people homozygous for this allele have Le(a–b–) red cells, but secrete Lewis antigens [176].

In Caucasian populations, $le^{202,314}$ and $le^{59,1067}$ are the two most frequent Lewis-negative alleles [174,180,181], whereas $le^{59,508}$ is the most frequent in black Africans and in Eastern Asia (including $le^{59,508,980}$ in Africans) [87,174, 177,181,182] (Table 2.8).

The positions of the inactivating mutations in *FUT3* suggest that the catalytic domain of the Lewis-transferase includes the region from amino acid residues 68 to 356. Expression of *FUT3* constructs that produce truncated proteins demonstrated that a protein consisting of amino acids 62 to 361 is enzymatically active, but shorter forms were inactive [183].

Table 2.8 Some *FUT3* alleles, the encoded amino acid substitutions, and their frequencies in three populations (data from [174]). Synonymous substitutions are not shown.

Symbol	Amino acids															Allele frequencies		
	5	16	20	68	105	149	151	162	170	191	223	270	325	327	356	Caucasian	Ghanaian	Mongolian
*Le**	Gly	Cys	Thr	Trp	Thr	Leu	Arg	Asn	Gly	Glu	Gly	Val	Thr	Arg	Ile	0.71	0.44	0.61
$le^{13,484,667}$	Ser	–	–	–	–	–	–	Asp	–	–	Arg	–	–	–	–	0	0.10	0
$le^{13,484,667,808}$	Ser	–	–	–	–	–	–	Asp	–	–	Arg	Met	–	–	–	0	0.04	0
$le^{13,484,667,974}$	Ser	–	–	–	–	–	–	Asp	–	–	Arg	–	Met	–	–	0	0.03	0
$le^{47,202,314}$	–	Ser	–	Arg	Met	–	–	–	–	–	–	–	–	–	–	0.03	0	0
$le^{59,508}$	–	–	Arg	–	–	–	–	–	Ser	–	–	–	–	–	–	0.02	0.22	0.24
$le^{59,508,980}$	–	–	Arg	–	–	–	–	–	Ser	–	–	–	–	Gln	–	0	0.02	0
$le^{59,202,1067}$	–	–	Arg	Arg	–	–	–	–	–	–	–	–	–	–	Lys	0.01	0	0
$le^{59,445}$	–	–	Arg	–	–	Met	–	–	–	–	–	–	–	–	–	0.01	0	0
$le^{59,508}$	–	–	Arg	–	–	–	–	–	Ser		–	–	–	–	–	0	0	0.05
$le^{59,571,1067}$	–	–	Arg	–	–	–	–	–	–	Lys	–	–	–	–	Lys	0	0	0.01
$le^{59,1067}$	–	–	Arg	–	–	–	–	–	–	–	–	–	–	–	Lys	0.04	0.01	0.03
$le^{202,314}$	–	–	–	Arg	Met	–	–	–	–	–	–	–	–	–	–	0.17	0.07	0.06
$le^{202,314,451}$	–	–	–	Arg	Met	–	Gly	–	–	–	–	–	–	–	–	0	0.02	0
Other *le*																0.01	0.05	0

*Includes all functional alleles.

α1,4-fucosyltransferase activity has been identified in a number of tissues and secretions: kidney, gastric mucosa, submaxilliary glands, ovarian cyst linings, saliva, milk (see [10]). α1,4-fucosyltransferase activity has not been detected in serum, red cells, lymphocytes, granulocytes, or platelets [170,184–186], suggesting that there is no haemopoietic origin for this enzyme. High levels of *FUT3* transcripts are present in colon, stomach, small intestine, lung, and kidney; lesser amounts are present in salivary gland, bladder, uterus, and liver [187].

2.3.4 Le^x, Le^y, and sialyl-Le^x

Le^x (CD15) and Le^y represent the Type 2 isomers of Le^a and Le^b, respectively (Table 2.3). An α1,3-L-fucosyltransferase catalyses the transfer of Fuc from a nucleotide donor to C-3 of the subterminal GlcNAc of Type 2 precursor, Type 2 H, Type 2 A, or Type 2 B to produce Le^x, Le^y, ALe^y, and BLe^y, respectively (Figure 2.1). In analogy with the Lewis structures, Le^x is not converted to Le^y by H-transferase or Se-transferase, and Le^y is not converted to ALe^y or BLe^y by GTA or GTB. (The antigen described here as Le^x differs from the original Le^x antigen, called Le^{abx} in this chapter, see Section 2.18.1.)

Fucosylation of a 2,3-sialylated acceptor produces sialyl-Le^x (sialyl-CD15) [188,189] (Table 2.3), a ligand for the selectin family of cell adhesion proteins [190,191] (Section 2.18.3).

2.3.5 Other fucosyltransferase genes

In addition to *FUT3*, four other genes encoding enzymes with α1,3/4-fucosyltransferase activity, *FUT4–FUT7* and *FUT9*, plus two others with α1,3-fucosyltransferase activity, *FUT10* and *FUT11*, have been identified [192]. *FUT3*, *FUT5*, and *FUT6* have about 90% sequence homology and form a cluster on chromosome 19p13.3 (*pter–FUT6–FUT3–FUT5–cen*) [193], as part of a linkage group including *FUT1*, *FUT2*, *ICAM4* (*LW*), and *BCAM* (*LU*) (Section 6.2.4). The *FUT6–FUT3–FUT5* cluster, and possibly the other fucosyltransferase genes, probably arose by successive duplications followed by translocations and divergent evolution from a single ancestral gene. *FUT8*, which encodes an α1,6-fucosyltransferase, may represent the ancestral gene [173].

Nine percent of Indonesians from Java have α1,3-fucosyltransferase deficiency as a result of inactivating mutation in *FUT6*. Ninety-five percent of these individuals have Le(a–b–) red cells, indicating linkage disequilibrium between *FUT3* and *FUT6* [194].

Part 3: ABO, H, and secretor

2.4 A_1 and A_2

The existence of subgroups of A, with red cells of one subgroup demonstrating weaker expression of A antigen than those of the other, was first recognised by von Dungern and Hirszfield [195] in 1911. Landsteiner and Levine [196] named the two major subgroups A_1 and A_2.

The usual way of interpreting the A_1 and A_2 subgroups is as follows:

Group	Antigens	Anti-A (group B serum)	
		Anti-A	Anti-A_1
A_1	A A_1	+	+
A_2	A	+	–

Sera from group B individuals appear to contain two antibody components, anti-A and -A_1. A_1 cells react with both components, whereas A_2 cells react only with anti-A. Adsorption of some group B sera with A_2 cells removes anti-A leaving behind anti-A_1 [195]; continued adsorption of group B serum with A_2 cells, however, eventually removes all antibody [197]. Regrettably, the term anti-A has two meanings: the antiserum that reacts with A and AB cells and one of the two antibody components present in group B serum. In this chapter, the precise meaning of 'anti-A' should be apparent from its context.

Anti-A_1 is present in the serum of some A_2 and A_2B people [198,199]. By agglutination of A_1 cells at room temperature, anti-A_1 was found in the serum of 1–2% of A_2 and 22–26% of A_2B individuals [200,201]. More sensitive techniques revealed anti-A_1 in higher proportions of A_2 and A_2B donors [202,203].

The best and most widely used anti-A_1 reagent is *Dolichos biflorus* lectin [204]. Raw extract of *Dolichos* seeds agglutinates A_1 and A_2 red cells, but at a suitable dilution the lectin will easily distinguish A_1 and A_1B from A_2 and A_2B. Red cells from group A babies usually react only weakly with *Dolichos* lectin and may not be agglutinated at all by human anti-A_1. It should be remembered that *Dolichos* lectin also agglutinates rare red cells with a very

Table 2.9 A_1A_2BO genotypes and serologically determined phenotypes.

Genotype	Phenotype
A^1/A^1, A^1/A^2, A^1/O	A_1
A^2/A^2, A^2/O	A_2
B/B, B/O	B
A^1/B	A_1B
A^2/B	A_2B
O/O	O

strong Sd^a antigen and Tn polyagglutinable red cells, regardless of ABO group (Chapters 31 and 33).

A_2 red cells have substantially higher expression of H antigen than A_1 cells.

When determined by serological means, the A^1 allele appears dominant over A^2 and the genotypes A^1/A^1 and A^1/A^2 cannot be discriminated by blood grouping techniques (Table 2.9).

2.4.1 A_1- and A_2-transferases (GTA_1 and GTA_2) and the genes that produce them

A-transferase (GTA) isolated from sera or gastric mucosa of A_1 individuals is more effective at converting group O red cells to A-active cells than that from A_2 people [205–208]. When A_2 enzyme is used, the reaction is much slower and under normal conditions O cells are only converted to A_2 phenotype. After extended incubation with A_2 enzyme, however, O cells may be agglutinated weakly by A_1-specific reagents [208]. A_1 enzyme can convert A_2 cells to A_1 phenotype [206,207]. GalNAc-transferases from A_1 and A_2 sources have the same specificity for low molecular weight acceptors and both synthesise the same A determinant [10]. Yet at pH 5.5, activity of GTA from A_1 serum (GTA_1), with low molecular weight substrate, is 5–10 times higher than that from A_2 serum (GTA_2) [209].

Serum GTA_1 and GTA_2 have different pH optima: 5.6 for GTA_1 and between 7 and 8 for GTA_2 [210]. Sera from heterozygous A^1/A^2 individuals can be distinguished from sera from A^1/A^1 or A^1/O people by pH optima and by isoelectric point [211]. At pH 7.2, GTA_2, the less efficient enzyme, has a K_m value about 10 times higher than that for GTA_1 [210]. *In vitro* conversion of O cells to A activity by GTA generally requires the presence of Mn^{2+} ions. If Mn^{2+} is substituted by Mg^{2+}, GTA_1 remains active, but GTA_2 does not [210].

The A^2 allele (*A201*) in people of European origin contains a deletion of one of the three cytosines at positions 1059–1061 (CCC to CC). This deletion is in the codon before the translation stop codon and causes a reading frameshift and loss of the stop codon, resulting in a gene product with an extra 21 amino acid residues at its C-terminus [118] (Figures 2.5 and 2.6). The A^2 allele also contains 467C>T, Pro156Leu, which is common in A^1 (*A102*) in East Asia and has no effect on enzyme activity. An *A* allele with 1016delC, but without 467C>T (*A206*), has been found in Chinese [123].

In East Asia, where A_2 phenotype is rare, the A^2 allele with 1016delC is also rare. The two most common alleles responsible for A_2 in Japan do not have 1016delC, but have different missense mutations within codon 352, only three codons before the normal stop codon: 1054C>T, Arg352Trp (*A202*) and 1054C>G, Arg352Gly (*A203*) [212,213]. A *B*-O^{1v} hybrid allele (*A204*), also quite common in Japan, gives rise to an A_1 phenotype when paired with *O*, but an A_2B phenotype when paired with *B*, presumably because of competition for a common acceptor between the A-active hybrid transferase and GTB [213]. This allele is responsible for an imbalance in A_2 and A_2B phenotype frequencies in Japan. The most common A^2 allele among A_2B donors in Taiwan contains 467C>T, Pro156Leu and 1009A>G, Arg337Gly (*A205*) [213,214].

Other alleles responsible for an A_2 phenotype are listed in dbRBC [215]. Over 20 alleles containing 1016delC with additional missense mutations were responsible for a variety of phenotypes, ranging from very weak to nearly A_2, with the majority displaying A_x-like characteristics [216]. A weak A phenotype known as A_{bantu}, found in about 4% of black South Africans [217], results from a hybrid of the common A^2 allele with 1016delC and an O^1-like allele (O^{1bantu}), with a cross-over region near exon 5 (*Abantu01*) [218]. Another similar hybrid allele, with exons 1–5 derived from a variant O^{1v} allele and exons 6 and 7 from A^2 (*A201*), also found in people of African origin, gave rise to an A antigen weaker than that of A_2 phenotype [219].

2.4.2 A_1 and A_2 determinants differ quantitatively and qualitatively

After the A_1 and A_2 subgroups were first described there was controversy over whether A_1 and A_2 cells differ purely in the number of A determinants or whether these antigens actually show structural differences. Numbers of antigen sites per red cell, estimated from a variety of techniques, can be summarised as follows: A_1, $8–12 \times 10^5$; A_2, $1–4 \times 10^5$; A_1B, $5–9 \times 10^5$; A_2B, 1×10^5 [220–226]. Repeated adsorption of anti-A_1 from group B serum with A_2 cells will remove all antibody, suggesting only a quantitative difference [197,227], but A_2 and A_2B individuals often make anti-A_1, suggesting that A_2 cells lack a determinant present on A_1 cells [198,199].

The majority of red cells from A_2 individuals showed faint fluorescence with fluorescent *Dolichos* lectin, while a few cells demonstrated very strong fluorescence; conversely, in a population of A_1 cells, most had strong reactivity while around 10% exhibited only faint fluorescence [228]. This may explain the 'mixed field' appearance of agglutination usually observed with anti-A_1 reagents.

The precise biochemical background to A_1 and A_2 is controversial, but it appears that A_1 red cells have both repetitive Type 3 A and Type 4 A glycolipids (Section 2.2.2), whereas A_2 red cells either lack both Type 3 A and Type 4 A or have Type 3A, but lack Type 4 A glycolipids [43,55,56,58,229–231]. Svensson *et al.* [231] detected abundant Type 3 A glycolipids in A_2 red cell membranes and, therefore, considered that the major difference between A_1 and A_2 phenotypes is the dominance of Type 4 A glycolipids in the A_1 phenotype, which are essentially absent in A_2. It is probable, therefore, that GTA_2 is unable to utilise Type 4 H as an acceptor substrate, possibly as a result of the extension of GTA_2 compared with GTA_1 (Figure 2.6). It is probable that anti-A_1 is specific for, or at least shows a preference for, Type 4 A structures. *Dolichos* lectin, however, detects GalNAc and, when present in sufficient concentration, agglutinates A_2 cells, so its use as a reagent for subtyping group A cells probably depends more on the quantitative than the qualitative difference between A_1 and A_2 phenotypes.

2.4.3 A_{int}

Landsteiner and Levine [196] recognised that the red cells of some group A individuals could not be defined as either A_1 or A_2, but fell into an intermediate category. A_{int} does not represent a true intermediate, however, as the level of H is as high as that found in A_2 and may be higher [217,232–234].

A_{int} is more common in black than white people. Of group A African Americans, 8.5% were found to be A_{int} compared with about 1% of group A white Americans [235]. Of group A black South Africans, 13.7% were A_{int} [217].

A unique form of GTA in A_{int} sera was detected, which differed from GTA_2 in having a high affinity for UDP-GalNAc and from GTA_1 in having a low affinity for 2′-fucosyllactose, a soluble analogue for membrane-bound H-substance [236]. One *A* mutation in an $A_{int}B$ individual is listed in dbRBC: 923A>G, Lys308Arg [215].

2.5 ABO phenotype and gene frequencies

Millions of people have been ABO grouped and the frequencies of the four phenotypes, A, B, AB, and O, differ substantially throughout the world, and often show marked variations even within quite small countries. In 1976, Mourant *et al.* [237] published the results of ABO tests on nearly 15 million people from populations of virtually every country in the world. As an example of ABO frequencies in Britain, a study of unrelated individuals from the South of England is shown in Table 2.10.

Populations with a high frequency of *O* (gene frequency greater than 0.7, i.e. 70%) are found in North and South America, and in parts of Africa and Australia, but not in most of Europe or Asia. Some native people of South and Central America are virtually all group O and probably were entirely so before the European invasion. The frequency of *A* is quite high (0.25–0.55) in Europe, especially in Scandinavia and parts of Central Europe. High *A* frequency is also found in the Aborigines of South Australia (up to 0.45) and in certain Native American tribes where the frequency reaches 0.35. A^2 is found mainly in Europe and Africa, but is either very rare or absent from indigenous populations throughout the rest of the world. The frequency of A^2 in Lapland reaches 0.37, but elsewhere in Europe it does not exceed 0.1. *B*, almost absent from Native Americans and most Australian Aborigines, probably was absent before the arrival of Europeans. High frequencies of *B* are found in Central Asia (0.2–0.3). In Europe, *B* frequency diminishes from about 0.15 in the east to less than 0.05 in the Netherlands, France, Spain, and Portugal (data compiled from [237]). For a diagrammatic representation of some examples of ABO phenotypes in different populations, see Figure 2.8.

Table 2.10 A_1A_2BO phenotype, gene, and genotype frequencies in the South of England [238].

Phenotype			Gene		Genotype	
	No.	Frequency		Calculated frequency		Calculated frequency
O	1503	0.4345	*O*	0.6602	*O/O*	0.4349
A_1	1204	0.3481	A^1	0.2090	A^1/A^1	0.0437
					A^1/O	0.2760
					A^1/A^2	0.0291
A_2	342	0.0989	A^2	0.0696	A^2/A^2	0.0048
					A^2/O	0.0919
B	297	0.0859	*B*	0.0612	*B/B*	0.0037
					B/O	0.0808
A_1B	91	0.0263			A^1/B	0.0256
A_2B	22	0.0063			A^2/B	0.0085
Total	**3459**	**1.0000**		**1.0000**		**1.0000**

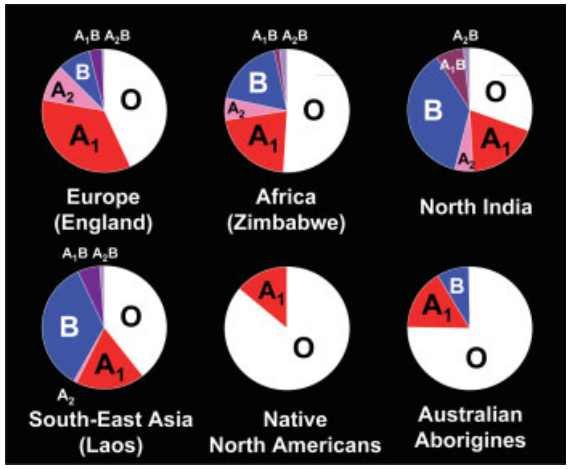


Figure 2.8 Diagram showing the distribution of ABO phenotypes in six selected populations.

Some gene frequencies determined by molecular methods are provided in Table 2.11. The frequencies for English donors correlate remarkably well with those calculated from serological data (Table 2.10), considering changes in the ethnicity of the donor populations over 60 years.

2.6 Secretion of ABO and H antigens

By 1926 it was apparent that A and B antigens were not confined to red cells, but were present in soluble form in seminal fluid and saliva [240]. In 1930, Putkonen [241] noted that a proportion of A, B, and AB individuals lacked A or B antigens from their body fluids. The ability to secrete A, B, and 'O' was found to be inherited in a Mendelian manner, genetically independent of ABO [76]. The locus controlling ABH secretion was called Secretor (*Se*, and subsequently *FUT2*): the ability to secrete (*Se*) is dominant over non-secretor (*se*). Although some other blood group antigens are also present in secretions, the terms 'secretor' and 'non-secretor' refer only to ABH secretion.

In secretor individuals of the appropriate ABO group, ABH antigens are detected in the secretions of the goblet cells and mucous glands of the gastrointestinal tract (saliva, gastric juice, bile, meconium), genitourinary tract (spermatic fluid, vaginal secretions, ovarian cyst fluid, urine), and respiratory tract, as well as in milk, sweat, tears, and amniotic fluid [32,242]. Secreted ABH antigens are mostly carried on mucins, glycoproteins of high molecular weight, but are also present in milk and urine as free oligosaccharides [10,33,34]. Secreted ABH

Table 2.11 *ABO* allele frequencies determined by PCR-based analyses of genomic DNA.

Population	No. of alleles tested	Alleles					References
		A^1	A^2	B	O^1	O^2	
Europeans	600	0.215	0.062	0.112	0.583	0.028	[129]
English	172	0.198	0.075	0.105	0.605	0.017	[130]
White Americans	240	0.188	0.017	0.108	0.671	0.017	[131]
Kuwaitis	166	0.136	0.030	0.166	0.660	0.009	[121]
Chinese (Han)*	417	0.213	0.002	0.209	0.572	0	[117]
Japanese	208	0.288	0	0.178	0.534	0	[239]

O^1 includes all alleles with 261delG.
*plus single examples of *cisAB06* and *O06* 87–88insGG.

antigens are expressed on Type 1, Type 2, and Type 3 structures [10,14,39,53].

Se and *se* are alleles of the endodermal α1,2-fucosyltransferase gene, *FUT2*. The symbol *se* represents numerous alleles containing inactivating mutations (Section 2.3.1.2 and Table 2.7). *Se* and *se* determine the presence or absence of H in secretions. A- and B-transferases are not under the control of the secretor gene, but are unable to catalyse the production of A and B substances in body fluids of non-secretors owing to lack of H, their acceptor substrate (Section 2.3.2). The study of dispermic chimeras has shown that in order to secrete A, an *A* gene and an *Se* gene must be expressed in the same cell, and the corresponding situation applies to cells that secrete B [243,244].

The simplest method for determining secretor status is by inhibition of haemagglutination. Saliva (previously boiled) is added to selected and appropriately diluted anti-A, -B, and -H (usually *Ulex europaeus* lectin), and inhibition determined by the failure of these mixtures to agglutinate A_2, B, and O cells, respectively.

2.6.1 Frequencies

In most European populations the frequency of secretors is about 80% [237]. Table 2.12 shows the results of secretor tests, with deduced gene and genotype frequencies, on over a thousand people from Liverpool. The frequency of the *Se* gene does not differ greatly from 0.5 in most ethnic groups, although in Australian Aborigines, Inuits, some Native Americans, and some Melanesians, the frequency approaches 1.0 [237]. In India there is more variation with a high frequency of *Se* in the North (up to 0.75) and low frequency in the South (0.22).

2.6.2 Quantitative aspects

A study of sibling pairs indicated that individual quantitative variation of salivary A, B, or H is, at least in part, inherited, and inherited in a polygenic manner [245]. The primitive salivary glands of a human fetus produce secretion rich in ABH antigens from the gestational age of about nine weeks [246] and ABH antigens are well developed in neonatal saliva [247,248]. A variety of techniques, mostly employing human anti-A or *Dolichos biflorus* lectin, has provided substantial evidence that A_1 saliva contains more A antigen than A_2 saliva [165,249–251]. Saliva from AB secretors contains less A and B than saliva from group A secretors and group B secretors, respectively [249–251], the result of competition between GTA and GTB for a common substrate.

Small quantities of H, A, and B substances can be detected in the saliva of most non-secretors [166, 252–254]. H production in non-secretor saliva is probably catalysed by the *FUT1* gene-specified α1,2-fucosyltransferase and not the *FUT2* gene product. Low levels of α1,2-fucosyltransferase in submaxillary gland preparations from non-secretors showed the Type 2 acceptor preference typical of *FUT1* gene-specified transferase [66].

2.6.3 Se^w

A weak secretor gene (Se^w or Se^{w385}), containing a missense mutation encoding Ile129Phe, is responsible for the Le(a+b+) red cell phenotype common in East Asia, Polynesia, and the Philippines [67,85–89] (Table 2.7). An α1,2-fucosyltransferase that is less efficient than the normal *Se* gene product competes less effectively with the Lewis-transferase for the Type 1 precursor substrate.

Table 2.12 Phenotype, gene, and genotype frequencies for secretor status of a random selection of people from Liverpool.

Phenotype			Gene		Genotype	
	No.	Frequency		Calculated frequency		Calculated frequency
Secretors	864	0.7728	*Se*	0.5233	*Se/Se*	0.2739
					Se/se	0.4989
Non-secretors	254	0.2272	*se*	0.4767	*se/se*	0.2272

Consequently, a greater quantity of the substrate is converted to Le^a so that less is available to be converted to Type 1 H and, subsequently, to Le^b (Section 2.3.3.1).

2.6.4 A, B, and H in plasma

A, B, and H are found in the plasma of secretors and non-secretors, although greater quantities are present in the former [79,255–257]. With anti-Type 1 H (serum from goats immunised with human saliva and adsorbed with immunoadsorbents coated with Type 2 H trisaccharide) and anti-Type 2 H lectin (*Ulex europaeus*), Le Pendu *et al.* [79] showed that plasma from ABH secretors contains Type 1 H and Type 2 H, but plasma from non-secretors contains only Type 2 H. They estimated that all of the Type 1 H and about one-third of the Type 2 H in plasma is controlled by the secretor system (*FUT2*), whereas most of the Type 2 H is independent of secretor and is presumably of haemopoietic origin. Plasma ABH substances are carried on glycosphingolipids and glycoproteins [258]. Their quantity is greatly affected by Lewis phenotype: Le(a–b–) ABH secretors have substantially more ABH determinants in their plasma than do Le(a–b+) ABH secretors [79,257–259].

Over a period of about two weeks, group O transfused red cells adsorb A and B antigens from the plasma of an AB recipient and become agglutinable with anti-A, -B, and -A,B [260]. A and B antigens adsorbed from plasma onto O red cells, *in vitro*, are glycosphingolipids and contain Type 1 chains [257].

2.7 Subgroups of A

In addition to the common phenotypes A_1 and A_2, numerous phenotypes with weak expression of A on the red cells have been found and a multitude of names have been adopted. Most of these phenotypes can be fitted into the following categories: A_3, A_x, A_{end}, A_m, A_y, and A_{el}. The serological characteristics of these phenotypes are shown in Table 2.13. All have normal or enhanced expression of H. Most result from inheritance of a rare allele at the *ABO* locus, usually involving missense mutations in exons 6 or 7, although other mechanisms do occur. Some alleles are listed in Table 2.14. The abnormal A phenotypes are only apparent when the variant gene is paired with *O* or *B*, not with A^1 or A^2. A_y probably results from germline mutation or from homozygosity for a rare gene at a locus independent of *ABO*. A_{end}, A_m, A_y, and A_{el} red cells are not agglutinated by most anti-A and are disclosed in routine testing because they resemble group O or B red cells, but no anti-A is present in the serum. A_x cells are agglutinated by group O (anti-A,B) serum and some monoclonal anti-A. In A subgroups the A antigen is more easily detected if the cells are protease treated. In many cases weak A subgroups have been found and associated with *ABO* mutations although serological analyses have been insufficient for categorising the phenotype, usually because of unavailability of secreted material. The subgroups are usually categorised as A_w (for weak). Olsson *et al.* [261] considered that, 'The relevance of categorical subgroup classification based on serological phenomena alone is becoming questionable; so use of the more general terms A_{weak} and B_{weak} is not unfounded'. The serological terms will continue to be used here, as they are still used in laboratories where detailed serological analyses are carried out.

Measurements of relative agglutinability, obtained by counting the number of cells agglutinated by anti-A in a cell counter, and site density, determined with radiolabelled rabbit IgG anti-A [223,224], are shown in Table 2.15. These quantitative techniques reveal substantial

Table 2.13 Serological and transferase characteristics of weak A subgroups.

	Reactions of cells with		Antibodies in serum			
Name	Anti-A	Anti-A,B	Anti-A	Anti-A_1	Antigens in saliva of secretors	A-transferase in serum
A_3	mf	mf	No	Sometimes	A H	Sometimes
A_{end}	mf	mf	No	Sometimes	H	No
A_x	–*/w	+	–/+	Usually	(A_x) H	Rarely
A_m	–*/w	–/+	No	No	A H	Yes
A_y	–*	–	No	No	A H	Trace
A_{el}	–*	–	Some	Yes	H	No

Red cells of none of the subgroups reacted with anti-A_1; all reacted with anti-H.
*Anti-A may be adsorbed onto and eluted from these cells.
(A_x), may require inhibition of agglutination of A_x cells for detection.
mf, mixed field agglutination; w, very weak agglutination.

Table 2.14 Some *ABO* alleles associated with weak A expression.

Phenotype	Allele	Nucleotide changes†	Amino acid changes†	References
A_3		None	None	[261,262]
A_3	*A301*	871G>A	Asp291Asn	[263]
A_{end} A_{finn}		IVS6+4A>G	Alternative splicing	[264]
A_{end} A_{bantu}	*Abantu01*	O^{1bantu}–A^2 hybrid	exon 4 del; Pro156Leu; 354fs+21aa	[218]
A_x	*Ax01*	646T>A	Phe216Ile	[212]
A_x	*Ax02*; B/O^2–O^{1v}	297A>G, 646T>A, 681G>A, 771C>T, 829G>A	Phe216Ile, Val277Met	[261,265]
A_x	*Ax03*; A^1–O^{1v}	646T>A, 681G>A, 771C>T, 829G>A	Phe216Ile, Val277Met	[261,265]
A_m	*Am01*	467C>T*, 761C>T	Pro156Leu*, Ala254Val	[266]
A_m	*Am02*	664G>A	Val222Met	[267]
A_{el}	*Ael01*	804insG	Phe269fs	[212,261,268]
A_{el}	*Ael02*	467C>T, 646T>A, 681G>A	Pro156Leu*, Phe216Ile	[212]
A_{el}	*Ael03*	804delG	Phe269fs	[269]
A_{el}	*Ael04*	IVS6+5G>A	No full transcript	[270]
A_{el}	*Ael05*	467C>T, 767T>C	Pro156Leu*, Ile256Thr	[271]
A_{el}	*Ael06*	425T>C, 467C>T	Met142Thr, Pro156Leu*	[272]
A_{el}	*Ael07*	A^1–O^{1v} hybrid	Pro156Leu*, Val277Met	[273]

fs, reading frameshift; aa, amino acids.
†Changes from the A^1 (*A101*) consensus sequence.
*Common in East Asian A^1 alleles (*A102*).

Table 2.15 Relative agglutinability with anti-A and A site density per red cell [223].

Phenotype	No. of subjects	Agglutinability	Antigen site density (A sites per red cell x 10^5)
A_1	4	100	10.5 (7.95–14.56)
A_2	10	96 ± 2	2.21 (1.29–3.53)
A_3	11	63 ± 10	0.35 (0.07–1.0)
A_{end}	7	10 ± 5	0.035 (0.011–0.044)
A_x	9	33 ±10	0.048 (0.014–0.10)
A_m, A_y	4	0	0.012 (0.001–0.019)
A_{el}	4	0	0.007 (0.001–0.014)

individual variation within a subgroup, but do determine a hierarchy in respect to red cell A antigen expression.

The serologically defined subgroups of A do not represent single genetic entities. In some cases A phenotype can differ according to whether the *trans* allele is *O* or *B*, either as a result of competition for substrate or allelic enhancement (Section 2.10).

2.7.1 A_3

The least rare of the weak A phenotypes is A_3. The frequency has been estimated as 1 per 1000 group A Danes [274], 9 in 150 000 French donors (0.0136% of group A) [275], and 2 in about 180 000 Canadians [276]. The main serological feature of A_3 phenotype is a characteristic 'mixed field agglutination' when red cells are incubated with anti-A and with most anti-A,B [277]. That is, small agglutinates are seen surrounded by a mass of unagglutinated, 'free' cells. On occasion A_3 serum contains anti-A_1. Group A substance is detected in the saliva of secretors.

Unlike A_{mos} described in Section 2.9, A_3 does not appear to be a mosaic of A_2 and O cells: in the A_3 phenotype anti-A can be eluted from the population of cells that was not agglutinated by it [276] and some anti-A,B agglutinate the whole population of cells [250]. Flow cytometry revealed a characteristic pattern of two main populations of cells either expressing A at a normal level for group A red cells or at a low level, similar to that of A_x cells, with a small number of cells expressing A antigen ranging between the two main populations [262]. It has been estimated that only 3–4% of the cells have sufficient sites to permit agglutination with anti-A [278].

Serum A-transferase was detected at low level in some A_3 individuals, with pH optima of either 6 or 7, typical of A_1 or A_2 enzymes, respectively [279–281]. Under optimum conditions the A_1 type of enzyme in the first category can convert O cells to A active cells, which do not display the characteristic A_3 agglutination pattern when incubated with anti-A and are agglutinated more strongly than are O cells converted with A_2 serum [279,281]. Surprisingly, in view of the high level of H antigen on the red cells, H-transferase levels in A_3 sera are generally considerably lower than H-transferase levels in A_1 or A_2 sera [281].

Sequencing of all seven exons of *ABO*, including intronic splice site sequences, plus the enhancer region, revealed no deviation from the consensus A^1 sequence in numerous individuals of Nordic origin with typical A_3 phenotype and A^1/O^1 or A^1/O^{1v} genotype [261,262]. Sequencing of exons 6 and 7 of the *A* gene from two A_3B individuals revealed an A^1 sequence with a single base change encoding Asp291Asn [263], but this was not a typical A_3 phenotype as shown by flow cytometry [262]. Eleven alleles encoding A_3 are listed in dbRBC, representing missense mutations in A^1 (*A101*), A^1 (*A102*), and A^2 alleles [215], though not all are necessarily associated with the characteristic mixed-field phenotype and so are not genuinely A_3.

2.7.2 A_{end} (A_{finnr}, A_{bantu})

A_{end} was the name given to a phenotype that resembles weak A_3 cells; A_{end} red cells give very weak 'mixed field' agglutination with some anti-A and -A,B [282]. The saliva of A_{end} secretors, however, contains H, but no A.

Anti-A_1 is present in some A_{end} sera. No GTA was detected in sera or red cell membranes of A_{end} individuals [279,280].

Two examples of A_{end} were found in testing 150 000 French donors (0.003% of group A) [275].

An A variant, which differs from A_{end} in only minor details, was found in Finns and named A_{finn} [283]. The frequency of A_{finn} in Finnish blood donors was estimated at about 1 in 6000 [283], but may be as high as 1 in 1000 in parts of southern Finland [284]. A_{finn} red cells display a characteristic pattern by flow cytometry, with most of the cells expressing no A antigen, while 2–4% expressed variable amounts of A [262]. A_{finn} individuals have an A^1 allele with a>g in the 5′ donor splice site of intron 6 [262,264]. Although skipping of exon 6 would introduce a reading frameshift and no active enzyme product, the mutation is not in the invariable splice site sequence, so a minor fraction of the RNA could be spliced normally.

A_{bantu} is another variation of A_{end}, found in about 4% of group A black South Africans [217], and in up to 8% of Bushmen and Hottentots [285]. Anti-A agglutinate A_{bantu} red cells more strongly than A_{end} cells. A_{bantu} results from a hybrid of the common A^2 allele and an O^1-like allele (O^{1bantu}), with a cross-over region near exon 5 (*Abantu01*) [218].This includes a deletion of a nucleotide in the 5′ intron 4 splice site, leading to the loss of 16 amino acids from the stem region of GTA, but does not include 261delG as exon 6 is derived from A^2.

2.7.3 A_x

The major serological characteristics of A_x phenotype [286] are:

1 the red cells are not agglutinated by most anti-A (group B) sera, yet are agglutinated by the majority of anti-A,B (group O) sera; no mixed field pattern is observed;

2 the serum usually contains anti-A_1 and occasionally an antibody that agglutinates A_1 and A_2 cells [287];

3 in addition to H substance, the saliva of A_x secretors contains a trace of A, which is best detected when A_x cells are used as indicator cells for inhibition of anti-A [288].

A_x phenotype is heterogeneous. Some other symbols (e.g. A_4, A_5, A_6, A_z, and A_o) have been used to describe subgroups of A that differ from the original A_x by only fine serological details.

Most sera from group B donors do not agglutinate A_x cells, although sera from group B volunteers immunized with A substance usually do [289]. Monoclonal anti-A reagents have been produced that are effective at detecting A_x cells, although, under certain conditions, these antibodies may also agglutinate some group B cells [290–292] (Section 2.11). Anti-A can be readily adsorbed onto and eluted from A_x cells.

In two separate studies, the frequency of A_x in France has been estimated as 1 in 77 000 (0.003% of group A) [275] and as 1 in 40 000 [281].

GTA cannot usually be detected in A_x serum or red cell membranes [212,279–281]. H-transferase activity in A_x sera is low [281].

The molecular genetics of A_x reflects the heterogeneity of the serological phenotypes. The most common A^x allele (*Ax01*) has the A^1 consensus sequence with 646T>A encoding Phe216Ile [212,261,293]. Twenty-one A^x alleles are listed in dbRBC; six of them (*Ax01–Ax06*) encode Phe216Ile [215]. Exon 7 of O^{1v} has the sequence encoding the amino acids important for A-specificity, but, like the typical A^x allele, also encodes Ile216. Consequently, hybrid genes in which exon 7 is derived from O^{1v} and exon 6 is derived from *A*, *B*, or O^2 alleles, and so lacks 261delG, produce an active GTA containing Phe216Ile, responsible for an A_x phenotype [261,265]. Three different cross-over regions in intron 6 were detected [261]. GTA containing Phe216Ile alone appears to produce more A antigen than GTA containing Phe216Ile plus Val-277Met (see Table 2.14) [262]. Several other amino acid substitutions have accounted for A_x phenotype [215] and one A_x New Zealander had an A^1-like allele encoding a nonsense mutation (Trp332Stop), which predicts the loss of 23 amino acids from the C-terminus of the GTA [261]. Of 10 alleles containing 1061delC, characteristic of A^2, plus other missense mutations, most displayed A_x-like patterns by flow cytometry [216].

The effects of allelic enhancement on A^x alleles are described in Section 2.10.2.

A very weak GTA, with higher activity at pH 8 than at pH 6 (A_2 type), was detected in the A_x mother of a baby who was A_2 at birth, but became A_x within 2 years [281,294].

2.7.4 A_m

A_m red cells are not agglutinated, or are agglutinated only very weakly, by anti-A and -A,B. Anti-A can be adsorbed onto and eluted from A_m cells. Saliva of A_m secretors, however, contains normal quantities of A and H substances. A_m serum does not usually contain anti-A_1.

A_m is inherited as a rare allele at the *ABO* locus [295–300]. The name A_m was originally coined for a new weak-A phenotype assumed to arise from homozygosity for a recessive regulator gene at a locus independent of *ABO* [301], but this phenotype is now called A_y and is discussed below.

One example of A_m was found in 150 000 French donors (0.0015% of group A) [275] and in 400 000 Chinese in Taiwan [302].

In most A_m samples the serum GTA had a pH optimum of 6 and the kinetic properties of GTA_1, whilst in serum from one A_m person the enzyme had a pH optimum of 7 and resembled GTA_2 [279,280,298,303]. In all cases enzyme activity was between 30 and 50% of that found in A_1 or A_2 sera and probably originated from tissues other than the haemopoietic tissue [304].

In a Japanese family the A_m father and child had an *A* gene that differed from A^1 (*A102*) by 761C>T, Ala254Val (*Am01*) [266], whereas A_m individuals from three generations of a family from Taiwan had an *A* gene that differed from A^1 by 664G>A, Val222Met (*Am02*) [267]. Whether these mutations severely reduce A expression on red cells, while permitting normal A expression in secretions, is not known. In the Taiwanese case GTA activity was virtually undetected in the serum and expression of cDNA encoding Val222Met (A^m) produced GTA with reduced activity compared with A^1 cDNA [267]. Furthermore, activity from the GTA expressed from A^m cDNA did not demonstrate different preferences for Type 1 and Type 2 H substrates, eliminating this as an explanation for normal A antigen expression in saliva, but not on red cells. An analogous phenotype, B_m, results from a deletion of an erythroid-specific regulator in intron 1 of *ABO* [141] (Section 2.8.3). It would be very valuable to test for similar mutations in A^m.

2.7.5 A_y

A_y phenotype is similar to A_m, but the most significant and definitive way in which A_y and A_m differ is by their mode of inheritance. A_y does not result from a rare allele at the *ABO* locus, but probably arises from a germline mutation of an *A* gene. Weiner *et al.* [301] reported two families: one A_y (then called A_m) propositus had a group O parent and A_1 and O siblings; the other was A_yB and had A_1B and B parents. Other similar families have been described since [305–307], yet none of the A_y propositi had an A_y sibling. In one family the A_y son of A^1/O and B parents had an A_yB son who, in turn, had an A_y son [308].

A_y differs from A_m phenotypically in the following ways: substantially less anti-A is eluted from A_y cells than from A_m cells incubated with the same serum; A_y secretor saliva contains considerably less A substance than A_m saliva; and A_y serum contains only a trace of GTA, whereas A_m serum contains readily detectable enzyme [279,280,303,309].

2.7.6 A_{el}

Under usual conditions A_{el} cells are not agglutinated by anti-A or -A,B, although they do bind these antibodies, as demonstrated by adsorption and elution [282,310–312]. Very low levels of A antigen were detected on A_{el} cells by flow cytometry [262] and immunogold electron microscopy [313,314]. Saliva from A_{el} secretors contains H, but no A substance. Serum from A_{el} individuals usually contains anti-A_1 and may also contain an antibody that agglutinates A_2 cells. No GTA has been detected in A_{el} serum or red cell membranes [212,279–281]. Serum H-transferase is weaker than that found in A_1 or A_2 serum [281]. No example of A_{el} was found in testing 150 000 French blood donors [275], but five were found among 400 000 Chinese from Taiwan [302]. As a result of allelic enhancement (Section 2.10.2), $A_{el}B$ cells may be weakly agglutinated by some monoclonal anti-A and may resemble B(A) phenotype (Section 2.11.1) [315].

The usual form of A^{el} (*Ael01*) has the A^1 consensus sequence except for a single G insert in a string of seven guanosines at nucleotides 798–804 [261,268]. This insert creates a reading frameshift, altering the amino acid sequence after Gly268 and abolishing the translation stop codon, so that the gene product is 37 amino acids longer than GTA_1 and 16 amino acids longer than GTA_2. A single nucleotide deletion at the same position was responsible for an A_{el} phenotype in an African (*Ael03*) [269]. The frameshift caused by a G insert at G798–804 in an otherwise normal A^2 allele was corrected by the 1059delC characteristic of A^2, but despite encoding a product of normal length this allele, O^3 or *O08*, was associated with no expression of A [145]. A Japanese individual with A_{el} red cells had an *A* (*A102*) allele encoding Phe216Ile (*Ael02*) [212]. Some other mutations associated with an A_{el} phenotype include other missense mutations in exon 7 [271,272], an intron 6 splice site mutation [270], and an A^1–O^{1v} hybrid with a recombination site within exon 7 in a blood group chimera with $A_{el}B_{el}$ phenotype [273] (Table 2.14). A mutation,1A>G, Met1Val, in the translation start codon of an A^2 allele was responsible for a phenotype referred to as $A_{el/weak}$ [216]; 2T>C, Met1Thr produced an A_3-like phenotype [316]. It must be presumed that these alleles produced weakly active, truncated GTA by initiation of translation at alternative start sites upstream of the usual initiator.

2.7.7 A_w

Many abnormal A antigens are referred to as A_w because the phenotype did not easily fit into any existing classification, often because sufficient serological testing was not

Table 2.16 Typical serological and transferase characteristics of weak B subgroups.

Name	Reactions of cells with			Anti-B in serum	Antigens in saliva of secretors	B-transferase in	
	Anti-B	Anti-A,B	Anti-H			Serum	Red cell membrane
B_3	mf	mf	+	No	B H	Yes	No
B_x	w	w	+	Yes	(B_x) H	No	No
B_m	–*/w	–/w	+	No	B H	Yes	Trace
B_{el}	–*	–	+	Sometimes	H	No	No

*Anti-B may be adsorbed onto and eluted from these cells.
(B_x), may require inhibition of agglutination of B_x cells for detection.
mf, mixed field agglutination; w, very weak agglutination.

possible. The A^w alleles commonly have single missense mutations in exon 6 or 7 of A^1 or A^2 alleles, though mutations have been found elsewhere in the gene. Many are listed in the dbRBC web site [215] or in several publications [159,216,261,262,317].

2.7.8 Do non-deletional *O* alleles produce any A antigen?

Most *O* alleles contain a single nucleotide deletion (261delG), preventing the production of any active transferase, but the allele known as O^2 or *O03*, lacks 261delG, but has two nucleotide differences from the A^1 exon 7 sequence that encode Arg176Gly (identical to that of GTB) and Gly268Arg [124,125] (Figures 2.5 and 2.6) (Section 2.3.2.2). Although initially considered an *O* allele, with Arg268 inactivating any potential GTA activity, there is now evidence supporting very low levels of GTA activity, though this is controversial (reviewed in [134]). The evidence is as follows: (1) minute quantities of anti-A adsorbed onto and eluted from red cells of individuals with O^2 [133,318]; (2) absence or reduced levels of anti-A and -A_1 in their plasma [133,318–320], which may cause typing problems on donors [319]; and (3) expression of A antigen, detected by adsorption and elution, on HeLa cells transfected with plasmids containing O^2 constructs [318]. Yazer *et al.* [320], however, were unable to adsorb and elute anti-A from O^2 red cells or to detect any A antigen on their surface by a very sensitive flow cytometry test.

An allele similar to O^2, encoding Gly268Arg and an additional Thr163Met substitution, was named *Aw08* as it also appeared to produce low levels of A antigen [318,321]. Some other rare non-deletional *O* alleles may also be responsible for production of marginal levels of A antigen [133,134].

2.8 Subgroups of B

Weak variants of B are very rare. They appear to be much rarer than weak A subgroups, although this probably reflects the relatively low frequency of the *B* gene in many populations. In Japan, for example, where the incidence of B is about half that of A, the frequencies of B_x and B_m are considerably higher than those of A_x and A_m [322].

Weak B subgroups have proved difficult to classify. Salmon [323] concluded that the best system for classifying B variants was by a loose analogy with the A variants: B_3, B_x, B_m, and B_{el}, plus B_w for those that do not fit any of the other four categories (Table 2.16), although others have suggested that the serological classification of B variants is no longer sustainable [261,262]. Some alleles encoding weak B antigens are listed in Table 2.17.

2.8.1 B_3

B_3 phenotype [323,330] is characterised by mixed field haemagglutination with anti-B and -A,B, by absence of anti-B in the serum, and by normal B antigen in the saliva (in secretors). B_3 was found in approximately one in 10 000 group B French donors [331] and in Chinese donors, one in 900 group B and one in 1800 A_1B [332].

GTB was detected in sera from B_3 individuals, but not in B_3 red cell membranes [333].

Eight B^3 alleles encoding single amino acid substitutions in GTB are listed in dbRBC [215]. One allele encoding Phe216Ile (*B302*) appears to represent a *B* allele with

Table 2.17 Some *ABO* alleles associated with weak B expression.

Phenotype	Allele	Nucleotide changes	Amino acid changes from *B* sequence	References
B_3	*B301*	1054C>T	Arg353Trp	[263]
B_3	*B302*	*B–O^{1v}–B* hybrid	Phe216Ile	[135]
B_3	*B303*	IVS3+5G>A	exon 3 del; del17–35	[324]
B_3	*B304*	247G>T	Asp83Tyr	[324]
B_3	*B305*	425T>C	Met142Thr	[325]
B_x	*Bx01*	871G>A	Asp291Asn	[212]
B_x	*Bx02*	905A>G	Asp302Gly	[326]
B_x	*Bx03*	541T>C	Trp181Arg	[326]
B_m	*B^m*	Intron 1 5.8 kb del		[141]
B_{el}	*Bel01*	641T>G	Met214Arg	[212]
B_{el}	*Bel02*	669G>T	Glu223Asp	[212]
B_{el}	*Bel03*	502C>T	Arg168Trp	[327,328]
B_{el}	*Bel04*	467C>T; 646T>A; 681G>A; 771C>T; 829G>A	Pro156Leu, Phe216Ile, Val277Met	[329]

a small region of exon 7 exchanged for that of O^{1v} [135]. A *B* gene (*B303*) containing a splice site mutation causing an in-frame skipping of exon 3 encodes a protein product lacking amino acids 17–35 [324].

2.8.2 B_x

B_x represents a heterogeneous group, but typical B_x red cells are weakly agglutinated by anti-B and -A,B [322,323]. The serum contains weak anti-B and the saliva of B_x secretors contains some B substance, which is often only detected by inhibition of agglutination of B_x cells by anti-B. GTB was not detected in serum or red cell membranes of B_x individuals [212,333].

Ten B^x alleles are listed in dbRBC, containing missense mutations in exon 7, though many more alleles labelled as B^w could possibly be classed as B^x [215].

2.8.3 B_m

B_m cells are not agglutinated by anti-B or -A,B; the B antigen is only detected by sensitive techniques such as adsorption and elution of anti-B [334,335]. The saliva of B_m secretors contains about as much B substance as that of a normal B secretor. Characteristically, sera from B_m individuals do not contain anti-B. In Japan B_m is relatively common for a B subgroup, with B_m and AB_m having a total frequency of 0.0244% [141].

Only very little GTB activity could be detected in B_m red cell membranes [336]. B_m sera demonstrated less than half of the GTB activity of B sera [105,335,337,338] and B_m saliva had normal GTB activity compared with that of B secretor saliva [105]. In families with B_m and A_1B_m members much higher levels of GTB activity were apparent in the A_1B_m sera than in the B_m sera, presumably a result of allelic enhancement [335,336] (Section 2.10.2).

A deletion of a 5.8 kb sequence in intron 1 of a *B* allele, which encompassed an erythroid-specific promoter site (Section 2.3.2.2) was present in 110 of 111 Japanese with B_m or AB_m phenotypes [141]. This deletion is predicted to ablate GTB production in haemopoietic tissue, but not in other tissues. The molecular basis for B_m in the other individual was not reported.

Homozygosity for a recessive gene that suppresses B in haemopoietic tissues has been proposed to explain abnormal inheritance of B_m-like phenotypes in a few families [339–341]. Red cells of the son of O and A_2B parents resembled B_m phenotype and should probably be called B_y, in analogy with A_y (Section 2.7.5). His serum contained normal H-transferase, but only about 70% of the normal level of GTB [341], the amount expected if all of it was non-haemopoietic in origin [304].

2.8.4 B_{el}

B_{el} red cells are not agglutinated by anti-B or -A,B [342,343]. They do bind anti-B, which can be detected in eluates. B is not present in the saliva of B_{el} secretors; anti-B may be present in the serum.

No GTB was detected in B_{el} sera or red cell membranes [212,333,343]. In a family with B_{el} and A_1B_{el} members, the A_1B_{el} red cells were weakly agglutinated by some anti-B

[343]. In another family, B_{el} was enhanced to B_3 in an *A/B* heterozygote [344] (see Section 2.10.2).

Eight B^{el} alleles are listed in dbRBC, five of them containing single missense mutations in exon 7, though many more alleles labelled as B^w could possibly be classed as B^{el} [215]. One B^{el} allele (*Bel04*) encodes three amino acid differences from normal GTB (Table 2.17), and probably represents multiple interallelic exchanges between *B* and O^{1v} [329]. Met214Arg encoded by *Bel01* is adjacent to the ^{211}Asp–Val–Asp^{213} motif that captures Mn^{2+} and is essential for enzyme activity [158]. (All transferases of this type contain an Asp–X–Asp motif.) Recombinant mutant GTB containing Met214Arg had a 1200-fold decrease in k_{cat} (the catalytic rate of an enzyme) compared with the normal GTB [345].

2.8.5 Other subgroups of B

Numerous other *ABO* alleles responsible for variant B phenotypes have been described, mostly as B^w [159, 215,261,317,346]. A structural analysis, based on the crystal structure of GTB, demonstrated that the mutations in variant *B* alleles are likely to disrupt molecular bonds important for enzyme function [346].

Four cases of B_w phenotype appeared to result from aberrant CBF/NF-Y motif sequences in the regulatory region upstream of exon 1 (Section 2.3.2.2) [148]. Two had *B* alleles with normal coding sequences, but two alleles had reduced numbers of 43-bp repeat units and in a third a novel CBF/NF-Y motif was present. The fourth was AB_w and whereas the *B* allele appeared normal, the *A* allele had an increased number of the enhancer elements, suggesting that enhanced GTA production resulted in weakened B antigen as a result of competition between GTA and GTB for acceptor substrate. A hybrid gene (*Bw26*) with exons 1–3 from O^2 and exons 4–7 from *B* differed from normal *B* by encoding Arg18Leu and having only one CBF/NF-Y repeat, compared with the four usually present in *B* [149]. Transcript levels from this gene were, however, about normal for a *B* allele, and Thuresson *et al.* [149] considered that the weak B expression resulted from the Leu18Arg substitution rather than the number of enhancer elements.

Immunofluorescence microscopy showed that GTB derived from a *B* allele is located almost entirely in the Golgi apparatus, whereas GTB from a B^w allele (*Bw21*) encoding Gly230Arg was evenly distributed throughout the cytoplasm [347]. This suggests that structural changes in ABO glycosyltransferases arising from amino acid substitutions may cause defective trafficking of the enzyme to the Golgi.

An inherited variant B antigen called B_v was characterised by the failure of B_v red cells to react with human anti-B reagents that had been adsorbed with rabbit red cells [348,349]. B_v red cells and secretions appear to lack normal human B antigen, but contain a B-like determinant, possibly the non-fucosylated B-like antigen on rabbit red cells (the 'Galili antigen' [350]). Sera of B_v individuals contain a form of anti-B; no GTB activity could be detected. Among 567 210 Hong Kong Chinese blood donors, 46 examples of B_v and eight examples of AB_v were found [349]. Of 18 Hong Kong Chinese with B_v red cells, 17 had a *B* allele containing 695T>C, Leu232Pro (*Bw11* [346]) and one had a *B* allele containing 721C>T, Arg241Trp (*Bw03* [261]); no mutation was detected in the coding region of the *B* gene of an AB_v individual [351].

2.9 A_{mos} and B_{mos}

In 1975 Marsh *et al.* [352] applied the names A_{mos} and B_{mos} to remarkably similar variants of A and B. In A_{mos}, agglutination tests with anti-A and -A,B revealed two separable populations of cells, one A_2, the other O. A_{mos} sera contained no anti-A, and the saliva contained H and possibly a trace of A. In addition to two A_{mos} families, B_{mos}, A_1B_{mos}, and $A_{mos}B$ phenotypes were described [352]. The inherited A+O and B+O mosaics previously reported in Japan probably represent earlier examples of A_{mos} and B_{mos} [353–355].

A_{mos} is inherited, apparently at the *ABO* locus; a characteristic that distinguishes it from most other forms of red cell mosaicism. All A_{mos} members within a family have about the same proportion of A and O cells, although these proportions vary substantially between different families. A_{mos} differs from A_3 serologically as the cells left unagglutinated with anti-A do not adsorb anti-A. The level of serum GTB activity in the B_{mos} members of a family was only about 7–20% of that of normal B controls [356].

2.10 *A* and *B* gene interaction

2.10.1 Allelic competition

It is well established that A antigen is weaker on A_2B cells than on A_2 cells and that A_1 is weaker on A_1B than A_1 cells; the effect of two different glycosyltransferases competing for the same acceptor substrate. Although generally less obvious, B is often weaker in A_1B than in B [357–360].

In some cases A^1/B genotype may be expressed as an A_2B phenotype [233,344,359,361–363] and A^2/B may be expressed as A_3B or A_wB phenotype [219,361,364]. In black populations the A_2B:A_1B ratio is often significantly higher than would be expected from the A_2:A_1 ratio [344,362]. In a study of 5000 African Americans, 80% of group A individuals were A_1 and 20% A_2, whereas 53% of the group AB individuals were A_1B and 47% were A_2B [362]. Similar discrepancies have been observed in white people [233,344], Chinese [332], and Japanese [213,363]. This imbalance in Japanese is due, at least in part, to an *ABO* allele (*A204*: Arg176Gly, Gly235Ser, Val277Met) that is expressed as A_1 in A^1/O genotype, but as A_2 in A^2/B genotype [213].

Sera from some A_2B black people contain GTA_1 and no GTA_2, plus GTB with activity considerably higher than that found in most group B sera [344,365]. Elevated GTB activity together with a GTA_1 was found in the sera of 50% of the A_2B African Americans [362]. This superactive GTB utilises the lion's share of available H sites, so that the GTA_1 cannot produce sufficient A antigen to provide the high site density required for A_1 status.

2.10.2 Allelic enhancement

'Le renforcement allélique' is a gene interaction, the reverse of allelic competition described in the previous section [288,366]. It is an enhancement of expression of weak *A* or *B* alleles in *A/B* heterozygotes. For example, A_x may be inherited from an A_2B parent [288,366–368] because the presence of a *B* allele enhances the expression of A^x to that expected of A^2. The effect of allelic enhancement is clearly visualised by flow cytometry [262]. In one family the A_x cells had 11 200 A sites per red cell, whereas the A_2B cells, with the same *A* allele, had 96 000 A sites [366]. Weak GTA activity was detected in the sera of people with an A_2B phenotype (genotype A^x/B) resulting from allelic enhancement; no enzyme was found in the serum of their A_x siblings who have the same *A* gene [366]. In a Taiwanese family, an A^x (*Ax11*) allele was expressed as A_x in an A^x/O^1 individual, but as A_3B in her A^x/B father [369]. Other families have shown that a *B* gene responsible for B_x phenotype was expressed more strongly in A_1B members [370,371]. In addition to *A* and *B*, the non-deletional O^2 allele has also been responsible for allelic enhancement. A^x alleles (*Ax03* and *Ax04*) behaved as *O* when paired with O^1 or O^{1v}, but as A^x when paired with *B* or O^2 [262,372].

The molecular mechanism for allelic enhancement is not known. One possible explanation is that ABO glycosyltransferases can form dimers [126] and heterodimer formation between a defective enzyme and a different full-length protein – an *A*, *B*, or O^2 product – can lead to functional rescue of the defective transferase [262,372].

2.11 Overlapping specificities of A- and B-transferases (GTA and GTB)

The glycosyltransferase products of the *A* and *B* alleles differ in their donor substrate specificity, although they share a common acceptor substrate. GTA and GTB, however, are not precise in their choice of donor substrate and there is a small degree of overlap in their specificity [373–376].

Under the appropriate conditions, enzyme from group B serum can catalyse the transfer of GalNAc from UDP-GalNAc to 2′-fucosyllactose (a low molecular weight analogue of H) to form an A-active structure. Concentrated GTB could even make group O cells strongly agglutinable with anti-A. If equivalent quantities of UDP-Gal and UDP-GalNAc were present only B activity could be detected; in the presence of UDP-Gal, thrice the quantity of UDP-GalNAc was required to produce A activity. As would be expected, when there is competition for substrate, GTB is far more efficient at catalysing the transfer of Gal than of GalNAc [373,375,376]. Likewise, GTA can, under appropriate conditions, catalyse the synthesis of B-active structures [108,374].

2.11.1 B(A) and A(B)

The observation in several laboratories that highly potent monoclonal anti-A reagents capable of agglutinating A_x red cells also weakly agglutinated some group B cells led to the recognition that the phenomenon described above, the ability of GTB to produce A determinants *in vitro*, may also be observed *in vivo*. Red cells from 25 of 3458 group B donors were reactive with one example of monoclonal anti-A [377]. The reaction of these B(A) cells with some anti-A could be inhibited by group A secretor saliva [377] and the A-activity removed by treatment of the cells with α-*N*-acetylgalactosaminidase, but not α-galactosidase [378]. People with B(A) red cells were mostly black and were shown to have highly active serum GTB, in some cases five to six times more active than that from most other group B individuals [377]. In B(A), the hyperactive α1,3-galactosyltransferase catalyses the transfer of sufficient GalNAc to its acceptor substrate to permit agglutination by certain anti-A.

Molecular genetic analyses have revealed at least six alleles responsible for B(A) phenotype, mostly in China

Table 2.18 Enzymes with dual A- and B-transferase activity: amino acid substitutions at the four positions (176, 235, 266, 268) characteristic of A and B and at positions 214 and 234.

Phenotype*	Alleles	Amino acids						Shorthand	References
		176	214	234	**235**	**266**	**268**		
A_1	*A101* or *A102*	Arg	Met	Pro	Gly	Leu	Gly	AAAA	
B	*B*	Gly	Met	Pro	Ser	Met	Ala	BBBB	
B(A)	*B(A)01 & B(A)03*	Gly	Met	Pro	**Gly**	Met	Ala	BABB	[269,293]
B(A)	*B(A)02*	Gly	Met	**Ala**	Ser	Met	Ala	BBBB	[379]
B(A)	*B(A)04*	Gly	**Val**	Pro	Ser	Met	Ala	BBBB	[380]
B(A)	*B(A)05*	Gly	**Thr**	Pro	Ser	Met	Ala	BBBB	[380]
cisAB A_2B_3	*cis-AB01*	Arg	Met	Pro	Gly	Leu	**Ala**	AAAB	[381]
cisAB A_2B_w	*cis-AB02*	Gly	Met	Pro	Ser	**Leu**	Ala	BBAB	[382]
cisAB A_2B	*cis-AB03*	Gly	Met	**Ser**	Ser	Met	Ala	BBBB	[383]
cisAB A_2B	*cis-AB04*	Arg	Met	Pro	Gly	**Met**	Gly	AABA	[384]
cisAB AB or B(A)	*cis-AB05* or *B(A)06*	Gly	Met	Pro	Ser	Met	**Gly**	BBBA	[215,385,386]
cisAB A_2B		Gly	Met	Pro	Ser	**Val**	Ala	BB^B	[387]

*Typical phenotype when paired with an *O* allele.
B(A)01 & B(A)03 differ only by synonymous changes.

and Japan (Table 2.18), a situation not concordant with the theory of a hyperactive GTB producing some A antigen. Three are *B* alleles apart from encoding an amino acid substitution at one of the four positions that differentiate GTA and GTB: *B(A)01* and *B(A)03* giving the BABB sequence and *B(A)06* (which is also named *cis-AB05*) BBBA [269,293,385,386]. The other three alleles encode substitutions at other positions, with *B(A)04* (Val214) and *B(A)05* (Thr214) encoding different amino acids substituting Met214, which is adjacent to the Asp–Val–Asp motif [345,379,380,385] (see Section 2.8.4). With all of these *B(A)* alleles, B(A) phenotype is more likely to have resulted from a shift in substrate specificity of the enzyme rather than greatly enhanced GTB activity.

One monoclonal anti-B brought about agglutination of 1.42% of group A red cell samples, all A_1, and this was considered to represent A(B) phenotype; B activity resulting from GTA_1 activity [388]. A(B) was not associated with elevated GTA activity, but A_1(B) cells did have elevated levels of H antigen and plasma H-transferase activity.

2.11.2 cisAB

In 1964, Seyfried *et al.* [389] described a Polish family in which inheritance of the ABO groups did not fit the single locus concept for ABO genetics. A and B appeared to have been inherited together in this family: an A_2B woman with a group O husband and a group O mother had two A_2B children. Numerous other similar families have been encountered since. Yamaguchi *et al.* [390] proposed the term cisAB for this phenotype. Fourteen cisAB samples were found from over a million Japanese blood donors, 0.012% of the 112710 group AB bloods tested [391].

2.11.2.1 Serological characteristics

Although the main feature of cisAB is its unusual mode of inheritance, the cisAB phenotype almost always differs from 'transAB' serologically. Serological characteristics differ from family to family, but remain consistent within a family [392,393]. Whereas B(A) red cells have normal B antigen expression and only trace levels of A, cisAB cells are readily agglutinable by both anti-A and -B.

1 The A antigen of cisAB is usually referred to as A_2, but cisAB cells generally express more A than A_2B and less than A_1B [392,394–396]. The genotype *cisAB/B* may be expressed as A_xB or A_3B [380,387]. The phenotype cisA_1B is not unknown [397,398].

2 B is almost always expressed weakly, often being described as B_3 [391]. The B antigen of cisAB may not

always be atypical; an A_2B mother of two group O children had an apparently normal B antigen [399].

3 An unusually high level of H, about the level found on A_2 cells, which is higher than that of normal A_2B cells [392].

4 Sera from cisAB people almost always contain weak anti-B. This antibody apparently recognises part of the B antigen lacking from cisAB cells [397,400]. Serum from the *cisAB/O* woman with normal B antigen did not contain anti-B [399].

5 Salivas from cisAB secretors contain normal quantities of A and large quantities of H, plus a very little B that is only detectable by inhibition of agglutination of cisAB cells by anti-B [392,393].

2.11.2.2 Molecular genetics and transferase activities

Although there have been suggestions that the cisAB phenomenon could result from the presence of both *A* and *B* alleles on the same chromosome, producing separate transferases [401], it is now clear that the usual cause is a mutated *A* or *B* gene producing an enzyme capable of transferring significant quantities of both GalNAc and Gal to the H acceptor substrate.

All of the *cisAB* genes listed in Table 2.18 represent *A* or *B* alleles containing single nucleotide changes encoding single amino acid substitutions, mostly at positions 266 or 268, the two most important residues for distinguishing between GTA and GTB activity. Two alleles (*cis-AB01* AAAB and *cis-AB04* AABA) are basically *A* alleles encoding the *B* amino acid at one of those positions and two (*cis-AB05* BBBA and *cis-AB02* BBBA) are *B* alleles encoding the *A* amino acid at one of those positions. The *cis-AB05* allele has also been named *B(A)06* [215], demonstrating a blurring of the distinctions between cisAB and B(A) phenotypes, though from their brief descriptions [385,386] cisAB appears most appropriate. Another *cisAB* allele is a *B* allele encoding a Met266Val [387]. One allele (*cis-AB03*) encodes Pro234Ser, which is not in any of the four discriminating positions [383,402]. The specificity reversal has been explained by the breakdown of van der Waals contacts that occur between Pro234 and Met266 of GTB, enabling the aliphatic tail of Met266 to adopt a configuration that opens the enzyme pocket to accommodate the larger GalNAc [403]. *B(A)02* encodes GTB with Ala234 [379].

The phenotype cisAB is most commonly found in East Asia and the most common allele is *cis-AB01* [403]. GTA and GTB activities of *cis-AB01*-transferase are 29 and 27% of normal GTA and GTB activities, respectively [403]. The alleles *cis-AB02*, *cis-AB04*, *cis-AB05* were also found in East Asians; *cis-AB03* and *cis-AB05* have been found in Caucasians.

2.12 H-deficient phenotypes

The H-deficient phenotypes are those rare phenotypes in which the red cells are totally or partially deficient in H antigen. H may or may not be present in secretions; that is, individuals with H-deficient red cell phenotypes may be ABH secretors or non-secretors. The various H-deficient phenotypes are summarised in Table 2.19.

2.12.1 Genetics of red cell H-deficient phenotypes

In 1955, Watkins and Morgan [404] suggested that H expression may be controlled by a gene at a locus independent from *ABO*, and that the Bombay phenotype could arise from homozygosity for a rare allele, *h*, at that locus. As described in Section 2.3.1, *FUT1* controls α1,2-fucosyltransferase activity in haemopoietic tissue and, consequently, H antigen expression on red cells, whereas *FUT2* controls α1,2-fucosyltransferase activity in secretory tissue. Red cell H-deficient phenotypes, whether in secretors or non-secretors, result from homozygosity or compound heterozygosity for mutations in *FUT1* that totally or partially inactivate the H-transferase in red cell progenitors. The ISBT database [405] lists 44 *FUT1* alleles. Two of them reflect an Ala/Val12 polymorphism, which is in the transmembrane domain and probably has no effect on H-transferase activity. Twenty-three of the alleles are responsible for weakened H-transferase activity; 21 of these encode amino acid substitutions, but one encodes a single nucleotide deletion (990delG) responsible for a stop signal at codon 336, near the C-terminus [406] and another a triplet insertion. The remaining 19 alleles produce inactive enzymes: 10 of these encoding amino acid substitutions and nine encoding early termination of translation either through nonsense mutation (7) or dinucleotide deletion (2). The dbRBC [215] lists 50 *FUT1* alleles. Some key references are [92,406–412].

Expression of the mutant alleles by transfection of cultured cells has shown that some alleles give rise to no α1,2-fucosyltransferase activity and some produce low levels of enzyme activity [92,93,406,407,409]. This explains the different levels of H expression found in H-deficient phenotypes.

Red cells of a Swiss non-secretor reacted with one anti-H, despite being a compound heterozygote for two *FUT1*

Table 2.19 H-deficient phenotypes.

		Antigens							Glycosyltransferases					
		Red cells*			Secretions				Serum			Red cells		
Type	Notation	A	B	H	A	B	H	Antibodies	A	B	H	A	B	H
H-deficient, non-secretor (Bombay)	O_h	–	–	–	–	–	–	anti-H	–	–	–	–	–	–
	O_h^A	–	–	–	–	–	–	anti-H	+	–	–	+	–	–
	O_h^B	–	–	–	–	–	–	anti-H	–	+	–	–	+	–
H-partially deficient, non-secretor	O_h †	–	–	–/w	–	–	–	anti-H	–	–	–/+	–	–	–
	A_h	+/w	–	–/w	–	–	–	anti-H	+	–	–/+	+	–	–
	B_h	–	+/w	–/w	–	–	–	anti-H	–	+	–/+	–	+	–
H-deficient, secretor (para-Bombay)	O_h^O-secretor	–	–	–/w	–	–	+	anti-HI	–	–	–/+	–	–	–
	O_h^A-secretor	+/w	–	–/w	+	–	+	anti-HI	+	–	–/+	+	–	–
	O_h^B-secretor	–	+/w	–/w	–	+	+	anti-HI	–	+	–/+	–	+	–
H_m (dominant)	OH_m	–	–	w	–	–	+	none	–	–	+	–	–	+
	AH_m	w	–	w	+	–	+	none	+	–	+	+	–	+
LADII		–	–	–	–	–	–	none	–	+	+	nt	nt	nt

*Tested by direct agglutination.
†only distinguished from 'atypical' O_h-non-secretor by family studies.
w, weak expression of antigen; nt, not tested.

alleles encoding premature stop codons (421A>G, Trp-140Stop and 826C>T, Gln276Stop) [411]. A possible explanation is that recombination between the two alleles could result in *FUT1* containing neither stop codon, a speculation previously put forward to explain weak A expression in an individual with compound heterozygosity for two different *O* alleles [173]

The structural loci *FUT1* and *FUT2* are very closely linked. In most cases, *FUT1* mutant alleles are associated with the same *FUT2* allele, even in unrelated individuals [93,406]. In six red cell H-deficient Japanese, nine 695G>A *FUT1* alleles were linked to *FUT2* Se^{w385}, whereas one 695G>A *FUT1* allele was linked to *FUT2 Se*; the other two *FUT1* alleles contained 721T>C [406].

Two families are described in which recombination between *FUT1* and *FUT2* may have occurred. One family contains red cell H-deficient, secretor and non-secretor members [413]. In the other family it can be inferred that a father has passed inactive *FUT1* and *FUT2* alleles to his five Bombay phenotype children, active *FUT1* and *FUT2* alleles to his four group B, secretor children, and active *FUT1* but inactive *FUT2* to his group B, non-secretor daughter [78,414].

2.12.2 Red cell H-deficient, non-secretor; the Bombay phenotype

In 1952, Bhende *et al.* [415] described the abnormal blood groups of three men from Mumbai whose red cells were group O, but H negative. All had anti-H in their serum. This rare phenotype later became known as the Bombay or O_h phenotype. Many other examples have been found since through the presence of anti-H in the serum.

2.12.2.1 Serological characteristics and genetics

O_h red cells are not agglutinated by anti-H, -A, -B, or -A,B. No H, A, or B antigens can be detected, by adsorption and elution techniques, on red cells with the 'typical O_h' phenotype [416]. Red cells of phenotypes that have been called 'atypical O_h', however, do bind anti-H, which can be detected in an eluate [417,418]. It may also be possible to adsorb and elute anti-A and/or anti-B from these cells [417,419,420].

As with most ABH non-secretors, O_h red cells are usually Le(a+b–), but may be Le(a–b–). O_h red cells never express Le^b.

No H, A, or B antigens are present in O_h saliva, which may contain Le^a, but never Le^b.

The serum of O_h individuals always contains anti-H, -A, and -B.

When describing Bombay phenotypes the appropriate superscript may be added to the O_h notation when the *ABO* genotype is determined by family study, by glycosyltransferase analysis, or by molecular genetical tests: O_h^O, O_h^A, O_h^B, O_h^{AB}.

The typical Bombay phenotype in people originating from India results from homozygosity for *FUT1* 725T>G, Leu242Arg, together with homozygosity for a deletion of *FUT2* [92,93].

Heterozygosity for an inactive *FUT1* allele may result in reduced H expression [421] (see Section 2.12.5.1).

Unlike Rh_{null} cells, there is no evidence to suggest that Bombay phenotype cells are haematologically abnormal. Autologous ^{51}Cr-labelled O_h red cells survive normally [422,423].

2.12.2.2 Glycosyltransferases

H-transferase has not been detected in the serum or red cell membranes of O_h individuals [101,184,424]. O_h sera and red cells contain GTA and GTB when *A* and *B* genes are present [101,205]. These enzymes are unable to act in the absence of their acceptor substrate (H antigen) and neither A nor B structure is produced. O_h red cells that have been made H-active *in vitro*, in the presence of H-transferase, can be converted to A- or B-active cells by GTA or GTB [425]. In families, sera from heterozygous *H/h* members have about half the H-transferase activity of serum from *H/H* homozygotes [426].

2.12.2.3 Frequency and distribution

The Bombay phenotype is very rare, but appears to be less rare in India than elsewhere with an O_h frequency of about 1 in 7600 Indians in Bombay, an *h* gene frequency of 0.0115 [427]. A rich source of two types of H-deficiency phenotype exists in Réunion Island in the Indian Ocean: typical O_h in the Tamil Indian population and partial red cell H-deficiency, non-secretor in the population of European origin [428]. O_h has also been found in other ethnic groups, including people of European origin [407,411,419,420], where the 'atypical Oh' phenotype may predominate, African Americans [429], and in Japanese, where homozygosity for Se^{w385} suggests that they are actually weak secretors [406].

2.12.3 Red cell H-partially deficient, non-secretor

Levine *et al.* [430] used the notation A_h to describe a phenotype in a non-secretor Czech woman whose red cells lacked H, but were weakly agglutinated by anti-A. The equivalent B phenotype, B_h, was also found in a Czech [431]. AB_h has also been described [428,432]. A_h, B_h, and AB_h have mainly been reported in people of European origin [428,430–434]. The term para-Bombay has been used for these red cell H-partially deficient, non-secretor phenotypes, but is better reserved for H-deficient and -partially deficient secretors.

2.12.3.1 Serological characteristics and genetics

The strength of A expression on red cells of some A_h individuals resembles weak A_2 [430,433], whereas those of others are more like A_x, being agglutinated by only a minority of anti-A sera [434–436]. Likewise, B_h red cells have weak B antigen [431,437]. Little or no H antigen is detected on these cells. No H, A, or B antigen is present in the saliva and, like red cells of most non-secretors, A_h and B_h cells are usually Le(a+b–), but may be Le(a–b–) [426,432]. The serum contains anti-H. A_h serum contains anti-B, but no anti-A, although anti-A_1 is usually present [430,435,436]; in B_h, anti-A is always present and anti-B may also be detected [431,437].

Many examples of A_h, B_h, and AB_h, as well as O_h, have been identified in the people of French origin on the small island of Réunion, off the East Coast of Africa [428,432]. O_h in this population arises from the same *FUT1* genotype as the A_h, B_h, and AB_h phenotypes, because they are present in the same families and have the same *FUT1* mutation, 349C>T encoding His117Tyr in the stem region of the enzyme (together with the European non-secretor allele, se^{428}, in *FUT2*) [93]. This 'Réunion O_h phenotype' can be distinguished from Bombay phenotype by the quantity of H on the cells [428]. Purified *Ulex europaeus* lectin agglutinated papain-treated Réunion phenotype cells, but not Bombay phenotype cells, and high-titred H antibodies found in the sera of Bombay phenotype Indians agglutinated Réunion phenotype cells. These same sera agglutinated red cells from some O_h Europeans [428]. The term O_h is ambiguous. It can represent homozygosity for an *h* allele that produces no active α1,2-fucosyltransferase (Bombay phenotype), or homozygosity for an *h* allele that produces weakly active α1,2-fucosyltransferase in people with no *A* or *B* gene and, therefore, no weak expression of A or B antigen. There is a series of weak *FUT1* alleles resulting in different degrees of red cell H deficiency.

2.12.3.2 Glycosyltransferases

Trace quantities of H-transferase activity were detected in sera from red cell H-partially deficient, non-secretor

individuals from Réunion Island [426], although no H-transferase activity could be detected in sera or red cell membranes from A_h or B_h individuals [101,184,424]. As with O_h^A and O_h^B, A_h and B_h sera contain *A* and *B* gene-specified glycosyltransferases, respectively [101,424].

Red cell H-partially deficient phenotypes arise from homozygosity for a mutant gene at the *FUT1* locus, which produces only a very weakly active H-transferase. Consequently, the small amount of H produced is completely converted to A or B. Mulet *et al*. [438] demonstrated that H is the precursor of B on the cells of a B_h individual. B_h (B+ H–) red cells treated with α-galactosidase extract of *Trichomonas foetus* lost their B antigen and became H-active. These B– H+ cells could then be converted to A_h (A+ H–) by A-transferase. If the α-galactosidase-treated B_h red cells (B– H+) were treated with H-degrading α-fucosidase from *T. foetus*, they could no longer be converted to A activity.

2.12.4 Red cell H-deficient, secretor

Red cells of people with another type of H-deficiency have little or no H, A, and B red cell antigens, yet they are ABH secretors, with secretions containing normal quantities of H, A, and B. In the first family showing that people lacking H from their red cells could secrete H, two brothers, whose red cells lacked H and bound anti-A, but were not agglutinated by it, secreted A and H; a third brother, with group O, H-negative red cells, secreted H alone [439]. A secretor of B and H with H-deficient red cells was subsequently found [440]. The terms O_h-secretor, A_h-secretor, and B_h-secretor are recommended to describe the phenotypes (Table 2.19).

2.12.4.1 Serological characteristics and genetics

Red cells of O_h-secretors are not agglutinated by most H antibodies, but they may be agglutinated weakly by the potent anti-H in some O_h sera and by other strong anti-H reagents [441]. Adsorption and elution of anti-H may or may not reveal H antigen on red cells of O_h-secretors [80,440].

O_h-secretor red cells are not usually agglutinated by anti-A or -B, but some O_h^A-secretor cells behave like A_x cells and are agglutinated by anti-A,B and very potent anti-A [439,442]. Sometimes the A antigen can only be detected by adsorption and elution of anti-A. A similar variation exists with B antigen strength in O_h^B-secretors [80,440,442,443].

Like those of most secretors, O_h-secretor red cells are usually Le(a–b+), but may be Le(a–b–). The Le(a+b+) phenotype, common in the Far East, was not found in 25 Taiwanese O_h-secretors [408], but was found in two of 51 Hong Kong Chinese O_h-secretors, about half the normal incidence [441].

H substance is present in saliva, in approximately normal quantities for an O secretor [80,439–444]. A and B substances are detected in normal quantities in the secretions when *A* and *B* genes are present.

The serum almost always contains an H-like antibody, which is generally weak and reacts only at low temperature. This antibody, called anti-HI, is not inhibited by secretor saliva and does not react with group O cord cells. Two thirds of O_h-secretors from Hong Kong had anti-HI or anti-H active at 37°C [441].

Numerous *FUT1* mutations have been found to be responsible for red cell H-deficiency in secretors [215, 405], most of them from East Asia. Probably the two most common in China contain dinucleotide deletions, 547delAG and 880delTT [408,412], whereas in Japan the two most common alleles appear to be 990delG and 721T>C, Tyr242His [406].

2.12.4.2 Glycosyltransferases

Originally no H-transferase was detected in O_h-secretor sera or red cell membranes, although the appropriate A- and B-transferases were present [424,445]. In four O_h-secretor sera H-transferase activity representing about 5–10% of that found in sera of people with normal H phenotypes indicated that this enzyme derived from secretory tissues [80].

2.12.4.3 Frequency and distribution

Most H-deficient secretors have been found in Eastern Asia – Chinese [408,412,441,446] and Japanese [406, 409,440] – but also very rarely in other ethnic groups including people of European origin [80,411,443,447], from the Middle East [80,411], and a Native American [448]. Some estimated frequencies: one in 5000 Thais [442], one in 8000 Taiwanese [449], and one in 15 620 Hong Kong Chinese [441]. Among 324 Lahu Chinese, a nomadic ethnic minority in China, seven (2.2%) were H-deficient secretors. All were either homozygous or heterozygous for one or both of two *FUT1* alleles: 328G>A, Ala110Thr and 658C>T, Arg220Cys [450].

2.12.5 Other H-deficient phenotypes

2.12.5.1 H_m

The primary characteristic of the H_m phenotype (Table 2.19) is its dominant mode of inheritance, the rare phenotype appearing in several generations of the same family [424,447,451,452]. H_m red cells are weakly

agglutinated by anti-H, but the H deficiency is not as dramatic as in Bombay or para-Bombay phenotypes. The saliva contains normal quantities of H and H-transferase is present in serum and red cell membranes. AH_m cells show depression of the A antigen. In one family the proposita was A_2, but had very little H on his cells; the GTA was of the A_1 type, but presumably insufficient H was available for A_1 antigen expression [424,445].

Two individuals with slightly weakened A or B antigens had H– red cells [421], a phenotype resembling H_m. Each had one *FUT1* allele with the consensus sequence and one inactive allele with either 684G>A (Met228Ile) or 694T>C (Trp232Pro). It is possible that the H_m phenotype simply results from heterozygosity for an inactive *FUT1* allele.

2.12.5.2 Leucocyte adhesion deficiency type II (LADII)

LADII (also known as congenital disorder of glycosylation type IIc, CDGIIc) is a generalised fucosylation defect associated with recurrent infections, short stature, mental retardation, and a distinctive facial appearance, but also with H-deficient (Bombay phenotype) red cells, ABH non-secretion, and Le(a–b–) red cell phenotype (reviewed in [453]). Transferase assays on one patient revealed normal levels of H- and Le-transferase activity in his serum and saliva, respectively [454]. The leucocyte adhesion defect results from a deficiency of sialyl-Le^x, a fucosylated ligand for selectins (Section 2.18.3). Treatment with oral L-fucose reverses most of the symptoms of LADII in some patients, although it has no effect on the red cell Bombay phenotype [455].

LADII results from homozygosity for mutations in the gene (*SLC35C1*) that encodes the GDP-fucose transporter, responsible for transfer of GDP-fucose, the donor substrate for various fucosyltransferases, from the cytosol to the lumen of Golgi apparatus where *N*-glycans are fucosylated. Six patients are reported: four Arab children [456] with *SLC35C1* 923C>G, Thr308Arg [457]; a boy of Turkish origin [458] with 439C>T, Arg147Cys [457,459]; a girl of Pakistani origin with 969G>A, Leu322stop [460]; and a patient of Brazilian origin with 588delG introducing premature termination of translation [461].

2.12.6 I and i expression in H-deficient phenotypes

The I and i antigen structures represent carbohydrate chains that are precursors of H, A, B, Le^a, and Le^b, so it is not surprising that I and i expression is elevated in H-deficient red cells (see Chapter 25). This effect has been demonstrated on O_h cells and on red cells of some H-deficient, secretors by agglutination titrations with anti-I and -i. Measurement of percentage agglutination by an electronic cell counter demonstrated significantly higher agglutination by anti-I with O_h, A_h, and B_h cells from non-secretors (90.2%), compared with control cells (73.5%) [462].

2.13 Acquired alterations of A, B, and H antigens on red cells

Since the ABO blood groups were shown to be inherited characters, numerous rare variants have been recognised, many of which have been described in this chapter. Most of these variants are inherited, resulting from mutant genes at the *ABO*, *FUT1*, and occasionally other loci. Some ABO anomalies, however, are acquired, generally as a result of infection or malignancy, or the effect of laboratory intervention.

2.13.1 Acquired B

Over a period of four years, Cameron *et al.* [463] identified seven patients with some kind of red cell B antigen, but with apparently normal anti-B in their sera. The anti-B did not react with the patients' own red cells. Cameron *et al.* [463] gradually came to appreciate that this B-like antigen was an acquired character, probably associated with disease. All seven patients were A_1, the secretors secreted A and H, but no B, and four had group O children and therefore had an A^1/O genotype.

Most individuals with acquired B are ill, although examples of acquired B in healthy subjects are recorded [464–466]. An estimated 64% of reported cases had diseases of the digestive tract, most of those being carcinoma of the colon [467]. In a survey of 200 patients (106 group O, 94 group A) with gastrointestinal disease, 10 cases of acquired B were found, all in group A patients [468].

Sera from patients with acquired B antigen contain GTA, but no GTB [469]. No *B* allele was present in the genome of patients with acquired B [261,470,471].

2.13.1.1 Serological characteristics

Acquired B is only found on group A cells. These are nearly always A_1, although A expression may be depressed [467]. A few examples of A_2 with acquired B have been found: in one case the cells became A_1 as B expression diminished [472]; in another the patient had serum GTA_2 [473]; and in two cases the patients had A^2/O genotypes [261]. One example of acquired B was associated with weak expression of A and H antigens [474].

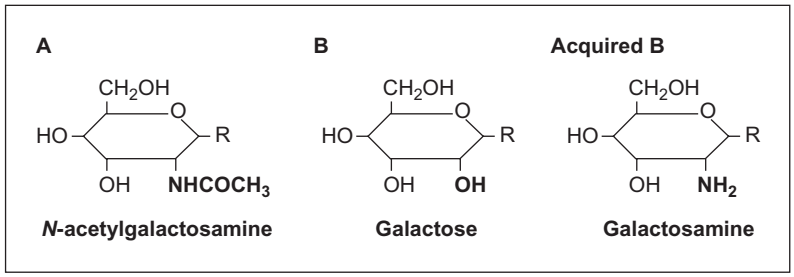


Figure 2.9 Terminal immunodominant sugars for A, B, and acquired B, demonstrating the similarity between Gal and galactosamine (deacetylated GalNAc). R, remainder of molecule.

Acquired B antigen is usually weak, but varies in different individuals and with time. Often a proportion of cells remain unagglutinated with anti-B. Sera from A_2 donors are better at detecting acquired B than are sera from A_1 donors [469]. Some group A and O sera contain a specific anti-acquired B, which does not react with normal B cells and can be separated from anti-B by adsorption and elution [465,475]. Anti-acquired B was produced by immunising a rabbit with acquired B red cells [465].

Some monoclonal anti-B react with acquired B cells [475–478] and monoclonal anti-acquired B has been produced by immunising mice with acquired B red cells [479,480]. A blood-grouping reagent containing a monoclonal anti-B clone (ES4) that strongly agglutinates acquired B cells, greatly increased the rate of detection of this phenotype [478]. A group A patient with acquired B was grouped as AB with reagents containing ES4 and suffered a fatal haemolytic reaction following transfusion with four group AB units [481]. The patient's weak anti-B was not detected by abbreviated compatibility testing. ES4 is no longer used as an anti-B reagent.

Serum from acquired B individuals contains anti-B, which does not react with acquired B cells. Saliva of acquired B secretors contains A and H, but no B.

Acquired B red cells are often polyagglutinable (see below).

2.13.1.2 Cause of acquired B

Evidence suggesting that acquired B might result from enzyme action on red cells included the conversion of group A cells to acquired B activity, *in vitro*, by bacterial filtrates [482] or by sera from individuals with acquired B [466,483,484]. This led Gerbal *et al.* [474] to hypothesise that bacterial deacetylases convert GalNAc, the A immunodominant sugar, to galactosamine, which is similar enough to Gal, the B immunodominant sugar, to react with some anti-B (Figure 2.9). A wealth of evidence, summarised below, confirms that deacetylation of GalNAc is the most common cause of acquired B [467–469,481,485].

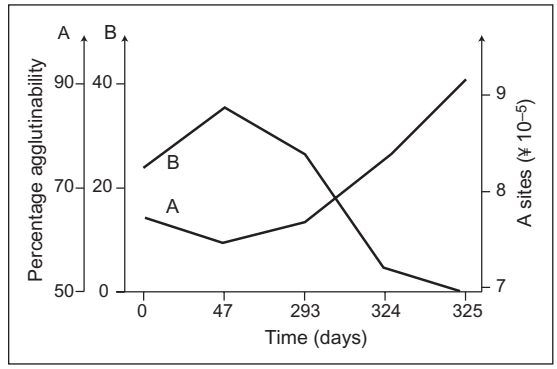


Figure 2.10 Graph demonstrating the inverse relation between A and acquired B expression, as measured by percentage agglutination and number of A sites, in a patient studied over several months. As acquired B expression increased, A antigen expression decreased, and vice versa. Adapted from [469].

1 Only group A cells acquire B antigen.

2 The strength of A antigen expression on acquired B cells is inversely related to the strength of the acquired B antigen [469] (Figure 2.10). Two populations of red cells from a patient with acquired B were separated with *Dolichos biflorus* lectin: the A_1 cells agglutinated by the lectin had only weak acquired B, whereas the remaining A_2 cells had strong acquired B expression [467].

3 Deacetylases have been isolated from the bacteria *Clostridium tertium* A and *Escherichia coli* K12 [486,487]. Acquired B cells could be created, *in vitro*, by treating A_1 cells with culture filtrate from *C. tertium* or from one of six strains of *E. coli* [467,469]. Group O cells were not converted to B activity.

4 Chemical acetylation of acquired B cells with acetic anhydride destroyed the B activity and raised the A activity back to that of normal A_1 cells [485].
5 A-trisaccharide [GalNAcα1→3(Fucα1→2)Gal] that has been chemically deacetylated inhibits the reaction of anti-B with acquired B, but not with normal B cells [488]. B-trisaccharide [Galα1→3(Fucα1→2)Gal], in which the hydroxyl group of carbon-2 of the α-Gal residue has been substituted by an amino group (see Figure 2.9), had the same effect [489]. Agglutination of acquired B cells with anti-B is dispersed by the addition of galactosamine [490].
6 Suspension of acquired B red cells in an acid medium (pH 6) reduces reactivity with anti-B [469], presumably because the NH_2 group of the galactosamine residue is converted to NH_3^+.

2.13.1.3 Polyagglutination

Acquired B red cells are usually polyagglutinable [467,491]; they are agglutinated, at least weakly, by most AB sera. This polyagglutination evolves in parallel with the acquired B phenomenon, but disappears before B activity during recovery; it is not apparent at pH 4.5 or below and it disappears after chemical acetylation of the cells [467,476,485]. Agglutination of acquired B cells by AB serum is inhibited by deacetylated A-trisaccharide, by amino-substituted B-trisaccharide, and by galactosamine [475,488,489].

It is possible that there are antibodies present in most human sera specific for the deacetylated A antigen that are responsible for the acquired B phenomenon. As polyagglutinable cells can be produced by deacetylation with *C. tertium* filtrate of O cells, as well as A cells [467], a different antigen from acquired B is probably involved, possibly involving glucosamine produced by deacetylation of GlcNAc [492].

Acquired B is a unique type of polyagglutination. Acquired B cells react with AB sera from which anti-T, -Tk, -Tn, -Cad, and anti-CDA II have been removed by adsorption [485,493] (see Chapter 33). However, other cryptantigens, responsible for other forms of polyagglutination, are often revealed on acquired B cells, especially Tk, but also T and Th [466,475,483,494,495].

2.13.2 Alterations in leukaemia patients

2.13.2.1 Serology

The association of weak A expression with acute myeloid leukaemia (AML), first recognised in 1957 [496], is well documented (reviewed in [497,498]). In some cases all red cells show weakness of A, whereas in others two populations of red cells are clearly apparent [499–502]. Two populations of red cells from a patient with acute monoblastic leukaemia were separated [499]. Initially only 2% were agglutinated with anti-A, but in remission the proportion of agglutinable cells rose to 65% before falling again shortly before death. In another patient 26% of the red cells were group AB, 12% A, 42% B, and 20% O [503]. Presumably the patient was genetically AB; 62% of his cells had lost their A antigen and 32% their B antigen. Leukaemia-associated changes in B and H antigens are also recorded [502–504]. Between 17% and 37% of patients with leukaemia have significantly lower A, B, or H antigenic expression compared with healthy controls [504–507]. By flow cytometry, 55% of A, B or AB patients with myeloid malignancies had decreased expression of A or B compared with healthy controls of the same ABO genotype; 21% of group O patients had reduced H [508]. In almost all cases the changes represent a loss or diminution of antigen strength and not the expression of a new red cell antigen, although one case is reported of a group O (O^{Iv}/O^{Iv}) patient with myelodysplastic syndrome acquiring an A antigen [261].

Although modifications of ABH antigens are usually associated with acute leukaemia, they are also often manifested before diagnosis of malignancy and therefore indicate preleukaemic states [497]. Loss of an ABH antigen in a patient with a haematological disorder is generally prognostic of AML [509]. For example, a 4-year-old girl whose red cells gave mixed field agglutination with anti-A initially had no sign of haematological disease, but was diagnosed with AML 18 months later [510].

Acute leukaemia has, on occasion, been associated with loss or weakening of Lewis antigens [499,509,511].

2.13.2.2 Transferases and epigenetics

Depression of A or B antigens in AML and in preleukaemic states is generally associated with a severe reduction in red cell GTA or GTB activity, but little or no reduction in red cell H-transferase activity [510,512,513]. In patients with separable populations of red cells, GTA or GTB activity was greatly reduced in the membranes of those cells that had lost their A or B antigens, but were normal in those that had not [512]. During clinical remission, the A antigen of one of the patients returned to normal, as did membrane GTA activity [512]. Thus, the loss of A or B expression in AML results from a defect or deficiency of the *A* or *B* gene products and not a defect in enzyme substrates.

About 58% of AML patients with ABH antigen loss, detected either by conventional serology or by flow

cytometry, had corresponding loss of expression of mRNA, suggesting gene inactivation, and 73% of these patients had hypermethylation of the *ABO* promoter [514]. Furthermore, *ABO* transcripts were re-expressed in leukaemic cell lines after treatment with demethylating agents. Consequently, at least one cause of silencing of the *ABO* gene in AML appears to be epigenetic modification of the *ABO* promoter.

Other mechanisms may also be involved. Gene deletion would be a rare cause as no loss of heterozygosity was detected in 28 AML patients with ABH antigen loss [514]. In a patient with erythroleukaemia, however, about 50% of the red cells had lost their A antigen and those cells also showed a very low level of adenylate kinase-1 an enzyme encoded by a gene close to *ABO* on chromosome 9 [515]. This was presumed to result from a chromosome lesion in the part of the chromosome containing both *ABO* and *AK1*. The *ABL1* oncogene maps between *ABO* and *AK1* on chromosome 9q34 and is at the breakpoint of the Philadelphia-chromosome, a leukaemia-specific reciprocal translocation involving paternal chromosome 9 and maternal chromosome 22 [516]. In four informative cases of A or B antigen loss during AML, the allele that was lost could only have been maternally derived [517]. The significance of this is unclear, but the results suggest that imprinting affects other loci on chromosome 9q34 other than *ABL1*.

Serum H-transferase activity is generally reduced in patients with AML [512,518–520], but increased in those with chronic granulocytic leukaemia [520,521].

2.13.3 Other acquired changes in ABO antigens

Acquired loss of A from a proportion of the red cells occasionally occurs in healthy, elderly individuals [522, 523]. Weakened A expression may also occur in pregnancy, and this is often most obvious in women with A^2/B genotype [261,262].

A premature baby (26 weeks) was typed as group B at birth, but became A_2B by day 73, presumably an effect of the prematurity [524]. A healthy child who was A_2 at birth, but later became A_x, is described in Section 2.7.3 [294].

Following transplantation of a liver from a group AB donor, a proportion of the red cells of a group O child became transiently group AB, as did group O transfused red cells [525]. The cause of this phenomenon is unknown, but could have been caused by hepatic transferase activity. Weak A activity on red cells of group A recipients of group O bone marrow transplants may result from adsorption of A substance from the plasma of the recipient [526].

2.13.4 *In vitro* enzymatic degradation of A, B, and H antigens

The exoglycosidases α-*N*-acetylgalactosaminidase (A-zyme) and α-galactosidase (B-zyme) cleave the A and B immunodominant monosaccharides GalNAc and Gal from A- and B-active oligosaccharides, to destroy A and B blood group activity, reveal H, and produce enzyme-converted group O (ECO) red cells (reviewed in [527–529]). In times of blood insufficiency there is often a particular shortage of group O. Large-scale production of ECO cells from A, B, and AB, RhD-negative cells would be a boost to the blood supply and would assist in providing suitable blood for patients with antibodies to very common antigens, patients with multiple antibodies to polymorphic antigens, and transfusion-dependent patients who should receive matched blood to help prevent them from making multiple antibodies.

Although α-galactosidase from green coffee beans (*Coffea canephora*) appeared to convert group B red cells to group O, the enzyme was not very efficient and required low pH for optimum activity. Phase I and phase II clinical trials, on healthy volunteers and patients, respectively, have shown that group B red cells treated with either native or recombinant α-galactosidase from coffee beans are safe and efficacious when transfused to group O or A subjects, once, in multiple-unit volumes, or on more than one occasion [530,531]. Early attempts at converting A red cells to O with α-*N*-acetylgalactosaminidases from chicken liver or from the bacteria *Ruminococcus torques* IX-70 and *Clostridium perfringens* were less successful. Although A_2 cells could be converted, A_1 cells remained stubbornly A-active, possibly because of the complex repetitive Type 3 A structures present on glycolipids of A_1, but not A_2, red cells (Section 2.4.2).

In 2007, Liu *et al.* [532] went fishing through 2500 fungal and bacterial isolates for more suitable enzymes. The upshot was the production of novel recombinant glycosidases, derived from *Elizabethkingia meningosepticum* and *Bacteroides fragilis*. The enzymes derived from these bacteria had high efficiency and substrate specificity for cleavage of the A and B immunodominant monosaccharides, respectively, under reaction conditions suitable for maintenance of red cell integrity and functions, and with properties that facilitate enzyme removal from the converted red cells by routine washing methods. Consequently, following a 60-minute incubation time with the

appropriate enzyme, whole units (200 ml) of A_1, A_2, B, and A_1B red cells expressed neither A nor B antigens as determined by licensed blood grouping reagents. The results of clinical trials with ECO red cells are still to be published [529].

An α-fucosidase isolated from *Aspergillus niger* abolished H activity on group O cells [533].

2.13.5 Modification of antigen expression by polyethylene glycol (PEG)

Another methodology investigated as a way to convert group A or B red cells to artificial group O cells is the use of PEG to mask the A and B determinants (stealth red cells). PEG is a non-ionic polyether that exists in many configurations. Pegylation of red cell surface glycoproteins could provide a coating of PEG molecules and water attracted by the PEG, which blocks access of antibodies. Coating of red cells with modified high molecular weight PEG molecules of different chain lengths has led to the production of red cells with substantially reduced surface antigen expression (reviewed in [534]). Unfortunately, PEG is immunogenic and antibodies to PEG shorten survival of PEG-treated red cells in rabbits and of pegylated proteins in humans [534]. Consequently, it is unlikely that PEG-modified 'universal' red cells will become part of transfusion practice.

2.14 ABH antibodies and lectins

2.14.1 Anti-A and -B

Anti-A and -B are almost always present in sera of people who lack the corresponding antigen from their red cells (Table 2.2). With the exception of newborn infants, deviations from this rule are extremely rare; only about 1 in 12 000 adults lack expected anti-A or -B [535]. Missing agglutinins may indicate a weak subgroup of A or B, a twin or tetragametic chimera, hypogammaglobulinaemia, or old age, though very rarely are missing agglutinins with no apparent explanation encountered.

Antibodies detected in the serum of neonates are usually IgG and maternal in origin [536], but may, on occasion, be IgM and produced by the fetus [537]. Maternal ABO haemagglutinins, which have the potential to cause fatal HTRs, were detected in 6.4% of neonates, but were cleared within the first month of life [538]. Generally, ABO agglutinins are first detected at an age of about 3 months and continue to increase in titre, reaching adult levels between 5 and 10 years [537,539].

Levels of A and B antibodies appear to be influenced mainly by environmental factors, genetics playing no more than a minor role [540,541]. Anti-A and -B are often referred to as naturally occurring, probably appearing in infants as a result of immunisation by A and B substances present in the environment. Springer *et al.* [542,543] found that chickens, which normally develop an antibody to human group B red cells within a few months of hatching, fail to do so if kept in a germ-free environment. Feeding human infants with killed bacteria (*Escherichia coli* O_{86}) stimulated increased anti-B activity [544]. Changes in diet were considered responsible for a decrease in anti-A and -B titres in Japanese donors over a period of 15 years [545].

Changes in the characteristics of anti-A or -B occur as a result of further immunisation by pregnancy or by artificial means, such as incompatible transfusion of red cells or other blood products. Typical changes, serologically detectable, are increase in titre and avidity of agglutinin, increase in haemolytic activity, and greater activity at 37°C. Such 'immune' sera are generally difficult to inhibit with saliva or with A or B substances.

Anti-A and -B molecules may be IgM, IgG or IgA; some sera may contain all three classes [537,546]. Anti-A and -B of non-stimulated individuals are predominantly IgM, although IgG and IgA may be present [547,548]. During a programme of immunising donors with human A or B glycoproteins, with the purpose of producing potent blood grouping reagents, some IgG anti-A or -B was detected in all donors prior to immunisation [549]. These donors, who had been selected for high titre antibodies, all showed an increase in IgG after stimulation; IgA anti-A and -B, which could not be detected in any of the sera pre-immunisation, was present in all sera post-immunisation. Table 2.20 shows some of the characteristics of IgM, IgG, and IgA ABO antibodies. IgG1 and IgG2 anti-A and/or -B were present in most sera from mothers of group A or B children; almost 40% of the sera also contained IgG3 and/or IgG4 anti-A/B [550]. IgG2 usually had a higher titre than antibodies of the other subclasses. A quantitative analysis of sera from 235 healthy blood donors showed IgG1 and IgG2 anti-A/B predominant, with IgG3 and IgG4 playing only a minor role [546].

ABO antibodies may be found in various body fluids including saliva, milk, cervical secretions, tears, and the contents of cysts [551–556]. They are primarily IgA [554] and generally most active in fluids from group O individuals.

Anti-A_1 is described in Section 2.4.

Table 2.20 Some characteristics of IgM, IgG, and IgA anti-A and -B (compiled mostly from [537]).

Characteristic	IgM	IgG	IgA
Present in sera of			
non-immunised donors	Yes	Sometimes	Rarely
immunised donors	Yes	Usually	Usually
Agglutinates red cells	Yes	Yes	Yes
Agglutination enhanced in serum medium	No	Yes	
Haemolytic	Yes	Yes	No
Binds complement	Yes	Yes	No
Titre increased in antiglobulin test	No	Yes	Yes
Inhibited by secretor saliva or purified glycoprotein	Yes, easily	Poorly	Yes, less easily than IgM
Thermal optimum	4°C	4-37°C	
Activity destroyed by 2-ME or DTT	Yes	No	Partially
Activity destroyed by heating to 56°C	Yes	No	No
Present in colostrum	Sometimes	No	Yes

2-ME, 2-mercaptoethanol; DTT, dithiothreitol.

An interesting antibody described in 1953 remains unique [557]. In a saline medium this antibody agglutinated only group A Rh D+ cells; A_1 D+ cells gave stronger reactions than A_2 D+ cells. O D+ and A D– cells were not agglutinated. When the reactivity of the antibody was enhanced by addition of albumin it behaved as anti-D. The antibody could be completely adsorbed by O D+ cells, but it was not adsorbed by A_1 D– cells. Perhaps this D-like antibody recognised conformational changes in the Rh complex occurring with the presence or absence of an A determinant on the Rh-associated glycoproteins (see Section 5.5.6).

2.14.2 Anti-A,B of group O serum

Sera from group O people do not simply contain two separable antibodies, anti-A and -B, but a cross-reacting antibody called anti-A,B; an antibody that detects a structure common to both A and B determinants [11,558–561]. If an eluate is made from group A cells incubated in group O serum the antibody in the eluate will agglutinate A and B cells [198]. The same effect is observed if group B cells are used. There is, however, no such effect if artificial anti-A+B is made by mixing group A and group B sera [562,563].

The cross-reactivity of group O sera is often asymmetrical; some group O sera eluted from A cells react with B cells, yet when eluted from B cells will not react with A cells [563]. In group O people immunised with A cells or A substance the cross-reacting antibody usually shows a preference for A cells [549,564]; that is, it has a higher binding constant for A cells than for B cells [565]. The reverse is true in group O individuals immunised with B antigen.

Anti-A,B are mostly IgG, but may be IgM or IgA [537].

2.14.3 Clinical significance of ABO antibodies

Transfusion of ABO major incompatible red cells (e.g. A to O, B to O, A to B, B to A), where antibody in the recipient will destroy the transfused red cells, will almost always result in symptoms of an HTR and may cause disseminated intravascular coagulation, renal failure, and death. Of 36 Americans who received more than 50 ml of incompatible blood, 23 (64%) manifested signs or symptoms related to the incompatible transfusion and 6 (17%) died [566]. (See [537] for details on transfusion reactions.)

In transfusion practice group O is often considered the 'universal donor' and transfusion of O blood to an A or B recipient considered a compatible transfusion. The presence of ABO antibodies in minor incompatible (e.g. O to A, O to B) whole blood transfusion, however, may lead to destruction of the recipient's red cells and an HTR (see [537]), though this can be avoided by screening for donors with high levels of anti-A or -B. There is increasing evidence that infusion of relatively large quantities of ABO incompatible plasma, as frequently occurs when

transfusing ABO incompatible platelets, could cause impaired cellular immune function, infection, and multiorgan failure by a mechanism unrelated to haemolysis [567]. This might result from tissue damage caused by the presence of ABO antibodies in the transfused plasma or the presence of circulating immune complexes comprising soluble ABO substances in the transfused plasma and the recipient's antibodies. Furthermore, in a large cohort of plasma recipients, mortality was significantly higher in those who received ABO compatible, non-identical plasma, than in those who received ABO identical plasma [568].

Anti-A_1 is rarely clinically significant and most examples are not active above 25°C. There are, however, a few reports of HTRs caused by anti-A_1 [569–572, 854].

When it occurs, HDFN caused by ABO antibodies is usually in A_1 or B babies of group O mothers. Very rarely, group B babies of A_2 mothers may be affected. About 15% of pregnancies in women of European origin involve a group O mother with a group A or B fetus, yet ABO HDFN requiring clinical intervention is rare, though minor symptoms involving a small degree of red cell destruction may be relatively common. Hydrops caused by ABO HDFN is exceedingly rare, but very occasionally exchange transfusion for the prevention of kernicterus is indicated. Severe ABO HDFN is uncommon, despite the presence of IgG ABO antibodies in the serum of most group O women, because of the relatively low density of A and B antigens on fetal red cells and the presence of soluble A and B substances in the fetal plasma, which neutralise maternal antibodies. The complement deficiency of fetal plasma may also play a part in the rarity of ABO HDFN as IgG anti-A that haemolyses red cells in the presence of complement will not lyse cord cells if neonatal serum is used as the source of complement [573]. Also see Section 25.8.

2.14.4 ABO autoantibodies

ABO autoantibodies are rare. In one English blood centre, only six of 4668 patients with autoantibodies studied over 32 years had autoantibodies with ABO specificity [574]. Some apparent autoanti-A and -B do not react with group A or B cord cells and so their true specificity is anti-AI or -BI (Section 25.7.6). Several examples of autoanti-A and -B have caused AIHA [574], one resulting in fatal haemolysis and kidney failure [575]. One autoanti-B was associated with acrocyanosis, but no AIHA [576]. Autoanti-A_1 has been reported, but not implicated in AIHA [574].

2.14.5 ABO and transplantation

ABO antigens are expressed throughout the body (Section 2.19) and so represent histocompatibility antigens that are very pertinent to transplantation. Anti-A and -B can cause hyperacute rejection of incompatible kidney, liver, and heart, yet major ABO incompatible (ABOi) kidney transplantation has now become common practice, reducing the burden on the donor pool and facilitating use of living kidney donors. To achieve a successful ABOi transplant, ABO antibody levels in the recipient are reduced by pre-, peri-, and post-operative plasma exchange, either with plasma of donor type, which will contain soluble A or B antigens, or with autologous plasma with ABO antibodies removed by passing the plasma through a column containing A- or B-active oligosaccharides on an insoluble matrix. The patient is treated with intravenous immunoglobulin (IVIg) and with anti-CD20 (Rituximab) to inhibit B-cell activity (reviewed in [566,577–580]). Eventually the antibodies levels return from their low levels, but the graft usually continues to function well as a result of accommodation, an acquired resistance of an organ to immune-mediated damage, the mechanism of which is still uncertain [579]. Owing to the extremely low level of expression of A antigen on non-erythroid tissues of A_2 donors, A_2 grafts can be treated as group O for transplantation. Infants do not produce ABO antibodies during the first months of life, so ABOi heart and lung transplants can be carried out without applying the special procedures required for ABOi transplantation in adults [581,582].

Anti-A and -B can usually be disregarded for tissue transplants, including cornea, skin, and bone [583].

About 40–50% of haemopoietic progenitor cell (HPC) transplants are ABOi. Whether major ABOi has any deleterious effect remains controversial. Complications of major ABOi are haemolysis of residual red cells in the graft, delayed erythroid engraftment, resulting in extended dependency on transfusion, and pure red cell aplasia (reviewed in [577,584–587]). The risks of delayed engraftment and pure red cell aplasia are increased in non-myeloablative procedures.

Passenger lymphocyte syndrome is a form of graft versus host disease in which lymphocytes of donor origin are transferred to the recipient of a minor ABOi solid organ or HPC transplant, and leads to haemolysis of the patient's own red cells (reviewed in [577,587,588]). In solid organ transplantation the risk is higher in lung and heart/lung transplants, with greater levels of lymphoid tissue engrafted, than in liver and kidney transplants. Typically the ABO antibodies are IgG, appear 7–10 days

after transplantation, and last for about one month. They are occasionally responsible for severe haemolysis and have caused acute renal failure and death. Passenger lymphocyte syndrome is more commonly a complication of minor ABOi HPC transplants, especially following non-myeloablative conditioning. Haemolysis may be severe and even fatal.

2.14.6 Monoclonal antibodies

Three years after Köhler and Milstein [589] described their method for *in vitro* production of monoclonal antibodies from hybridomas of murine myeloma cells and lymphocytes from immunised mice, Barnstable *et al.* [590] reported the first monoclonal blood group antibody, anti-A. This antibody resulted from immunising a mouse with human tonsil lymphocyte preparations.

Numerous other monoclonal anti-A and -B followed: some produced deliberately by immunising mice with group A or B red cells or purified substance [360,476, 477,591–595]; others accidentally by immunising with other cells or with biomolecules expressing A. Some monoclonal anti-A bind preferentially to the A-terminal trisaccharide whereas others detect an epitope involving the oligosaccharide backbone. The former type of anti-A are more effective at agglutinating A_2B red cells with weak A expression and are more suitable for use as reagents [596,597]. Monoclonal antibodies behaving as anti-A_1 have also been reported [55,593,598].

Human monoclonal anti-A and -A_1 have been generated by Epstein-Barr virus (EBV) transformation of lymphocytes obtained either from hyperimmunised plasmapheresis donors [599] or from splenic tissue after *in vitro* stimulation with group A red cells [600].

Monoclonals that react with both A and B cells (anti-A,B) have been produced after immunising mice with A substance, group A red cells, or AB red cells [290,593, 594,601]. Some of these antibodies react more strongly with A cells than with B cells [290,601].

A Fab-phage was isolated from a human IgG1 phage-display library derived from splenocytes from a group O donor by panning with group B red cells. The 'antibody' agglutinated B, but not A or O red cells, but displayed interaction with A and B epitopes by inhibition techniques [602]. Other anti-A and -B scFv fragments are reported [603].

Details of numerous ABO monoclonal antibodies submitted to four international workshops are described in the workshop reports [604–607].

2.14.7 Anti-H

H antibodies detect the precursor of A and B antigens. They characteristically agglutinate group O and A_2 cells more strongly than A_1 and B cells.

Typically, H antibodies are inhibited by secretor saliva and react with group O cord cells, although often less strongly than with O adult cells. Morgan and Watkins [608] distinguished anti-H, which is inhibited by secretor saliva, from 'anti-O', which is not. The latter specificity is now generally called anti-HI (Section 2.14.8).

Antibodies specific for Type 1 H (Table 2.3) are often referred to as anti-Le^d or -Le^{dH} [259,609] (Section 2.18.2.1).

2.14.7.1 Anti-H in Bombay sera

Anti-H is generally present in the sera of people with H-deficient, non-secretor (Bombay, O_h, A_h, and B_h) phenotypes. These anti-H vary greatly in strength, ability to agglutinate cord cells, degree of inhibition by O saliva, and IgG content. Sera with the greater IgG content show least difference in strength between O cord and O adult cells and are least readily inhibited by saliva. O_h sera contain both anti-Type 1 H and -Type 2 H [610,611]. Réunion phenotype individuals (red cell H-partially deficient, non-secretors), however, produce a large quantity of anti-Type 1 H, but only little anti-Type 2 H, presumably because a small quantity of Type 2 H antigen is present on their red cells [611].

Anti-H in sera of red cell H-deficient, non-secretors has the potential to cause HTRs [437,612] and only H-deficient red cells are suitable for transfusion. Only 2% of group O cells injected into an O_h patient survived 24 hours [613]. In an A_h patient, 67% of A_1 cells were destroyed within 1 hour of injection, despite being only weakly agglutinated at 37°C by the serum of the patient [614]. Anti-H in an O_h mother caused severe HDFN [615], but all of 16 babies of O_h phenotype mothers in South Africa were either mildly affected or unaffected by HDFN and no exchange transfusions were required [612].

2.14.7.2 Other sources of human anti-H

Anti-H in the serum of people who do not have H-deficient red cell phenotypes, usually found in ABH non-secretors, are generally weak and only reactive at low temperatures [616]. An exceptionally potent anti-H from an A_1 Le(a–b+) person (Toml) was inhibited by secretor saliva, including the patient's own saliva, reacted with cord cells, and did not react with O_h cells [617]. Mono-

clonal IgM autoanti-H in a patient with lymphoma was responsible for fatal AIHA [618].

2.14.7.3 Monoclonal anti-H

Numerous mouse monoclonal H antibodies have been produced following immunisation by a variety of immunogens [52,229,230,604–607,619,620]. Unlike human anti-H, murine monoclonal anti-H are often not inhibited by secretor salivas, or at least are inhibited by only a minority of secretor salivas [619]. In one set of 11 H-like monoclonal antibodies, all reacted with either monofucosyl Type 2 H or difucosyl-Le^y, or with both structures; none reacted with the Type 1 structures [52]. Those antibodies reactive with only Type 2 H reacted with red cells, but not with salivary substances, presumably because of a predominance of difucosylated structures in saliva; those specific for the difucosylated Le^y structure did react with salivary structures, but not with red cells; and those reactive with both monofucosylated and difucosylated structures reacted with red cells and with saliva. Mollicone *et al.* [620] subdivided 28 monoclonal anti-Type 2 H into seven categories, based on their cross-reactivities with synthetic oligosaccharides.

2.14.7.4 Anti-H from other sources

Anti-H has been made in animals (including chickens, cattle, buffalo, goats, and sheep [621]) by immunising with O red cells or with purified H substance, and adsorbing with O_h cells. H-specific lectins are described in Section 2.14.9.4.

2.14.8 Anti-HI and -Hi

Anti-HI (or -IH) agglutinate red cells carrying both H and I [622]; they do not agglutinate, or agglutinate only very weakly, H-deficient cells (non-secretor or secretor) or I-deficient cells (cord and adult i cells). Anti-HI are usually weak antibodies reacting only at low temperatures.

In line with the observation of Sanger [616] that anti-H is only made by ABH non-secretors, the H-like agglutinin found in the serum of H-deficient secretors is generally anti-HI. Anti-HI in the serum of H-deficient secretors is not generally considered clinically significant. Although it has been responsible for rapid destruction of small quantities of radiolabelled group O red cells, it was predicted that transfusion of whole units of blood would result in near normal survival [623].

Autoanti-HI are generally benign, but anti-HI of broad thermal range have caused acute HTRs in group A_1 patients following transfusion of A_2 red cells [624–626] and in a group B woman following transfusion of 100 ml of group O red cells [627].

An autoagglutinin in the serum of an A_1 woman, which behaved like anti-H but was not inhibited by secretor saliva and reacted exceptionally strongly with group A_2 adult i cells, was called anti-Hi [628]. Three more examples have been reported since [629].

2.14.9 Lectins

The name lectin originally described plant extracts capable of agglutinating red cells [630], before Goldstein *et al.* [631] broadened this definition to, 'A sugar-binding protein or glycoprotein of non-immune origin, which agglutinates cells and/or precipitates glycoconjugates'. Thus, the vast array of haemagglutinating substances found in plant (mostly seed) extracts and in some animals such as snails, fish, and snakes can all be termed lectins.

The agglutinating activity of lectins is inhibited by simple sugars, usually monosaccharides. It is assumed that these sugars represent the binding site for the lectin on the cell surface. Through the use of lectins, Morgan and Watkins [632] obtained some of the early information on the nature of the A, B, and H antigens. Some plant extracts contain more than one lectin.

The variety of lectins with A, B, or H specificity are too numerous to itemise here. Most are seed extracts, predominantly from plants of the family Leguminosae, although many other sources exist. Lectins with anti-A, -B, and -H specificity are found in the fruiting bodies of many fungi [633]. A few lectins are listed in Table 2.21. For reviews see [634–637].

2.14.9.1 Anti-A

Renkonen [638] found anti-A activity in the seeds of *Vicia cracca*, the first blood group lectin to be recognised. Group A specificity has been found since in many seeds including *Dolichos biflorus*, an extremely useful blood grouping reagent because it agglutinates A_1 cells far more readily than A_2 cells and so, when appropriately diluted, distinguishes A_1 and A_1B from A_2 and A_2B [204]. *Dolichos* lectin is specific for terminal GalNAc [639] and so will also agglutinate Tn+ and Sd(a++) cells (see Chapters 31 and 33). *Dolichos* lectin probably differentiates A_1 and A_2 red cells on the basis of quantitative rather than structural differences [230].

The eggs and albumin glands of several species of snails, mostly of the family Helicidae, contain anti-A (GalNAc) activity and have often been used in automated

Table 2.21 Some lectins with A, B, A,B, or H activity.

Species	Source	Blood group activity	Monosaccharide specificity	Comments
Dolichos biflorus	Seed	Anti-A_1	GalNAc	Anti-Tn, -Cad also
Phaseolus limensis	Seed (lima bean)	Anti-A		
Phaseolus lunatus	Seed (lima bean)	Anti-A	GalNAc	
Helix pomatia	Snail	Anti-A	GalNAc	
Helix hortensis	Snail	Anti-A	GalNAc, NeuAc	
Fomes fometarius	Tree fungus	Anti-B		Anti-P^k also
Ptilota plumosa	Seaweed	Anti-B		
Salmo salar	Salmon roe	Anti-B	Gal	Anti-P also
Sophora japonica	Seed	Anti-A,B		Reacts strongly with En(a–) cells. Anti-B strongest.
Phlomis fructosa	Seed (Jerusalem sage)	Anti-A,B	GalNAc, Gal	
Bandeiraea simplicifolia	Seed	Anti-A,B	GalNAc, Gal	BSI, one of 3 lectins. Anti-B strongest
Ulex europaeus	Seed (gorse)	Anti-H	I Fuc II GlcNAc*	Lectin most commonly used for detecting H secretion
Lotus tetragonolobus	Seed	Anti-H (-HI)	Fuc	
Anguilla anguilla	Eel serum	Anti-H (-HI)	Fuc	
Cystisus sessifolius	Seed	Anti-H	GlcNAc*	
Laburnum alpinum	Seed	Anti-H	GlcNAc*	

*Probably requires terminal Fuc residue.

ABO grouping [635,640,641]. Lectins from the albumin glands of *Helix pomatia* are a heterogeneous mixture of polypeptides encoded by several separate genes [642].

2.14.9.2 Anti-B

B-specific lectins are less abundant than A-specific lectins. They are found, together with anti-H, in the arils (seed coats) of various species of *Evonymus* [643], in the fungus *Fomes fomentarius* [644], and in the seaweed *Ptilota plumosa*. Anti-B activity is also found in the roe of various species of fish, especially those of the salmon and herring families [645–647]. These lectins are D-galactose-specific and may also show some P, P1, and P^k specificity owing to the galactosyl determinants common to these antigens [647,648].

2.14.9.3 Anti-A,B

Several seed extracts agglutinate A and B cells but not O cells. In some cases this may be due to one lectin cross-reacting with both A and B structures. BSI, one of at least three lectins in *Bandeiraea simplicifolia* seeds, comprises five isolectins made up of different proportions of two subunits. Both subunits have a high affinity for Gal but one of the subunits also binds strongly to GalNAc [649]. A and B activity of *Phlomis fructicosa* lectin was inhibited by GalNAc, whereas Gal only inhibited B activity [650]. If separate A- and B-specific molecules are found in these lectins, the notation anti-A,B is inappropriate, anti-A+B being more suitable.

2.14.9.4 Anti-H

Lectins in the seeds of common gorse (*Ulex europaeus*) behave as anti-H [651] and this is the most widely used and probably best reagent for identifying secretor status from salivas of group O individuals. At least two lectins are present in *U. europaeus* seed extracts [652]: *Ulex* I is inhibited by L-fucose; *Ulex* II is not inhibited by L-fucose, but is inhibited by di-*N*-acetylchitobiose, a sugar with a GlcNAc residue [653]. Both *Ulex* I and *Ulex* II are H-specific and both fail to react with group O red cells treated with α-L-fucosidase [654]. It seems likely that *Ulex* II reacts with subterminal GlcNAc in the H structure, but only in the presence of terminal L-fucose.

Other H-specific lectins fall into two classes [632,655]: **1** those, like *Ulex* I, that are inhibited by L-fucose, for example *Lotus tetragonolobus* seeds and eel serum;

Table 2.22 Interaction of Lewis and secretor genes and the resulting red cell and secreted phenotypes (in group O individuals).

Genotype			Antigens in secretions	
Lewis	Secretor	Red cell phenotype	Le^a	Le^b
Le/Le or *Le/le*	*Se/Se* or *Se/se*	Le(a–b+)	+	+
Le/Le or *Le/le*	Se^w/Se^w or Se^w/se	Le(a+b+)	+	+
Le/Le or *Le/le*	*se/se*	Le(a+b–)	+	–
le/le	Any	Le(a–b–)	–	–

2 those, like *Ulex* II, that are inhibited by GlcNAc derivatives, for example seeds of *Cystisus sessilifolius* and *Laburnum alpinum*.

Ulex I is more readily inhibited by oligosaccharides with Type 2 chains, including those with difucosyl structures, than those with Type 1 chains [609,610,656,657]. *Lotus tetragonolobus* lectin is strongly specific for Type 2 chains and does not react with Type 1 chains [658], which explains why it is not a useful reagent for inhibition tests. *U. europaeus* seed extract has a similar reaction strength with either I-positive or I-negative (adult i) cells, but *L. tetragonolobus* lectin and eel serum lectin behave like anti-HI, with little activity for adult i or cord cells [610,659]. *Cystisus sessilifolius* and *Laburnum alpinum* lectins occupy an intermediate position, reacting with adult i cells less strongly than with I-positive cells [659].

Part 4: Lewis system

2.15 Le^a and Le^b antigens and phenotypes

The structure and biosynthesis of the Lewis antigens are described in Part 2 of this chapter. The details discussed here are mainly related to the serological expression of Lewis antigens, although some structural matters are addressed.

2.15.1 Red cells

A general rule applies to red cell Lewis phenotypes of European and African people. Adults with an *Le* gene are Le(a–b+) or Le(a+b–); if they are secretors of ABH their red cells are Le(a–b+); if non-secretors they are Le(a+b–). People homozygous for *le* have Le(a–b–) red cells (Table 2.22). There are, as might be expected, exceptions to this rule. Many anti-Le^b, often referred to as anti-Le^{bH}, fail to agglutinate A_1 Le(a–b+) cells (see Section 2.17.2.1) and A_1 Le(a–b+) cells may be falsely typed as Le(a–b–). Red cells from fetuses, cord samples, and neonates are generally Le(a–b–). Infants may be transiently Le(a+b+) before becoming Le(a+b–). Lewis-positive women may become transiently Le(a–b–) during pregnancy (Section 2.15.6).

Flow cytometry appears to be a more reliable method for determining Lewis phenotypes of red cells than conventional serological techniques. The following results were obtained with commercial anti-Le^b reagents on red cells of Europeans genotyped for *FUT2* and *FUT3*: A_1 Le(a–b+), 71% positive; B Le(a–b+), 95%; O and A_2 Le(a–b+), 99%; Le(a–b–) and Le(a+b–), <10% [660].

The red cell phenotype Le(a+b+), with both Le^a and Le^b strongly expressed, is very rare in European and African adults, but is common in East and Southeast Asia, and in the Pacific region [167,168,237,661,662]. The explanation for this is the presence of a weak *Se* (*FUT2*) gene and the rarity of *se* (explained in Section 2.6.3). Some anti-Le^b reagents do not detect the Le^b of Le(a+b+) red cells by some techniques, which may have led to failure to recognise Le(a+b+) in some studies [22].

Some Le^a expression may be detected on the red cells of O or A_2 Le(a–b+) adults with selected anti-Le^a if sensitive enough techniques are used, such as an IAT with enzyme-treated cells [537]. Le(a–b+) red cells were destroyed, *in vivo*, in a patient with potent anti-Le^a [663].

2.15.2 Secretions

Lewis antigens are easily detected in human saliva by haemagglutination inhibition. Anti-Le^a is inhibited by saliva of individuals with Le(a+b–) or Le(a–b+) red cells,

the former inhibiting more strongly than the latter [161,664]. Le^b is present in saliva of individuals with Le(a–b+) red cells [665]. Le^a and Le^b is present in saliva of people with Le(a+b+) red cells [167,662] (Table 2.22).

Lewis antigens have also been detected in, and isolated from, human milk [37], gastrointestinal juices [666,667], urine [666,668,669], seminal fluid [666,670], ovarian cyst fluid [9,10], and amniotic fluid [671].

2.15.3 Plasma

Le^a is easily detected in plasma of individuals with Le(a+b–) red cells [664]. Unlike saliva, only a trace of Le^a may be detected in plasma of people with Le(a–b+) red cells [672]. Le^b is present in plasma of individuals with Le(a–b+) red cells [672–674].

The site of synthesis of plasma Lewis substances is unknown. Recipients of transplants of bone marrow [675,676], kidney [668], or liver grafts [677] maintain their own red cell Lewis phenotypes, even though Lewis antigens of donor origin are detected in the urine of kidney recipients and in the bile of liver recipients. In view of the very high levels of intestinal Le^a active glycolipids, Hanfland and Graham [609] suggested that plasma Lewis substances might originate from the intestine. Non-secretor patients with coeliac disease have reduced quantities of urinary Le^a antigen [669]. Evans *et al.* [669] proposed that Le^a in urine and plasma derives from large Le^a-active molecules in the small intestine, which are digested to smaller molecules and absorbed into the blood stream. Some of these small molecules are subsequently excreted *via* the kidney. In coeliac disease these molecules cannot be absorbed by the intestine, resulting in reduced levels of Le^a substance in the urine. Following regeneration of the intestinal mucosa, normal quantities of urinary Le^a are detected. All of eight patients with intestinal failure, seven of whom had resection of the ileum and 80% of the jejunum, had Le(a–b–) red cells, statistically significant from the 6% expected [678]. Furthermore, Le(a–b–) red cells of a patient with failure of the small intestine became Le(a–b+) following an intestinal transplant [679], further evidence that Lewis glycolipids in the plasma, and consequently on the red cells, are intestinally derived. Henry *et al.* [22] consider that other exocrine organs, such as liver, kidney, and pancreas, may contribute to Lewis-active plasma glycolipids, explaining the differences in plasma and intestinal glycolipid profiles.

Trace amounts of glycolipids with the Le^b structure have been identified in plasma from individuals with Le(a–b–) red cells, suggesting some α1,4-fucosyltransferase activity [680.681]. This probably occurs either because the Lewis-fucosyltransferase encoded by an *le* allele is not completely inactive or because one of the α1,3-fucosyltransferases, possibly the product of *FUT5*, has low-level α1,4-fucosyltransferase activity [681].

Enhanced quantity of sialylated-Le^a (sLe^a or CA 19-9) in plasma is associated with cancer of the pancreas and colon, and has been used to support diagnosis (see Section 2.19.4).

2.15.4 Uptake of Lewis antigens by red cells

Red cells do not synthesise carbohydrate chains with a Type 1 backbone and consequently cannot synthesise Lewis antigens. Plasma lipids exchange slowly but freely with lipids in the red cell membrane; the composition of phospholipids and fatty acids in red cell membranes resembles that in plasma [682]. Consequently, plasma glycosphingolipids bearing Lewis structures become incorporated into the red cell membrane [47].

Sneath and Sneath [672] first recognised that Le(a–b–) red cells acquire Le^a and Le^b during incubation in plasma from Le(a+b–) and Le(a–b+) individuals, respectively, and that Le(a+b–) and Le(a–b+) red cells lose their Lewis antigens during incubation in Le(a–b–) plasma. These experiments were carried out with one volume of packed red cells shaken continuously in 10 volumes of plasma at 35°C for at least 3 days, with the plasma being changed daily. Le^a antigen cannot be removed from Le(a+) red cells simply by repeated washing of the cells [663].

Group O red cells transfused into an A_1 Le(b+) recipient were not agglutinated by anti-A_1Le^b on the first and second days post-transfusion, but were weakly agglutinated on days four and five, and were strongly agglutinated on day six [683]. Two days after transfusion of Le(b+) cells to an Le(a–b–) patient, the transfused cells still reacted strongly with anti-Le^b, but after seven days the transfused cells gave a scarcely detectable reaction with anti-Le^b [684].

Glycosphingolipids isolated from plasma were extremely effective at converting Le(b–) red cells to Le(b+); only five minutes incubation at 37°C made Le(b–) cells agglutinable with anti-Le^b [47]. Group O Le(a–b–) red cells exposed to glycosphingolipid fractions prepared from plasma of A_1 Le(a–b+) donors were agglutinated by anti-A_1Le^b [257]. These glycosphingolipids, once adsorbed, could not be washed off with saline. About one

third of the total Le^b-active glycolipid in whole blood is associated with the red cells, the rest being in the plasma [685]. Lewis antigens in saliva are glycoproteins and cannot be adsorbed onto red cells [674].

Twin chimeras have permanent bone marrow grafts derived, *in utero*, from their twin. Their peripheral red cell population is often a mixture of their own cells and those of their twin, yet, unlike other blood groups, the chimera's own genes determine the Lewis phenotype of both populations of red cells, further proof that Lewis antigens are not haemopoietic in origin. In one chimeric twin, a secretor of H, all her red cells were Le(a–b+), despite half of her cells being derived from her non-secretor brother; all the red cells of her non-secretor brother were Le(a+b–), including those from his secretor sister [686].

A_1Le^b can only be produced by individuals with *Le*, *Se*, and A^1 genes. Group A_1 red cells produced by grafted tissue in a group O secretor host are not agglutinated by anti-A_1Le^b, even though they carry both A_1 and Le^b antigens, because the A_1 originates from the graft and the Le^b originates from the host [304,683,687]. One chimeric twin made anti-A_1Le^b even though 50% of her red cells were A_1 Le(b+) [683]. The antibody reacted with none of her own red cells, but did react with all of those of her chimeric twin brother, a secretor of A and H. Recipients of successful bone marrow transplants acquire the blood groups of the donor with the exception of Lewis, which remains that of the recipient [675,676].

2.15.5 Development of Lewis antigens

Le^a and Le^b cannot usually be detected on cord red cell samples by direct agglutination [13]. When more sensitive serological techniques are used, such as IAT or agglutination of enzyme-treated cells with potent antibodies, traces of Le^a and possibly Le^b may be detected on some cord or fetal red cells [663].

Lewis antigens start to appear on red cells soon after birth [688]. Le^a develops first; red cells of infants with an *Le* gene generally become Le(a+) during the first few months of life. In white people, 80% of infants are Le(a+) at 3 months of age, this number dropping to the adult level of 20% by 2 years [689–691]. During this period the red cell phenotype Le(a+b+) is not uncommon [691,692]. By 6 years of age the proportion of Le(b+) reaches the adult level [691].

Appearance of Le^a and Le^b in the plasma of infants correlates with the appearance of those antigens on the red cells [635] and cord red cells can be made Le(b+) by incubation in plasma from an Le(b+) adult [674]. At the source of production of plasma Lewis substances in infants with an *Se* allele, the Le-fucosyltransferase appears to become active before the *Se* (*FUT2*) specified fucosyltransferase, resulting in the appearance of Le^a in the plasma before Le^b.

In Chinese, 50% of cord samples were found to be Le(a–b+) and 50% Le(a–b–) [680]. The Le(a–b+) cells became Le(a+b+) before reverting to Le(a–b+); the Le(a–b–) cells either became Le(a+b+) and maintained that phenotype or remained Le(a–b–).

Lewis antigens in the saliva of neonates with an *Le* gene are the same as those detected in adult salivas: ABH secretors have Le^a and Le^b in their saliva; ABH non-secretors have only Le^a [692]. Lewis substances in amniotic fluid corresponded to the Lewis and secretor phenotypes of the fetus and were present as early as 15 weeks' gestation [671].

2.15.6 Lewis antigens during pregnancy

Agglutinability of red cells with anti-Le^a or -Le^b may be reduced during pregnancy [693–695] and pregnant women with a transient Le(a–b–) phenotype may produce Lewis antibodies. In one survey the frequency of the Le(a–b–) red cell phenotype was 11% in non-pregnant women, but 36% in women at time of delivery [695]. Women who were A_1 most often became Le(b–) during pregnancy. The change in Lewis phenotype occurred as early as the 24th week of the pregnancy and Le^b antigen was detectable again 6 weeks after delivery. The concentration of Le^b glycolipid in plasma decreased only slightly during pregnancy, so, unlike the situation in infants, changes in Le^b red cell expression during pregnancy do not appear to result directly from the quantity of Le^b in the plasma. Hammar *et al.* [695] suggest that, as a result of the increased concentration of plasma lipoprotein that occurs during pregnancy, more Lewis determinants become attached to plasma lipoproteins and consequently less are available to become bound to the red cell surface. Levels of Le^b-active oligosaccharides secreted in the urine of Le(a–b+) women increase during pregnancy and lactation [694].

2.16 Antigen, phenotype, and gene frequencies

2.16.1 Red cells

Based on 1796 tests on red cells, the frequency of Le^a in England was 22.38%. This frequency is very similar to

Table 2.23 Frequencies of Lewis phenotypes in three populations.

Phenotype	Population		
	Europeans & white Americans [696–698]	African Americans [673,697]	Hong Kong Chinese [699]
Le(a–b+)	70–72%	52–55%	62%
Le(a+b–)	19–22%	19–23%	0%
Le(a+b+)	0%	0%	27%
Le(a–b–)	4–11%	22–29%	11%

that found in other European countries [237] and in white Americans [696,697]. Table 2.23 shows phenotype frequencies for Europeans and white Americans obtained from tests with anti-Le^a and -Le^b on O and A_2 cells. The frequency of the *Le* gene varies from 0.67–0.80 in white populations.

Two studies on African Americans revealed higher frequencies for the Le(a–b–) phenotype (Table 2.23) [673,697], giving a frequency of 0.46–0.53 for *Le*. The incidence of Le(a–b–) was as high as 35% in West Africans (*Le* = 0.59), with 15% Le(a+) (only O and A_2 tested [700]).

The Le(a+b+) red cell phenotype, rare in European and African people, is relatively common in East Asia, Southeast Asia, the Pacific region, and Australasia [237]. Le(a+b+) has an incidence of 27% in Hong Kong Chinese [699] (Table 2.23), between 16% and 25% in Taiwan Chinese [662], between 10% and 40% of Polynesians [701], and 10% in Australian Aborigines [702]. Le(a+b–) is rare or absent in Chinese [662,699,703].

Frequencies of *FUT3* alleles derived from DNA analysis are shown in Table 2.8.

2.16.2 Secretions

Frequency of the *Le* gene can be determined by inhibition tests, using saliva to inhibit agglutination of Le(a+b–) red cells by anti-Le^a. Salivas of individuals with an *Le* gene contain Le^a; those of *le/le* individuals do not. From saliva tests the following estimates of *Le* gene frequency have been obtained: 0.82 in English; 0.69 in Swedes; about 0.45 in Africans; 0.70 in Australian Aborigines; and 0.36–0.57 in native South Americans [237]. These are similar frequencies to those determined by red cell testing.

2.17 Lewis antibodies

2.17.1 Anti-Le^a

2.17.1.1 Human anti-Le^a

Anti-Le^a is not uncommon; haemagglutination tests in Denmark and France revealed anti-Le^a in about one in every 300 sera [704]. Agglutinating alloanti-Le^a is only found in people with Le(a–b–) red cells, possibly only those who are ABH secretors [673], and less often in group O individuals than in people of other ABO groups [704]. An exception to this rule appears to occur in Taiwan, where 10 Le(a–b+) patients with anti-Le^a were found; possibly the result of a different molecular background of some Le(a–b+) phenotypes in that population [705].

Le^a antibodies are usually 'naturally occurring' and predominantly IgM. Very rarely is anti-Le^a purely IgG [537,706]. Most potent anti-Le^a sera contain an IgG component detectable by radioimmunoassay or enzyme-linked immunoadsorbent assay [706–708], but seldom detectable by an antiglobulin test with anti-IgG [537].

Anti-Le^a usually cross-react with Le^b oligosaccharide and will sometimes react with the Lewis disaccharide Fucα1→4GlcNAc or with Le^x, the Type 2 isomer of Le^a (Table 2.3) [709]. IgM anti-Le^a have broader specificity for synthetic oligosaccharides than IgG anti-Le^a [710].

Despite the common occurrence of anti-Le^a, human antibodies potent enough to be useful as reagents are rare. As most sera containing anti-Le^a either contain weak anti-Le^b or cross-react with Le^b, methods of enhancing reactivity may lead to 'false positive' reactions with Le(a–b+) cells. Agglutination of red cells with anti-Le^a is generally strongest at low temperatures, often not occurring at all at 37°C. Anti-Le^a usually fix complement [711,712]; a two-stage complement-addition antiglobulin test is often a successful way of detecting anti-Le^a. Some Le^a antibodies lyse Le(a+) cells in the presence of complement. Another method for increasing the strength of reaction with anti-Le^a is to use protease-treated cells.

Some anti-Le^a are lymphocytotoxic [713,714].

2.17.1.2 Monoclonal anti-Le^a

Numerous murine monoclonal anti-Le^a have been produced [604–607]; some make excellent blood grouping reagents [715] and commercially produced monoclonal anti-Le^a are generally used.

Of the four monoclonal anti-Le^a described by Young *et al.* [716], one detected the Le^a trisaccharide Galβ1→3(Fucα1→4)GlcNAc, another the tetrasaccharide Galβ1

→3(Fucα1→4)GlcNAcβ1→3Gal, and two were directed mainly towards the disaccharide Fucα1→4GlcNAc. All monoclonal anti-Le^a cross-react with the Type 1 precursor Galβ1→3GlcNAc (Le^c) [717]. Some monoclonal anti-Le^a cross-react with the T disaccharide Galβ1→3GalNAc and agglutinate Le(a–) T-activated red cells [718] (see Chapter 33).

2.17.2 Anti-Le^b

2.17.2.1 Human anti-Le^b

The original anti-Le^b described by Andresen [13] only reacted specifically with group O Le(b+) or A_2 Le(b+) red cells, whereas the anti-Le^b reported by Brendemoen [665] reacted with all Le(b+) red cells. The two types of Le^b antibodies were named anti-Le^{bH} and anti-Le^{bL} by Ceppellini *et al.* [719].

Anti-Le^{bH}, which cross-reacts with H, can only be used reliably to type O or A_2 cells as it often fails to agglutinate A_1 or B, Le(a–b+) cells. Anti-Le^{bH} is inhibited by saliva from all ABH secretors regardless of whether their red cells are Le(a–b+) or Le(a–b–).

Anti-Le^{bL} agglutinates Le(b+) red cells regardless of ABO group. It is only inhibited by salivas of ABH secretors who also secrete Le^a; that is, not those who have Le(a–b–) red cells. Only anti-Le^{bL} are suitable as anti-Le^b blood grouping reagents.

Like anti-Le^a, anti-Le^b are found most often in people with Le(a–b–) red cells; unlike anti-Le^a, these people are generally non-secretors of ABH [673]. There are reports, however, of group A_1 or A_1B Le(a+b–) people who have made anti-Le^b of the anti-Le^{bH} type [665,720]. Compared with anti-Le^a, anti-Le^b is a relatively infrequent antibody. Tests on the sera of 72 000 Parisians revealed 259 anti-Le^a, 29 anti-Le^{bH}, and only 2 anti-Le^{bL}.

Anti-Le^b show many of the serological characteristics described for anti-Le^a above. Anti-Le^b are generally IgM. Of 14 anti-Le^b sera tested with synthetic oligosaccharides, four cross-reacted with Le^a trisaccharide and seven cross-reacted with Le^y tetrasaccharide (the Type 2 isomer of Le^b), four of these also reacting with H trisaccharide [709] (see Table 2.3).

Some anti-Le^b are lymphocytotoxic [775,721].

2.17.2.2 Monoclonal anti-Le^b

Many monoclonal anti-Le^b have been produced, some anti-Le^{bL}, some anti-Le^{bH}, and some giving intermediate reactions (anti-Le^{bH,L}) [604–607]. All anti-Le^{bL} and -Le^{bH,L} cross-react with Le^a; all anti-Le^{bH} and anti-Le^{bH,L} cross-react with Type 1 H (Le^d) and Le^y [717]. Despite these cross-reactivities, when the appropriate methods are adopted some monoclonal anti-Le^b make excellent blood grouping reagents.

2.17.3 Anti-ALe^b

Anti-A_1Le^b, an antibody that reacts only with the red cells of A_1 Lewis-positive secretors [A_1 Le(a–b+)], was first found in the serum of a group A_1B Le(a–b–) man [722]. Other examples have been described since [683,723]. Anti-ALe^b may be lymphocytotoxic [714]. Monoclonal anti-ALe^b may or may not cross-react with ALe^y, the Type 2 isomer [724,725].

Anti-BLe^b has not been reported.

2.17.4 Clinical significance of Lewis antibodies

Despite being relatively common antibodies, only a few examples of HTRs have been attributed to anti-Le^a [726–729], and even less to anti-Le^b [730–733]. This is probably because most Lewis antibodies are not active at 37°C, because Lewis antigens in the donor's plasma neutralise the recipient's Lewis antibodies, and because Le^a or Le^b antigens elute from red cells transfused into an Le(a–b–) recipient.

Lewis antibodies that are only active *in vitro* at temperatures below 37°C do not cause increased clearance of antigen-positive transfused red cells *in vivo* [734]. Consequently, patients with Lewis antibodies may be transfused with red cells that are compatible by IAT at 37°C.

Lewis antibodies do not cause serious HDFN, presumably because Lewis antigens are present in fetal secretions, but generally not on fetal red cells.

2.17.5 Lewis antibodies and renal transplantation

Kidney cells, particularly cells of the distal tubule epithelium and the endothelium of arteries, glomerula capillaries, and veins, biosynthesise and express Lewis antigens. Consequently, Lewis antibodies are histocompatibility antibodies in renal transplantation [668,735,736]. Two-year graft survival rates were found to be significantly lower in Le(a–b–) recipients than in Le-positive recipients [735] and in several retrospective studies [737–739] and a prospective study [740] Lewis-identical donor-recipient pairs were shown to have better graft survival than Lewis-incompatible pairs. Several cases are reported in which Lewis antibodies, anti-Le^a and -Le^b, were implicated as the possible cause of renal transplant rejection [741,742]. Anti-Le^a was detected by a highly sensitive kinetic enzyme-linked immunosorbent assay in all of eight Le(a–b–) patients with failed renal grafts, but not

in the single Le(a–b–) recipient of an Le(a–b–) graft [743]. Most of these antibodies could not be detected by haemagglutination.

In contrast, in two other prospective studies and one retrospective study [744–746], no significant difference in survival between Lewis-matched and Lewis-mismatched donor-recipient pairs was detected, although Le(a–b–) patients appeared to be at higher risk of graft failure when receiving HLA mismatched kidneys. These data led to the recommendation that Lewis phenotype should not be a basis for selection of kidneys for engraftment [745].

Anti-Le^a, presumably of graft origin, has been blamed for renal failure in bone marrow transplant recipients [676,747]. Autoanti-Le^{bH} in an Le(a–b+) patient who had received two kidney transplants which had both been rapidly rejected, agglutinated the patient's own red cells and was inhibited by his own saliva [741,748].

2.18 Other antigens associated with Lewis

2.18.1 Le^{abx}, the antigen originally called Le^x

An antigen named Le^x by Andresen and Jordal [691,749,750], present on Le(a+b–) and Le(a–b+) cells, but not Le(a–b–) cells, differs from inseparable anti-Le^{ab} because it reacts with 90% of umbilical cord red cell samples, despite cord red cells being Le(a–b–). These 90% are from infants with an *Le* gene, soon to develop red cell Le^a or Le^b [751]. As the symbol Le^x is commonly used for CD15, the monofucosyl Type 2 isomer of the Le^a trisaccharide (Section 2.18.3), the antigen originally called Le^x will now be referred to as Le^{abx}.

Anti-Le^{abx} reacts equally strongly with A_1, A_2, and O, Le(abx+) cells [749,750]. Anti-Le^{abx} is inhibited by saliva, amniotic fluid, and serum from Lewis-positive individuals [751].

Le^{abx} antibodies appear to be heterogeneous: some detect the Lewis disaccharide Fucα1→4GlcNAc and some detect larger determinants common to Le^a and Le^b [709,752,753]. These determinants must be more accessible on cord cells to anti-Le^{abx} than to anti-Le^a.

2.18.2 Le^c and Le^d

Le^c and Le^d are antigens on Le(a–b–) red cells from ABH non-secretor adults and ABH secretor adults, respectively. The symbols Le^c and Le^d are somewhat inappropriate as neither structure is produced by a Lewis gene-specified transferase, and Le^d is now known to be Type 1 H.

Like Le^a and Le^b, Le^c and Le^d are not intrinsic to the red cell but are incorporated into the red cell membrane from the plasma [754].

The phenotype Le(a–b–c–d–) is extremely rare and is associated with a deficiency of an α1,3-fucosyltransferase [755].

2.18.2.1 Le^d or Type 1 H (210002)

As a result of immunising a goat with saliva from an Le(a–b+) person and adsorbing the serum with Le(a+b–) cells, Potapov [756] identified two antibodies: anti-Le^b and a new antibody that agglutinated Le(a–b–) red cells from ABH secretors, but not those from non-secretors. He called this second antibody anti-Le^d, leaving anti-Le^c for the predicted antibody that would react with Le(a–b–) cells from non-secretors. Potapov proposed the following four Lewis phenotypes and corresponding genotypes:

Le(a+b–c–d–)	*Le/Le* or *Le/le*	*se/se*
Le(a–b+c–d–)	*Le/Le* or *Le/le*	*Se/Se* or *Se/se*
Le(a–b–c+d–)	*le/le*	*se/se*
Le(a–b–c–d+)	*le/le*	*Se/Se* or *Se/se*

Two years later human anti-Le^c was identified [757].

Le^d and Type 1 H are synonymous. Anti-Le^d reacts with oligosaccharides containing the Type 1 H determinant, but not the Type 2 H determinant (Table 2.3). Type 1 H active glycosphingolipids isolated from plasma of O Le(a–b–) secretors inhibited goat anti-Le^d [609]. Anti-Le^d, produced by immunising rabbits with synthetic Type 1 H oligosaccharide, reacted with tissues of Le(a–b–) secretors [758].

Type 1 H in the plasma of Le(a–b–) ABH secretors does not get converted to Le^b and so plenty is available to become adsorbed onto the red cell in detectable levels, giving rise to the Le(a–b–c–d+) red cell phenotype. Le^d in group A and B individuals is carried on Type 1 A (ALe^d) and Type 1 B (BLe^d), respectively (see Table 2.3).

Monoclonal anti-Type 1 A (anti-ALe^d) has been used in a biochemical analysis of red cell glycolipids [759]. Lymphocytotoxic murine monoclonal anti-A_1Le^d did not agglutinate red cells, but lysed A_1 Le(d+) red cells after addition of rabbit complement [760].

Antibodies that react with red cells of O and A_2 Le(a–b–) secretors, but not with those of A_1 Le(a–b–) secretors have been called anti-Le^{dH} [259].

2.18.2.2 Le^c (210001)

The symbol anti-Le^c was initially used for a weak antibody, made by injecting a rabbit with Le(a–b–) secretor saliva, that reacted with Le(a–b–) cells from both secretors and non-secretors [761,762] and so may have represented anti-Le^c+Le^d in the later notation [756]. Anti-Le^c of the type predicted by Potapov [756], an antibody that only reacts with red cells of Le(a–b–) non-secretors, was found during the fourth pregnancy of an O Le(a–b+) woman, a secretor of H, Le^a, and Le^b [757]. A second example of human anti-Le^c was found in a group A Le(a–b+) man who had never been transfused [763]. Other examples of anti-Le^c have subsequently been raised in goats by injection of Le(a–b–) non-secretor saliva and adsorption of the immune serum with Le(c–) red cells [259,764,765].

The structure of Le^c is less clearly defined than that of Le^d. Graham *et al.* [259] suggested that Le^c could represent the non-fucosylated Type 1 chain Galβ1→3GlcNAc, the precursor of Le^a and Type 1 H, unmodified by Le- or Se-fucosyltransferases. Further evidence that the Le^c determinant contains this disaccharide was provided when antibodies with anti-Le^c specificity were raised in rabbits immunised with Galβ1→3GlcNAc [765]. Furthermore, antibodies raised in goats immunised with Le(a–b–) non-secretor salivas and affinity-purified with Galβ1→3GlcNAc adsorbents behaved as anti-Le^c. Hanfland *et al.* [766] found that the glycosphingolipid fractions isolated from O Le(a–b–) non-secretor plasma that were most effective at inhibiting anti-Le^c of either human or goat origin carried a branched oligosaccharide chain, one branch containing Type 1 precursor Galβ1→3GlcNAc, the other a monofucosylated Type 2 structure. They concluded that unsubstituted Type 1 chain alone does not represent Le^c, although it does play a part. Further evidence that a monofucosylated Type 2 structure is an essential part of the Le^c determinant is provided by the very rare Le(a–b–c–d–) phenotype, which has only been found in two individuals, both with α1,3-fucosyltransferase-deficiency [755].

It is likely that anti-Le^c represents a heterogeneous group of antibodies that determine different structures abundant in the plasma of individuals who lack both *Le* and *Se* genes and which are incorporated into the red cell membrane.

2.18.3 Le^x, Le^y, and sialyl-Le^x

Le^x (CD15) and Le^y, the Type 2 isomers of Le^a and Le^b, are not really blood group antigens as they are not detectable on red cells [43]. Their structure and biosynthesis have been described in Part 2 of this chapter, and they are discussed briefly here because of their biochemical relationship to ABH and Lewis antigens (Table 2.3). Like Le^b and Le^d, the presence or absence of Le^y in secretions is principally dependent on alleles at the *FUT2* locus.

Human alloantibodies to the Le^x and Le^y determinants have not been found, although some anti-Le^a cross-react with Le^x and some anti-Le^b cross-react with Le^y [709]. Murine monoclonal antibodies to the Le^x, Le^y, and ALe^y determinants have been described [42,51,724,759,767].

Sialyl-Le^x (sLe^x, CD15s), the sialylated derivative of Le^x antigen, and, to a lesser extent, sialyl-Le^a, function as ligands for the lectin-like N-terminal domains of selectins, a family of cell adhesion molecules on endothelium (E-selectin), leucocytes (L-selectin), and platelets (P-selectin) (reviewed in [19,190,191]). Attachment of selectins to their carbohydrate ligands is important in the binding of leucocytes and platelets to endothelium, particularly at the region of inflammation.

Part 5: Tissue distribution, disease associations, and functional aspects

2.19 Expression of ABH and Lewis antigens on other blood cells and in other tissues

2.19.1 Leucocytes

A, B, and H antigens may be detected on lymphocytes, but they are acquired from the plasma and their expression is, therefore, under the control of *FUT2* [675, 768–771]. Cytotoxic anti-A react with lymphocytes from A secretors, but not with lymphocytes from A non-secretors; the strongest reactors are lymphocytes from A Le(a–b–) secretors [769]. The same antibodies reacted with group O lymphocytes exposed to a glycosphingolipid fraction prepared from plasma of A Le(a–b–) secretors. Lymphocytes cultured in serum from A or B individuals acquired A or B antigens [714]. Lymphocytes do not contain α1,2-fucosyltransferase [102,772]. Like red cells, lymphocytes adsorb Lewis antigens from the plasma [714].

Granulocytes and monocytes do not express ABH or Lewis antigens regardless of secretor phenotype [771, 773,774]. No α1,2-fucosyltransferase activity was detected in granulocytes or monocytes [102,186]. Granulocytes, monocytes, and lymphocytes do, however, have α1,3-fucosyltransferase activity [102] and express Le^x and sialyl-Le^x on their surface; determinants that function as

ligands for selectins, a family of cell adhesion molecules [190,191].

2.19.2 Platelets

ABH antigens are present on platelets and megakaryocytes [772,775–777]. Type 2 structures are endogenous and under control of *FUT1*; Type 1 ABH structures are adsorbed onto platelets from the plasma and are dependent on secretor phenotype [772]. Platelet ABH antigens are expressed on glycolipids and on the platelet glycoproteins, which include GPIIb/IIIa, GPIb/IX, GPIa/IIa, GPIc, GPIV, GPV, CD31 (PECAM), and CD109 [778]. Platelets from A_2 people carry almost no A antigen and can be considered group O for platelet transfusion (but with no anti-A) [779–781]. Platelets contain H-, A-, and B-transferases [185], and release of α1,2-fucosyltransferase during clotting may represent a major source of this enzyme in plasma [102,520]. Around 4–7% of A_1 and B Japanese and white Americans are high expressors (HPX) of platelet A and B antigens, with a 2- to 3-fold increase of antigen expression [782,783]. Flow cytometry demonstrates two subclasses of HPX: type 1 have platelets with high expression and platelets with moderate to low expression of ABO antigens, whereas in type 2 all the platelets have high ABO expression [783]. Although HPX appears to be inherited, the molecular background remains unknown.

ABO plays an important part in safe and efficacious platelet transfusion (reviewed in [778,784–786]). Major ABO incompatibility does not preclude platelet transfusion, as it does for red cells, and is often ignored. There is, however, ample evidence for some degree of platelet refractoriness, following ABO major incompatible platelet transfusions, particularly in children and in patients requiring long-term support. Minor ABO incompatibility, usually involving transfusion of group O platelets to a non-group O patient, may cause haemolysis of the patient's red cells, sometimes resulting in an acute HTR, which on rare occasions has proven fatal. One way to avoid this is to screen donors for high-titre anti-A and -B, and this is done in some countries, particularly in Europe and Japan [778]. In addition, there is evidence that infusion of relatively large quantities of ABO incompatible plasma could cause impaired cellular immune function, infection, and multiorgan failure [567] (see Section 2.14.3).

ABH antigen expression on platelets may be enhanced by platelet activation and apheresis and prolonged storage of A_1 platelets has led to higher levels of A antigen on the cell surface [780].

Anti-B appeared to be responsible for neonatal alloimmune thrombocytopenia in two group B babies with type 2 HPX platelets and a group O mother with high-titre IgG anti-B [787].

Le^a, assimilated into the membrane from the plasma, can be detected on platelets [788].

2.19.3 Other tissues

ABH and Lewis antigens are widely distributed in the human body and are often called histo-blood group antigens (reviewed in [14,17,21,789]). A scheme devised by Oriol and his colleagues [21] to summarise the genetic control of ABH and related antigens in various tissues is shown in Figure 2.11. Although a few exceptions exist, the following general rules prevail.

1 In tissues derived from ectoderm and mesoderm, ABH antigens are under the control of *FUT1* (H). Type 2 ABH structures are expressed, often together with Le^x and/or Le^y antigens. In addition to haemopoietic tissue, these tissues include skin, primary sensory neurons, vascular endothelium, and renal glomeruli and convoluted tubules.

2 In tissues of endodermal origin – such as digestive and respiratory mucosae and renal urinary epithelium – ABH, Lewis, and Lewis-related structures may be Type 1 or Type 2 and are primarily controlled by *FUT2* (Secretor) and *FUT3* (Lewis).

Exceptions to these rules include the following: some deep areas of digestive mucosa where, despite being of endodermal origin, histo-blood group antigens are independent of *FUT2* and *FUT3*; parts of the mammary and sweat glands (tissues of ectodermal origin), where ABH and Lewis antigens are governed by *FUT2* and *FUT3*.

Ravn and Dabelsteen [789] have devised an alternative scheme, in which it is the process of differentiation of epithelial cells, rather than embryonic origin, that determines whether H antigens are controlled by *FUT1* or *FUT2*.

Le^a and Le^b have been detected, immunochemically and by structural analyses, in small intestine and colon of individuals with Le(a–b–) red cells and whose saliva contains no Le-transferase [790–792]. See Section 2.15.3 for a possible explanation.

2.19.4 Tumours

ABH antigens are often absent from glycoproteins and glycolipids of malignant tissue of the gastrointestinal tract, oral cavity, uterine cervix, lung, prostate, breast and bladder, despite being present in the surrounding epithelium. In certain types of carcinoma loss of ABH

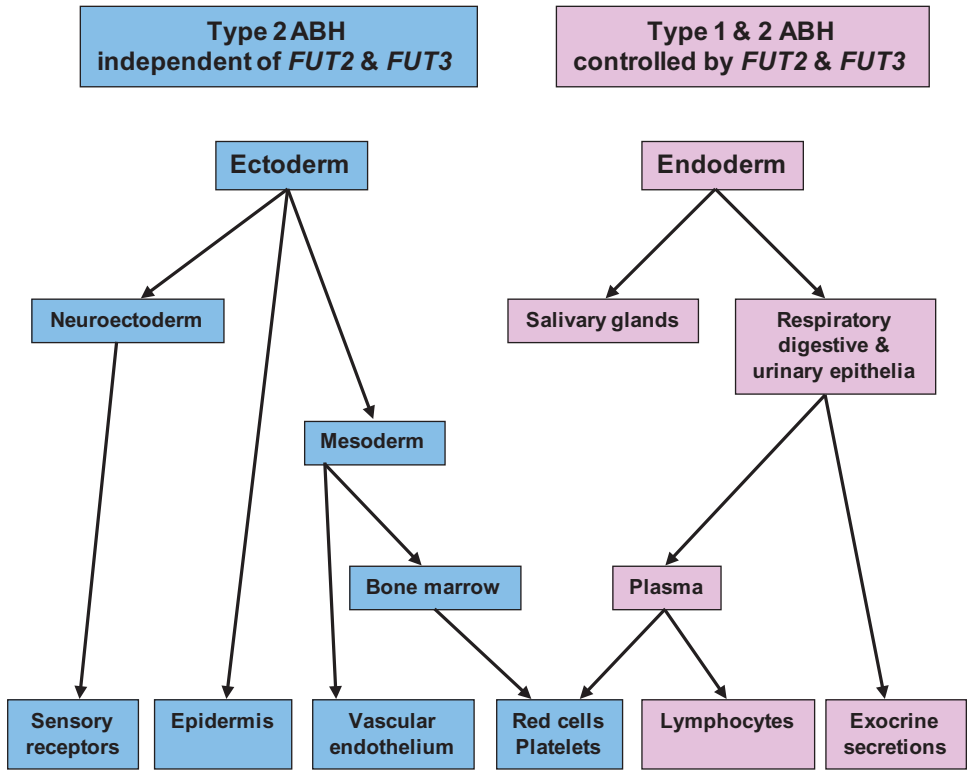


Figure 2.11 Scheme devised by Oriol [21] to demonstrate the genetic control of ABH and related antigens in human tissues derived from ectoderm, mesoderm, and endoderm. Some exceptions to this model are described in the text.

antigens precedes formation of distant metastases and hence is associated with poor prognosis [18,793]. Loss of A or B antigens from tumour cells may increase their motility and, consequently, their ability to form metastases. In addition, this absence of A or B antigens generally arises from disappearance of A- or B-transferase activity and results in an accumulation of H, Le^b, Le^y, sialyl-Le^a or sialyl Le^x. Sialyl-Le^a and sialyl Le^x are ligands for selectins, and their presence promotes the metastatic process by facilitating interaction with distant organs. ABO antigen loss results from downregulated transcription of *ABO*, and no A or B mRNA can be detected in high-grade tumours. At least two different mechanisms may be involved, possibly occurring in tumours derived from different sources:

1 loss of heterozygosity (allelic loss) involving deletions within chromosome 9q34, which contain the *ABO* locus in addition to tumour suppressor genes; and

2 hypermethylation of the CpG island of the *ABO* promoter region, which down-regulates transcription [794–796].

See Section 2.13.2 for discussion on ABH antigen loss in leukaemia patients.

Type 1 and Type 2 ABH structures and Le^b, Le^x, and Le^y antigens are present on fetal distal colon; they are not present in healthy adult distal colon, but are often re-expressed in adults in carcinoma of the distal colon [797]. Malignancy in the distal colon is associated with increased activity of α1,2-fucosyltransferase of H and Se types, indicating that α1,2-fucosyltransferases control, at least partially, oncodevelopmental expression of ABH and related antigens in colon [798].

Tumour-associated carbohydrate antigens, which include T, Tn, sialyl-Tn (Section 33.2.3), Le^x, sialyl-Le^x, sialyl-Le^a, Le^y, and Globo (Type 4) H, have been considered potential targets for the production of vaccines as agents of immunotherapy for cancer [799].

Up-regulation of glycosyltransferases in tumours may result in increased levels of certain carbohydrate structures in the plasma [800]. The quantity of circulating sialylated-Le^a (sLe^a), otherwise known as the CA19-9 antigen, is widely used as a marker to support diagnosis

of colorectal, pancreatic, and gastric cancer, and as an aid to prognosis after potential curative surgery [44,801–803]. CA19-9 levels vary according to Lewis and secretor genotypes, complicating cancer diagnosis; Le(a–b–) (*le/le*) individuals have no circulating CA19-9 [802]. A marker for cancerous regions of the colon of Le(a–b–) patients is sialyl-Le^c (DU-PAN-2), a precursor of sialyl-Le^a [802].

Another phenomenon associated with malignancy is the incompatible A antigen, occasionally expressed on tumours of group O or B people [804]. About 10% of colonic tumours from group O patients, shown to be homozygous for the O^1 allele, express A antigen and contain A-transferase activity [805,806]. The molecular basis of this O to A conversion is not known, but that alternative splicing of *ABO* RNA, resulting in loss of exons 5 and 6, would introduce no frameshift or translation-termination codons [110]. The putative gene product would be a truncated glycosyltransferase lacking the G261 nucleotide deletion, characteristic of the O^1 allele, and with a potential for A-transferase activity (see Section 2.3.2.2). The higher incidence of gastric and ovarian adenocarcinomas in group A people [807] could result from suppression of development of tumours bearing an A antigen by the anti-A naturally present in group O and B people.

2.20 Associations with disease

Some examples of associations between ABO group and disease have already been mentioned, notably HDFN (Section 2.14.3), leukaemia (Section 2.13.2), cancer (Section 2.19.4), acquired B resulting from bacterial infection (Section 2.13.1), and leucocyte adhesion deficiency type II (Section 2.12.5.2). A multitude of other associations between ABO and diseases have been reported, mostly based on observed ABO phenotype frequency discrepancies between patients with the disease and the healthy population (reviewed in [23,807,808]). Susceptibility to infection by numerous pathogenic micro-organisms is associated with ABO phenotype or secretor status [23].

2.20.1 Bacterial infections

Group O individuals are more susceptible than non-O to the severe effects of infection with enterotoxigenic *Escherichia coli* [809], responsible for millions of cases of gastrointestinal infections annually, and for some strains of the cholera bacterium, *Vibrio cholerae* [810]. This effect could result from a binding preference of bacteria-derived heat labile toxins for A- or B-active structures over H antigen [811].

Helicobacter pylori is a gram-negative bacterium present in over 50% of the world's population and associated with development of chronic gastritis, gastric and duodenal ulcers, and adenocarcinoma, though most infected individuals show no clinical symptoms [812,813]. In 1993, Borén *et al.* [814] provided evidence that Le^b mediates attachment of certain strains of *H. pylori* to gastric mucosa cells. Bacterial binding to gastric mucosa cells was abolished by the presence of Le^b-active soluble glycoproteins and by Le^b-specific monoclonal antibodies. Gastric mucosa cells expressing Le^b bound *H. pylori* P466, whereas cells lacking Le^b did not. Type 1 H also mediated *H. pylori* attachment, but to a much lesser degree. Le^a, ALe^b, and BLe^b did not bind the bacteria in this South American population, which could explain why group O individuals have an increased probability of developing gastric and duodenal ulcers [815]. Subsequent studies, however, have shown that other strains of *H. pylori* from around the world are less specific for Le^b in group O and will also bind ALe^b or BLe^b [816–818].

H. pylori expresses protein adhesins that confer close adherence to the gastric epithelium. These include blood group antigen-binding adhesin (BabA), which binds Le^b (and ALe^b and BLe^b) on mucous cells [819], and sialic acid-binding adhesin (SabA), which binds sialyl-Le^x glycolipids, rarely expressed in normal gastric mucosa [820]. Carbohydrate chains of lipopolysaccharides of the outer cell wall in most strains of *H. pylori* express Le^x, sialyl-Le^x, Le^y, Type 1 H, Le^a, Le^b, and A [821]. These antigens undergo phase variation, the random, reversible high-frequency switching of phenotype. In contrast to previous suggestions, molecular mimicry between *H. pylori* and host glycoconjugates does not appear to play a significant role in host evasion or autoimmunity [813,821].

ABH non-secretors appear to be more susceptible than secretors to infections by *Haemophilus influenzae*, *Neisseria meningitides*, and *Streptococcus pneumoniae* and urinary tract infection by *E. coli* [817].

2.20.2 Viral infections

Norovirus (NoV), a member of the Caliciviridae family, is the commonest cause of acute epidemic gastroenteritis (winter vomiting disease), affecting millions of people worldwide (reviewed in [817,818,822,823]). Despite being highly contagious, ABH non-secretors are resistant to symptomatic infection with most NoV strains, as demonstrated by challenge studies with human volunteers

[824] and by analyses of institutionalised outbreaks [825]. In three sporadic outbreaks of acute gastroenteritis in Sweden, 49% of symptomatic individuals were homozygous secretors (*Se/Se*), 51% were heterozygous (Se/se^{428}), and none was a non-secretor (se^{428}/se^{428}), compared with a non-secretor rate of 29% in asymptomatic individuals and 20% in Swedish blood donors [825]. Furthermore, recombinant NoV capsid proteins bound to intestinal epithelia from secretor, but not from non-secretor, individuals [826], suggesting that their receptors are controlled by *FUT2*. In thin-layer chromatography, capsid proteins from the Norwalk NoV bound to glycoconjugates with Types 1 and 2 H and A, Le^b, ALe^b, Le^y, and ALe^y, but not to B or BLe^b structures [823,827].

Non-secretor (se^{428}/se^{428}) genotype also appears to confer some degree of resistance to HIV-1 infection [817].

Rotaviruses are the most common cause of severe diarrhoea in infants and young children. A cell-attachment protein (VP8*) of a human rotavirus recognised A antigen and virus infectivity of a human cell line was abrogated by anti-A and infectivity was enhanced in Chinese hamster cells when genetically modified to express A antigen [828].

2.20.3 Malaria

There is convincing evidence that a major selection pressure driving the evolution of the ABO polymorphism has been provided by *Plasmodium falciparum* malaria. The global distribution of ABO phenotypes shows that O is more common, relative to non-O, in those parts of the world where *P. falciparum* infection is endemic [829,830]. Published evidence for group O individuals being more susceptible to *P. falciparum* infection is contradictory, but literature reviews, meta-analyses and a genome-wide association study provide very strong evidence that group O is associated with milder disease and group A with more severe disease, particularly in children [829–833, 855].

P. falciparum-infected red cells express on their surface the *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), 'sticky knobs' that are manufactured by the parasite and bind A and, possibly to a lesser extent, B antigens on uninfected red cells to produce clusters of infected and uninfected red cells called rosettes [832,834]. Rosettes still form with group O red cells, with the involvement of other receptors such as CR1 (see Section 20.7), but they are smaller and weaker than in non-O red cells. A and B antigens are also involved in sequestration, the adherence of infected red cells to the vascular endothelium [829,830,834]. Rosetting and sequestration protect the parasite by delaying the circulation of the infected cells towards their destruction in the spleen, but also play a key role in the severe and fatal cerebral malaria that kills so many children in affected areas of the world.

Since transfusion of A or B red cells to patients with severe malaria could promote rosetting, it has been proposed that it might be preferable to give O red cells to non-O patients with severe malaria requiring transfusion [834].

Although there are many other selective factors that have affected ABO evolution, including the bacterial and viral infections and thrombotic diseases described in this section, there can be little doubt that the ABO profile was shaped through blood group expression on red cells by the most virulent form of malaria, described as 'the strongest known force for evolutionary selection in the recent history of the human genome' [835].

2.20.4 Clotting

For well over half a century statistical associations between clotting anomalies and ABO phenotype have been recognised, with thrombotic disease more common in A than in O individuals and bleeding more common in O than in A [808]. The coagulation glycoproteins von Willebrand factor (VWF) and Factor VIII (FVIII) form a tight, non-covalently bound complex in the plasma. VWF has two major functions: to mediate the capture and interaction of platelets at sites of vascular damage and to stabilise plasma FVIII and carry it to sites of vascular injury. Plasma from non-O individuals contains about 25–30% more VWF than that from group O individuals, with AB > A > B > O > O_h (Bombay) (reviewed [836, 837]). VWF quantities are also affected by ABO genotype, with A/O^1 and B/O^1 individuals having lower VWF plasma levels than *A/A* and *B/B* individuals, respectively. ABH-active oligosaccharides are located on the complex *N*-glycans of VWF.

Plasma VWF levels are regulated in part by the metalloprotease ADAMTS13, which cleaves VWF at Tyr1605–Met1606, facilitating clearance from the plasma. Reduced cleavage can lead to vascular occlusion, whereas excessive cleavage results in increased bleeding. Proteolysis was significantly faster for group O compared with non-O VWF. The cleavage site on VWF is flanked by *N*-glycans at Asn1515 and Asn1574. These *N*-glycans express ABH antigens. It is likely that the additional GalNAc and Gal residues on the A- and B-active oligosaccharides affect VWF conformation, reducing access of ADAMTS13 to the VWF cleavage site and reducing clearance of VWF from the plasma. The resultant higher levels of plasma

VWF associated with non-O account for increased prevalence of venous and arterial thrombosis in non-O individuals. Associations of plasma FVIII levels with ABO phenotype are probably secondary affects of VWF levels [836,837].

A systematic review and meta-analysis confirmed historical associations between some vascular disorders and non-O phenotype: myocardial infarction, angina, peripheral vascular disease, cerebral ischaemia of arterial origin, and venous thromboembolism (VTE) [838]. The importance of *ABO* as a risk factor for VTE and in coronary artery disease was further confirmed by genome-wide association studies [839,840]. All of these associations result from the effect of *ABO* on VWF.

2.20.5 Pancreatic cancer

Since the 1950s numerous statistical analyses have indicated that group A individuals are at a higher risk than group Os for a variety of forms of cancer [808], although a recent meta-analysis suggested that the association between ABO and cancer is limited to exocrine pancreas malignancy [841]. Pancreatic cancer is a common cause of cancer-related mortality in the developed world, owing mainly to its late diagnosis and poor response to therapy. In 2009, in two large, independent populations, ABO was statistically associated with the risk of pancreatic cancer, with the highest risk observed for group B, intermediate risks for A and AB, and the lowest risk for O [842]. An increased risk was observed for A_1 over O, but not A_2 over O [843]. In the same year a genome-wide association study revealed that a SNP in intron 1 of *ABO*, in complete linkage disequilibrium with the *O* allele, is associated with lower risk of pancreatic cancer [844]. No significant effect on risk was associated with secretor status [843]. Risch [845] proposes that ABO phenotype influences the behaviour of *H. pylori* (Section 2.20.1), which affects gastric and pancreatic secretory function, which, in turn, influences the pancreatic carcinogenicity of dietary- and smoking-related *N*-nitrosamine exposures, and hence risk of pancreatic cancer.

2.20.6 Fucosidosis

Fucosidosis is a rare lysosomal storage disease with an autosomally recessive mode of inheritance, often fatal within the first 5 years of life. It is characterised by an accumulation of fucosylated glycolipids and glycoproteins in neural and visceral tissues, as a result of an α-L-fucosidase deficiency resulting from inactivating mutations [846,847]. Fucosidosis patients may have enhanced expression of Lewis antigens on their red cells and in their saliva [848–850]. Two siblings with fucosidosis were both H secretors and yet had Le(a+b+) red cells, with very high levels of red cell and salivary Le^a and Le^b expression [849]. Both had normal H activity, suggesting that the deficient fucosidase is specific for the α1→4 linkage to GlcNAc found in Lewis active structures and not the α1→2 linkage to Gal of H-active structures (see Table 2.3). These results suggest that biosynthesis of fucosylated structures in healthy individuals depends on a balance of fucosyltransferase and fucosidase activities.

2.21 Functional aspects

The role played by sialyl-Le^x and, to a lesser extent, sialyl-Le^a as ligands for lectin-like cell adhesion molecules, selectins, has been mentioned in Sections 2.3.4 and 2.18.3. Otherwise, almost nothing is known about the functions of ABO and Lewis antigens.

The red cell membrane has about 10^6 molecules of band 3 (anion transporter), 7×10^5 molecules of the glucose transporter, and about 10^6 molecules of polyglycosylated lipids [851]. All these molecules, plus some others of lower abundance, carry ABH antigens. The ABH-active oligosaccharides contribute to the glycocalyx or cell coat, an extracellular matrix of carbohydrate that protects the cell from mechanical damage and attack by pathogenic micro-organisms [851,852].

Carbohydrate structures on cell surfaces are exploited by pathogenic micro-organisms to gain entry to the cell or to facilitate parasite survival within the infected cell. Carbohydrate polymorphisms have almost certainly arisen in an attempt to evade microbial infection and have subsequently been maintained over at least 13 million years by a variety of conflicting selective forces (Section 2.20) [853].

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3 MNS Blood Group System

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3.1 History and introduction

MNS, the second blood group system discovered, is probably second only to Rh in its complexity. The 46 antigens of the MNS system are listed in Table 3.1.

The first antibodies to the M and N red cell antigens were found in rabbits immunised with human red cells. This was the result of a deliberate search by Landsteiner and Levine [1–4] in 1927 for more human blood groups, at a time when A and B were the only red cell antigens known. Human alloanti-M and -N are relatively uncommon antibodies and generally not clinically significant. Landsteiner and Levine [3,4] showed that M and N are inherited as the products of alleles, and this was soon confirmed by further family studies [5,6]. MN is polymorphic in all populations tested: the frequencies of the common phenotypes in white people are M+ N– 28%, M+ N+ 50%, and M– N+ 22%.

In 1947, Walsh and Montgomery [7] found an alloantibody, anti-S, detecting an antigen related to M and N. As a result of testing 190 English blood samples, Sanger *et al.* [8,9] found that 86% of S+ samples were M+, whereas only 63% of S– samples were M+, a highly significant difference. The relationship between MN and S was clearly not allelic, but could result from very closely linked loci. Anti-s, an alloantibody detecting the product of an allele of *S*, was reported in 1951 by Levine *et al.* [10]. Very close linkage between *MN* and *Ss* was subsequently confirmed by family studies [11]; very few examples of recombination between these loci are documented. Ss is polymorphic in most populations. Phenotype frequencies in white people are as follows: S+ s– 11%, S+ s+ 44%, and S– s+ 45%. Greenwalt *et al.* [12] found that about 1% of African Americans are S– s– and lack the high frequency antigen named U [13,14]. S– s– is extremely rare in Europeans. Complexities involving S– s– associated with weak expression of U soon became apparent. Table 3.2 shows the common MNSs phenotypes and genotypes, and their frequencies in white English and African American populations.

M and N determinants are carried on glycophorin A (GPA), the major red cell sialic acid-rich glycoprotein (sialoglycoprotein, SGP). M differs from N in the amino acid composition of the extracellular tip of GPA: M has Ser1 and Gly5; N has Leu1 and Glu5 (counting amino acids from the N-terminus of the mature protein, residues 20 and 24 counting from the translation-initiating

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Table 3.1 Antigens of the MNS system (system 002).

Number	Name	Characteristics
MNS1	M	Polymorphic; GPA 1-5 (20-24) Ser-Ser*-Thr*-Thr*-Gly-
MNS2	N	Polymorphic; GPA 1-5 (20-24) Leu-Ser*-Thr*-Thr*-Glu-
MNS3	S	Polymorphic; GPB Met29 (48)
MNS4	s	Polymorphic; GPB Thr29 (48)
MNS5	U	HFA associated with presence of S or s
MNS6	He	LFA; GPB 1-5 (20-24) Trp-Ser*-Thr*-Thr*-Gly-
MNS7	Mi^a	LFA; probably product of junction of A2 and BΨ3 (or altered A3)
MNS8	M^c	GPA 1-5 (20-24) Ser-Ser*-Thr*-Thr*-Glu-
MNS9	Vw	LFA; GPA Thr28Met (47), Asn26 (45) not glycosylated
MNS10	Mur	LFA associated with expression of *GYPB* pseudoexon
MNS11	M^g	LFA; GPA 1-5 (20-24) Leu-Ser-Thr-Asn-Glu-
MNS12	Vr	LFA; GPA Ser47Tyr (66)
MNS13	M^e	Determinant common to GPA.M and GPB.He
MNS14	Mt^a	LFA; GPA Thr58Ile (77)
MNS15	St^a	LFA; product of junction of exons B2 or A2 and A4
MNS16	Ri^a	LFA; GPA Glu55Lys (74)
MNS17	Cl^a	LFA; inherited with *Ms*
MNS18	Ny^a	LFA; GPA Asp27Glu (46)
MNS19	Hut	LFA; GPA Thr28Lys (47), Asn26 (45) not glycosylated
MNS20	Hil	LFA; product of junction of exons A3 and B4 with s
MNS21	M^v	LFA; GPB Thr3Ser (22)
MNS22	Far	LFA; possibly inherited with *MS* or *Ns*
MNS23	s^D	LFA; GPB Pro39Arg (58)
MNS24	Mit	LFA; GPB Arg35His (54)
MNS25	Dantu	LFA; probably product of junction of exons B4 and A5
MNS26	Hop	LFA; GPA Arg49Thr* (68)
MNS27	Nob	LFA; GPA Arg49Thr* (68) + GPA Tyr52Ser (71)
MNS28	En^a	Heterogeneous – HFAs on GPA
MNS29	ENKT	HFA; GPA, antithetical to Nob (MNS27)
MNS30	'N'	HFA; GPB 1-5 (20-24) Leu-Ser*-Thr*-Thr*-Glu-
MNS31	Or	LFA; GPA Arg31Trp (50)
MNS32	DANE	LFA; Pro-Ala-His-Thr-Ala-Asn in GP(A-B-A).Dane
MNS33	TSEN	LFA; product of junction of exons A3 and B4 with S
MNS34	MINY	LFA; product of junction of exons A3 and B4 with S or s
MNS35	MUT	LFA; generally behaves as anti-Mur+Hut
MNS36	SAT	LFA; probably product of junction of exons A4 and B5
MNS37	ERIK	LFA; GPA Gly59Arg (78)
MNS38	Os^a	LFA; GPA Pro54Ser (73)
MNS39	ENEP	HFA; GPA, antithetical to HAG (MNS41)
MNS40	ENEH	HFA; GPA, antithetical to Vw (MNS9)
MNS41	HAG	LFA; GPA Ala65Pro (84)
MNS42	ENAV	HFA; GPA, antithetical to MARS (MNS43)
MNS43	MARS	LFA; GPA Glu63Lys (82)
MNS44	ENDA	HFA; GPA-B-A, antithetical to DANE (MNS32)
MNS45	ENEV	HFA; GPA Val62Gly (81)
MNS46	MNTD	LFA; GPA Thr17Arg (36)

*O-glycosylated. HFA and LFA, high and low frequency antigens.
Numbers in parentheses representing amino acid position counting from the translation-initiating methionine.

Table 3.2 Common MNSs phenotypes and deduced genotypes and their frequencies in white European and African American populations.

	Europeans*		African Americans†	
Phenotype	Genotype	%	Genotype	%
M+ N– S+ s–	*MS/MS*	5.7	*MS/MS* or *MS/Mu*	2.1
M+ N– S+ s+	*MS/Ms*	14.0	*MS/Ms*	7.0
M+ N– S– s+	*Ms/Ms*	10.1	*Ms/Ms* or *Ms/Mu*	15.5
M+ N– S– s–		0	*Mu/Mu*	0.4
M+ N+ S+ s–	*MS/NS*	3.9	*MS/NS*, *MS/Nu*, *Mu/NS*	2.2
M+ N+ S+ s+	*MS/Ns* or *Ms/NS*	22.4	*MS/Ns* or *Ms/NS*	13.0
M+ N+ S– s+	*Ms/Ns*	22.6	*Ms/Ns*, *Ms/Nu*, *Mu/Ns*	33.4
M+ N+ S– s–		0	*Mu/Nu*	0.4
M– N+ S+ s–	*NS/NS*	0.3	*NS/NS* or *NS/Nu*	1.6
M– N+ S+ s+	*NS/Ns*	5.4	*NS/Ns*	4.5
M– N+ S– s+	*Ns/Ns*	15.6	*Ns/Ns* or *Ns/Nu*	19.2
M– N+ S– s–		0	*Nu/Nu*	0.7

*Frequencies from tests on 1000 white English people [15].
†Frequencies compiled by Race and Sanger [16] from tests on 1322 African Americans.
u represents all genes that result in no expression of S or s.

methionine – see Section 3.2.2). Carbohydrate, especially sialic acid, also plays a part in the expression of M and N antigens.

S and s are carried on another red cell SGP, glycophorin B (GPB). The S/s distinction arises from Met29Thr (48) in GPB. The first 26 amino acid residues from the extracellular terminus of GPB are identical to those of N active GPA (GPA.N). Consequently, GPB also demonstrates N activity (often referred to as 'N'), which is detected on the red cells of homozygous *M/M* individuals by some anti-N.

Red cells of individuals homozygous for the very rare MNS-null gene M^K lack all MNS antigens and have no GPA or GPB. Cells of another very rare phenotype, called En(a–), lack GPA and, consequently, MN antigen expression (apart from the 'N' antigen carried on GPB). En(a–) cells express normal Ss antigens but lack a variety of GPA-borne high frequency antigens collectively named En^a. En(a–) cells also lack Wr^b, expression of which results from an interaction between GPA and the red cell glycoprotein band 3 (Chapter 10). S– s– U– cells are deficient in GPB, but express normal MN antigens. GPA- and GPB-deficient phenotypes mostly result from gene deletions.

There are numerous low frequency red cell antigens associated with the MNS system (Table 3.1). Some are known to result from amino acid substitutions and/or glycosylation changes in GPA or GPB, but many are associated with abnormal hybrid glycophorin molecules comprising partly of GPA and partly of GPB. These hybrid glycophorins are presumed to have arisen as a result of chromosome misalignment followed by unequal crossing-over or gene conversion involving *GYPA* and *GYPB*, the genes encoding GPA and GPB.

GYPA and *GYPB* are homologous and, together with *GYPE*, a third homologous gene that may produce glycophorin E, they constitute a gene cluster on chromosome 4 at 4q31.22.

3.2 Biochemistry and molecular genetics

3.2.1 Glycophorins

Numerous intrinsic membrane proteins and glycoproteins are anchored within the phospholipid bilayer of the red cell membrane. Some of the glycoproteins are heavily glycosylated and rich in sialic acid (*N*-acetylneuraminic acid) and are called sialoglycoproteins or glycophorins (Table 3.3). Two of these glycophorins carry the MNS determinants: glycophorin A (GPA), M or N; glycophorin B (GPB), S or s. For reviews see [17–20]. Glycophorins C and D, which carry the Gerbich antigens, are genetically unrelated to the MNS system and are described in Chapter 18.

Glycophorins traverse the red cell membrane once and consist of a polypeptide backbone with its carboxy-terminus (C-terminus) inside the cell and its amino-terminus (N-terminus) outside the membrane (Figure 3.1). Attached to the polypeptide chain are two types of carbohydrate structures: *N*-linked oligosaccharides (*N*-glycans) and *O*-linked oligosaccharides (*O*-glycans). *N*-glycans are generally complex carbohydrate chains attached to the amide-nitrogen of asparagine, usually through GlcNAc. The tripeptide Asn-Xaa-Thr/Ser (where Xaa is any amino acid except proline) is a prerequisite for *N*-glycosylation. GPA has one *N*-glycan (Figure 3.2); GPB is not *N*-glycosylated. The *O*-glycans on glycophorins are smaller molecules and are attached to the

Table 3.3 Red cell glycophorins and some notations used in early publications.

Glycophorin		Gene	MW kDa	Blood group antigens	Other notations		
Glycophorin A	GPA	*GYPA*	43 000	M/N En^a	CD235A	α	PAS-2
Glycophorin B	GPB	*GYPB*	25 000	S/s 'N'	CD235B	δ	PAS-3
Glycophorin E	GPE	*GYPE*					
Glycophorin A dimer	GPA_2		86 000	M/N En^a		α	PAS-1
Glycophorin B dimer	GPB_2		50 000	S/s 'N'		δ	
Glycophorin AB heterodimer	GPAB		68 000	M/N S/s En^a 'N'		αδ	PAS-4
Glycophorin C	GPC	*GYPC*	40 000	Ge3 Ge4	CD236C	β	PAS-2′
Glycophorin D	GPD	*GYPC*	30 000	Ge2 Ge3		γ	

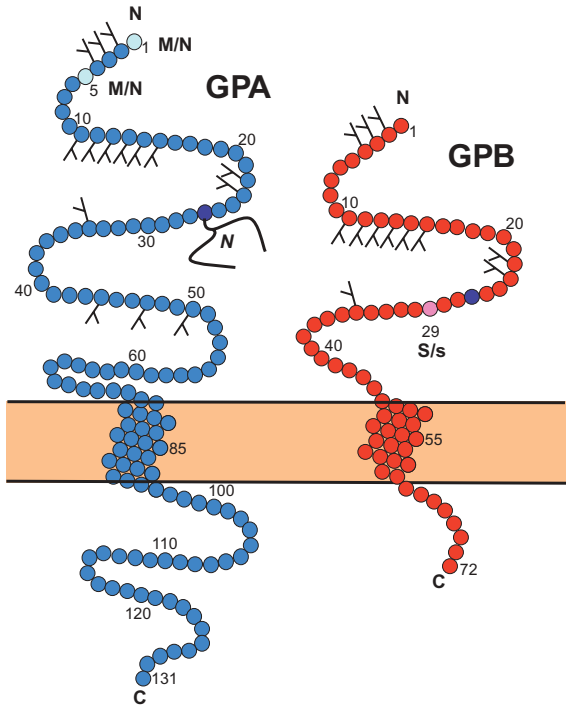


Figure 3.1 Diagrammatic representation of glycophorin A (GPA) and glycophorin B (GPB), and their situation in the red cell membrane, showing the positions of the M/N polymorphism at positions 1 and 5 of GPA, the S/s polymorphism at position 29 of GPB, and the *N*-glycan at Asn26 of GPA.

hydroxyl-oxygen of serine or threonine. They typically have the disialotetrasaccharide structure shown in Figure 3.2, although other structures have been identified [17], some of which express ABH activity [21]. All carbohydrate chains are attached to the extracellular domain of the polypeptide backbone (Figure 3.1).

Glycophorins, especially GPA and GPB, probably exist in the membrane in their monomeric (GPA and GPB) and dimeric (GPA_2 and GPB_2) forms, and as a heterodimer (GPAB) (Table 3.3). Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) and immunoblotting of membranes from M+ N+ S+ s+ red cells demonstrates that anti-M immunostains GPA, GPA_2 and GPAB, anti-N immunostains GPA, GPB, GPA_2, GPB_2 and GPAB, and anti-S and -s immunostain GPB, GPB_2 and GPAB [22–24].

GPA is closely associated with band 3, the Diego blood group antigen, and both molecules must be present for expression of the Wr^b (DI4) antigen (Sections 10.4.2 and 3.15). GPA and GPB are part of the band 3/Rh macrocomplex, which contains tetramers of band 3, trimers of the Rh proteins and the Rh-associated glycoprotein, ICAM-4 (LW), and CD47, and is linked to the cytoskeleton through ankyrin and protein 4.2 (Section 10.7 and Figure 10.2)

3.2.2 Glycophorin A (CD235A)

GPA is the most abundant red cell sialoglycoprotein and, together with band 3, the most abundant red cell membrane glycoprotein. The number of copies of GPA per red cell has been estimated to be about 1×10^6 [25].

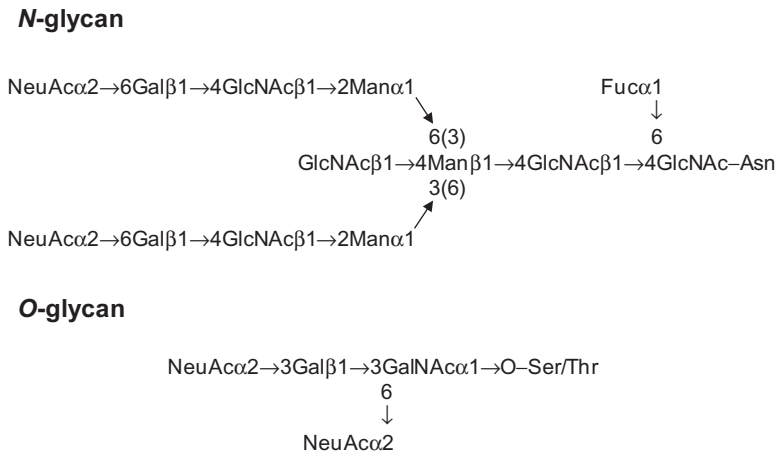


Figure 3.2 Predominant *N*-glycan of GPA and *O*-glycans of glycophorins. For abbreviations see Table 2.4.

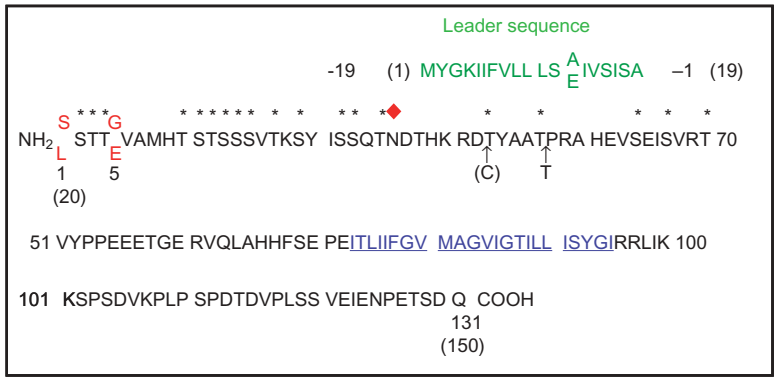


Figure 3.3 Amino acid sequence of glycophorin A (see Table 1.2 for code). Amino acids are numbered from the N-terminal residue of the mature protein with the numbers from the N-terminal Met of the nascent protein in parentheses. The leader sequence is cleaved after insertion of the protein into the membrane. Amino acids at positions –7, 1, and 5 for GPA.M and GPA.N are shown above and below, respectively. * represents probable sites of *O*-glycosylation. ◆ represents site of *N*-glycosylation. The membrane spanning domain is underlined. T, major trypsin cleavage site on intact cells; (C), partial chymotrypsin cleavage site.

GPA consists of 131 amino acids, organised into three domains:

1 an extracellular N-terminal domain of 72 amino acids;
2 a hydrophobic membrane-spanning domain of 23 amino acids; and
3 a C-terminal cytoplasmic domain of 36 amino acids.

The extracellular domain contains a high proportion of serine and threonine residues and is heavily glycosylated with about 15 *O*-glycans and a single *N*-glycan. GPA is generally present in the membrane in dimeric form, with the polypeptides associated at the hydrophobic membrane-spanning domain [26,27]. The prevalence of glycines and β-branched amino acids in the GPA transmembrane domain, but also residues in the extracellular region Ala65–Glu72, are important for stable dimer formation [28–30].

The amino acid sequence of GPA is shown in Figure 3.3 and Figure 3.1 is a diagrammatic representation of how it may appear in relation to the red cell membrane. Most of the amino acid sequence for GPA was resolved by degradation amino acid sequencing techniques [17,31,32]. The complete sequence in Figure 3.3 was deciphered from the nucleotide sequence of *GYPA* cDNA isolated by Siebert and Fukuda [33]. The amino

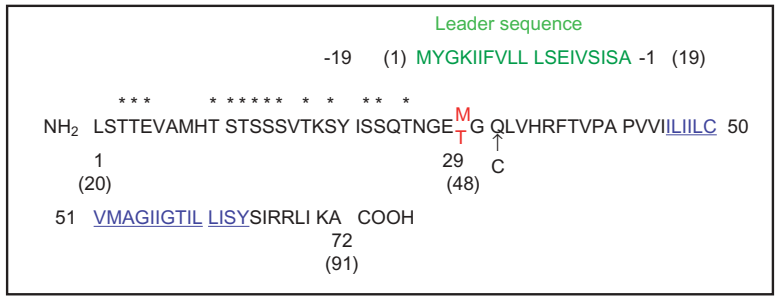


Figure 3.4 Amino acid sequence of glycophorin B (see Table 1.2 for code). Amino acids are numbered from the N-terminal residue of the mature protein with the numbers from the N-terminal Met of the nascent protein in parentheses. The leader sequence is cleaved after insertion of the protein into the membrane. Amino acids at position 29 for GPB.S and GPB.s are shown above and below, respectively. * represents probable sites of *O*-glycosylation. The membrane spanning domain is underlined. C, chymotrypsin cleavage site.

acids numbered −1 to −19 represent a leader sequence, which ensures correct insertion of the whole molecule into the cell membrane and is cleaved after membrane insertion. The tradition for numbering amino acids in GPA and GPB differs from that used for most other proteins, in that they are numbered from the N-terminal residue of the mature protein. For convenience of understanding, that tradition will be maintained in this chapter, numbering amino acids with counting starting at the N-terminal methionine of the nascent protein is often provided in parentheses.

The asparagine residue at position 26 (45) bears an *N*-linked oligosaccharide, a branched structure of approximate MW 3 kDa [34,35]. The predominant *O*-glycan of glycophorins is the branched tetrasaccharide shown in Figure 3.2, comprising two molecules of sialic acid, one Gal, and one GalNAc [36], although 1–6% of the molecules express ABH activity through one or more the sialic acid (NeuAc) residues being replaced by α1,2-fucose, plus GalNAc or Gal [37]. Other variations of this molecule have been recognised, including monosialotrisaccharides and trisialopentasaccharides [17,38]. Glycosylation of GPA is incomplete and variable; only about 15 of the 21 extracellular serine or threonine residues are glycosylated [21,32,39] and variation in *O*-glycosylation of different GPA molecules occurs within the same individual [25,40].

3.2.3 Glycophorin B (CD235B)

GPB is closely related in structure to GPA. It consists of 72 amino acids that, like GPA, fit into three domains:

1 an N-terminal glycosylated extracellular domain of 44 amino acids;

2 a hydrophobic membrane-spanning domain of 20 amino acids; and

3 a very short C-terminal cytoplasmic tail of eight amino acids.

The amino acid sequence shown in Figure 3.4 was deduced from the nucleotide sequence of *GYPB* cDNA [41,42]. Figure 3.1 shows a diagrammatic representation of GPB in the membrane. GPB has about 11 *O*-glycans and is devoid of *N*-glycosylation.

The first 26 amino acids from the N-terminus of the mature GPB protein are identical to those of the N antigenic form of GPA (GPA.N). This accounts for the N activity of GPB, usually denoted 'N' to distinguish it from the N activity of GPA.N. Unlike GPA, the N-terminal amino acids of GPB are not cleaved by trypsin treatment of intact red cells, so 'N' is a trypsin-resistant N antigen. The only difference between the first 26 amino acid residues of GPA.N and GPB is that Asn26 is *N*-glycosylated in GPA, but not in GPB [43]. This is because, unlike GPA, GPB does not have the requisite serine or threonine residue at position 28. GPA and GPB show other homologies. Amino acid residues 59–67 and 75–100 of GPA closely resemble residues 27–35 and 46–71 of GPB [17,44]. Also, the leader sequences of GPA and GPB are almost identical.

There are an estimated 1.7–2.5 × 10^5 molecules of GPB per red cell [25]. S+ s− red cells have about 1.5 times as much GPB as S− s+ cells, with S+ s+ cells having an intermediate quantity [17,45].

3.2.4 Cloning and organisation of the genes for GPA, GPB, and GPE

Siebert and Fukuda [33] synthesised mixed oligonucleotides corresponding to amino acid sequences in the

C-terminal region of GPA and used them to prime the synthesis of *GYPA* cDNA from a K562 cell-line cDNA library. *GYPA* cDNA from this library was then isolated with mixed oligonucleotides representing the central region of GPA. *GYPB* cDNA was isolated from a K562 cDNA library by the use of two oligonucleotide probes, one specific for a *GYPA* sequence and the other representing a sequence common to *GYPA* and *GYPB* cDNA [42]. Subsequently full-length *GYPA* and *GYPB* cDNA clones were isolated from human reticulocyte cDNA libraries [41] and from *GYPA* cDNA from a human fetal liver library [46].

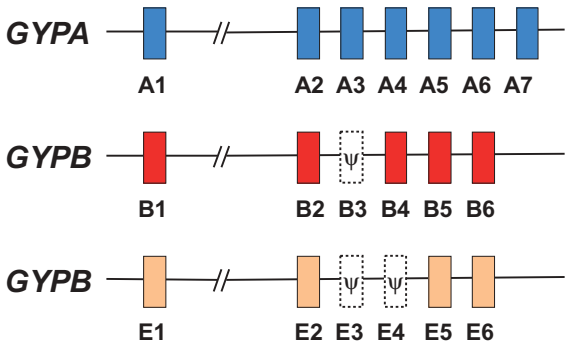


Figure 3.5 Genomic organisation of *GYPA*, *GYPB*, and *GYPE*. Boxes represent exons and pseudoexons (ψ). The pseudoexons are numbered so that homologous exons maintain the same number in all three genes [18].

GPA and GPB are encoded by discrete, single-copy genes specific to each polypeptide [47]. *GYPA* is about 40 kb and contains seven exons [48,49] (Figure 3.5, Table 3.4). Exon A1 codes for most of the leader peptide and is separated by a large intron of about 30 kb from exon A2, which encodes the remainder of the leader peptide and the first 26 amino acids of the extracellular domain. Exons A3 and A4 encode the remainder of the extracellular domain, exon A5 the transmembrane portion, and exon A6 and part of exon A7 the cytoplasmic portion of the polypeptide. Most of the seventh exon is not translated. Three *GYPA* mRNA transcripts, of 2.8, 1.7, and 1.0 kb differing from each other in the lengths of their 3′ untranslated regions, have been identified in erythroleukaemic cell lines [33,50,51], fetal liver [46], and reticulocytes [33,41].

GYPB has only five exons [48,49] (Figure 3.5). Exons B1 and B2 are almost identical to exons A1 and A2 of *GYPA*. The third exon, numbered B4 to demonstrate homology with exon A4, encodes the S/s polymorphism. Exon B5 encodes most of the C-terminal part of the polypeptide and exon B6 the C-terminal amino acid residue, the remainder of exon B6 being untranslated (Table 3.4). A sequence within the second intron of *GYPB* is homologous to exon 3 of *GYPA*. This 'pseudoexon' is not translated because the gt invariable splice site sequence at the 5′ end of intron 3 is mutated to tt [48], and other changes in intron 2 may also affect splicing. So the 'pseudoexon' is spliced out of *GYPB* mRNA, together with the regions homologous to the second and third introns of *GYPA* [52]. GPB, therefore, lacks a segment

Table 3.4 Structural organisation of *GYPA*, *GYPB*, and *GYPE*. Amino acid residues encoded by each exon are numbered from the N-terminal residue of the mature protein (with numbers from the N-terminal methionine of the nascent protein in parentheses). The exons are numbered according to the system used by Huang and Blumenfeld [18] in which pseudoexons are numbered so that homologous exons maintain the same number in all three genes.

GYPA		*GYPB*		*GYPE*	
A1	5′ UT, −19 to −8 (1–12)	B1	5′ UT, −19 to −8	E1	5′ UT, −19 to −8
A2	−7 to 26 (13–45)	B2	−7 to 26	E2	−7 to 26
A3	27–58 (46–77)	B3	Pseudoexon	E3	Pseudoexon
A4	59–71 (78–90)	B4	27–39 (46–58)	E4	Pseudoexon
A5	72–100 (91–119)	B5	40–71 (59–90)	E5	27–58 (45–77)
A6	101–126 (120–145)	B6	72 (91), 3′ UT	E6	59 (78), 3′ UT
A7	127–131 (146–150), 3′ UT				

homologous to amino acid residues 27–58 of GPA. The *GYPB* pseudoexon may be translated in rare phenotypes where a functional acceptor splice site is transplanted into *GYPB* from *GYPA* by gene conversion [18] (see Section 3.11).

During isolation of *GYPA* and *GYPB*, a closely associated gene, *GYPE*, was discovered [49,53,54]. The three genes show 90% nucleotide sequence homology, the coding regions demonstrating more diversity than the non-coding introns [18,48]. *GYPE* is present in all human DNA investigated including that from En(a–), S– s– U–, homozygous M^K, and homozygous *GYP(A–B)*Hil* (Mi.V) individuals [49,53,55–57]. *GYPE* has a similar genomic structure to that of *GYPB*, but contains four exons and two pseudoexons [54,57] (Figure 3.5, Table 3.4). The predicted polypeptide has 78 amino acids including a 19-residue leader peptide. The mature cell surface glycoprotein protein would be 59 amino acid residues long, carry 11 *O*-glycans and no *N*-glycan, have a MW of 17 kDa, and express M antigen. Anstee [58] speculated that a red cell membrane component of approximate MW 20 kDa, revealed by monoclonal anti-M on immunoblots of membranes from red cells of all MN groups, might be GPE.

The MNS genes were initially located on chromosome 4q28-q31 by an accumulation of linkage analyses [59] and *in situ* hybridisation [46,56,57]. The three genes are situated on chromosome 4q31.21 in the order 5′-*GYPA*-*GYPB*-*GYPE*-3′ and are over 95% identical to each other from the 5′ flanking region to an *Alu* repeat sequence 1 kb downstream of the exon encoding the transmembrane domain [54]. They are about an equal distance apart and occupy 330 kb of genomic DNA [60]. These genes appear to have evolved from a common ancestral gene through homologous recombination events involving *Alu* sequences [54] (see Figure 3.13). A putative precursor fragment downstream from *GYPA* has been isolated [61].

The proximal promoters for the three glycophorin genes had very similar sequences and the three genes exhibited similar transcriptional activities [62,63]. *GYPA* promoter activation is dependent on the assembly of a multifactorial complex containing SCL, a haemopoiesis-specific transcription factor essential for erythropoiesis, and the transcription factors Sp1, GATA-1, E47, Ldb1, and LMO2 [64]. *GYPB* mRNA transcript was less stable than *GYPA* transcript, however, and *GYPE* transcript was very unstable [63]. Post-transcriptional regulation, therefore, may be responsible for the very different quantities of the three protein products at the cell surface. Regulatory factors controlling transcription of *GYPB* have been analysed in detail [65–67].

For reviews on the molecular genetics of glycophorins see [18–20,68].

3.3 MN and Ss polymorphisms

3.3.1 M and N antigens (MNS1 and MNS2)

The amino acid sequence of GPA demonstrates polymorphic variation at positions 1 (20) and 5 (24), represented serologically as the MN blood groups. GPA isolated from M+ N– individuals has serine as the N-terminal residue of the mature protein and glycine at the fifth position; GPA from M– N+ individuals has Leu1 and Glu5 (Table 3.5) [39,69,70]. Both forms of GPA can be isolated from M+ N+ individuals. The terminal serine of GPA.M is not glycosylated; amino acid residues 2, 3, and 4 of GPA.M and GPA.N are *O*-glycosylated. The Ser/Leu1 polymorphism results from 59C/T (TCA/TTA) creating an *Sfa*NI restriction site in the *GYPA*M* allele and an *Mse*I site in *GYPA*N*; the Gly/Glu5 change results from two SNPs, 71G/A, 72T/G (GGT/GAG), creating a *Bsr*I site in *GYPA*M* and a *Dde*I site in *GYPA*N* [66]. In addition, there is an Ala/Glu polymorphism associated with M/N at position –7 (13) in the leader peptide (Figure 3.3) [41]. A total of 17 nucleotide differences in exons 1, 2, and 7 and introns 1–4 distinguish the standard *GYPA*M* and *GYPA*N* alleles [71]. Another *GYPA*M* allele, common in Asians, shares characteristics of both standard *GYPA*M* and *GYPA*N* [71].

Although the amino acid residues at positions 1 and 5 of GPA are primarily responsible for the MN polymorphism, glycosylation is also important in the serological expression of the M and N antigens. Many anti-M and

Table 3.5 Some N-terminal pentapeptides of GPA and GPB.

Glycophorin A	Human M	Ser–Ser*-Thr*-Thr*-Gly-
	Human N	Leu–Ser*-Thr*-Thr*-Glu-
	Human M^g	Leu–Ser–Thr–Asn–Glu-
	Human M^c	Ser–Ser*-Thr*-Thr*-Glu-
	Chimpanzee	Ser–Ser–Thr*-Thr*-Glu-
Glycophorin B	Human 'N'	Leu–Ser*-Thr*-Thr*-Glu-
	Human He	Trp–Ser*-Thr*-Ser*-Gly-

**O*-glycosylated.

-N do not bind sialidase-treated red cells [22,72–75]. This could result from an alteration in steric presentation of receptors dependent on an interaction between sialic acid and amino groups [76,77]. GPA contains a small number of non-galactosylated *O*-glycans, which may be partially sialylated. The number of these residues on Ser2, Thr3, and Thr4 is substantially higher in GPA.N than in GPA.M [78]. The role of sialic acid and amino acid sequence in M and N specificity is discussed further in Section 3.4.2.

3.3.2 S and s antigens (MNS3 and MNS4)

The S/s polymorphism is represented by a single amino acid substitution in GPB at position 29 (48); GPB.S has Met29 and GPB.s has Thr29 [45], (*GYPB*S*, 143T; *GYPB*s*, 143C; exon B4). A synthetic peptide representing residues 25–33 of GPB.S inhibited anti-S poorly and the equivalent s-specific peptide did not inhibit anti-s at all [79], suggesting that the S/s antigen sites are more complex than just the amino acid residue at position 29. Anti-S sera are heterogeneous; a synthetic peptide representing residues 25–43 of GPB.S inhibited six of 16 anti-S [80] and three human monoclonal anti-S demonstrated different serological characteristics (Section 3.18.8) [81,82].

A 251C/G polymorphism in exon 5 of *GYPB*s* encodes Thr/Ser58 in the membrane-spanning domain of GPB. *GYPB*S* has 251G [83].

3.3.3 Antigen, gene, and phenotype frequencies

All the early frequency studies, and very many others since, were performed with anti-M and -N alone [84,85]. In most populations, including most of Europe, Africa, and East Asia, the frequency of the *M* allele is between 50 and 60% and the *N* allele between 40 and 50%. A higher frequency of *M* is found in East Baltic countries, including European Russia, and in most of South Asia and western Indonesia. Highest *M* frequencies, over 90%, are found among the Inuit and some Native Americans. Lowest *M* frequencies are in the Pacific area and among Australian Aborigines. In regions of Papua New Guinea incidence of *M* drops below 2%.

The different S antigen frequencies between people of the three MN phenotypes (Table 3.6) led Sanger *et al.* [8,9] to recognise the association between MN and S; if there was no association the frequency of S+ would be the same in M+ N–, M+ N+, and M– N+ individuals.

There are four common haplotypes in white people, *MS*, *Ms*, *NS*, and *Ns*. Anti-s has often been considered too scarce to be used in large population studies, but, because *u*, a silent allele at the *Ss* locus, is extremely rare in white people, the S– phenotype can be considered to result from homozygosity for *s* in white populations and haplotype frequencies can be deduced. In Europeans, *MS* and *Ms* have similar frequencies, but *Ns* is about five or six times more common than *NS*. In white British donors the following haplotype frequencies were calculated: *MS*, 25%; *Ms* 29%; *NS* 7%; *Ns* 39% [15,16].

S is less common in the Far East than it is in Europe [84]: 52 624 Taiwanese were all s-positive [86]. S is virtually absent from Australian Aborigines [84].

Although Ss antigens are almost always present in white people, the phenotype S– s– is not uncommon in people of African origin (see Table 3.2). The presence of S and/or s is associated with the high frequency antigen U. S– s– cells are either U– or have a variant form of U (Section 3.6). For the purposes of describing gene frequencies *u* will be used here to represent a silent gene at the *GYPB* (*Ss*) locus (Table 3.2). Table 3.7 shows

Table 3.6 Some approximate phenotype frequencies in the MNS system for people of northern European extraction (after [16]).

	Ss phenotype		
MN phenotype	All (%)	S+ (%)	s+ (%)
All	100	55	89
M+ N–	28	72	78
M+ N+	50	56	92
M– N+	22	31	97

Table 3.7 Frequencies of *MNSs* haplotypes in black populations, deduced from serological testing.

Haplotype	USA (1000) [16,84,85,87]	Senegal (459) [84,88]
MS	0.1001	0.0244
Ms	0.3496	0.0492
Mu	0.0454	0.0747
NS	0.0614	0.0640
Ns	0.3744	0.2940
Nu	0.0691	0.1137

All tested with anti-M, -N, -S, and -s.
u represents all genes that result in no expression of S or s.

Table 3.8 MN and Ss genotype frequencies on four populations of American blood donors, obtained by testing on the BeadChip array [89].

		Genotypes *GYPA*			Genotypes *GYPB*		
Ethnic group	No. tested	*M/M*	*M/N*	*N/N*	*S/S*	*S/s*	*s/s*
Caucasians	1243	0.34	0.44	0.22	0.14	0.40	0.46
African Americans	690	0.41	0.32	0.27	0.07*	0.24*	0.69*
Hispanic	119	0.39	0.44	0.17	0.13	0.32	0.55
Asian	51	0.27	0.57	0.16	0.08	0.12	0.80

* includes silent and variant alleles.

frequencies, deduced from serological tests, for the six most common haplotypes in African Americans and in West Africa.

Table 3.8 shows genotype frequencies for four populations determined by molecular testing [89].

3.3.4 Inheritance

A wealth of family evidence has proven that *MN* and *Ss* behave as two very closely linked loci with virtually no recombination occurring between them [11,90,91]. The inheritance of MNSs in black families is complicated by *u* (Section 3.6), but, from the point of view of analysing families, *u* (initially called S^u) can be considered an allele at the *Ss* locus, recessive to *S* and *s*.

With the knowledge that *MN* and *Ss* represent two discreet gene loci encoding different proteins, it should be no surprise that recombination, presumably as a result of crossing-over, occurs between them, although documented examples of such recombination are rare. In one family an M– N+ S– s+ father and M+ N+ S+ s+ mother had three M– N+ S– s+, three M+ N+ S+ s+, and one M+ N+ S– s+ children [92]. The mother must be *MS/Ns* because she has three presumed *Ns/Ns* children and three presumed *MS/Ns* children (because the father is probably *Ns/Ns*); yet the other child appears to be *Ms/Ns*. Thus the mother appears to have passed *Ns* to three children, *MS* to three children, and *Ms* to another. This anomaly of inheritance may be explained by any one of several genetic mechanisms – suppression, deletion, mutation, or recombination – but Chown *et al.* [92] favour recombination between *MS* and *Ns* producing an *Ms* oocyte in the mother. Six other families are described in which MNSs inheritance anomalies could result from recombination [93].

3.4 Effects of enzyme treatment on the MNSs antigens

3.4.1 Proteases

Various proteolytic enzymes have proved very useful in the serological identification, analysis, and definition of antigens belonging to the MNS system. The effects on isolated sialoglycoproteins of proteases, glycanases, and various peptide bond-splitting chemicals such as cyanogen bromide, have been extremely valuable in elucidating the biochemical structure of these glycoproteins and of some of the antigens associated with them. Certain proteases, such as trypsin and chymotrypsin, are highly specific for the peptide bonds they cleave, although access of enzymes may be blocked by the presence of neighbouring oligosaccharides or, when intact cells are treated, by the red cell membrane or other membrane-bound components.

Effects of enzymes on low frequency MNS antigens are reviewed in [94].

3.4.1.1 Trypsin

Trypsin catalyses the hydrolysis of peptide bonds on the carboxyl side of lysine and arginine residues. There are at least seven trypsin cleavage sites on GPA, at amino acid residues 30, 31, 39, 61, 97, 101, and 102; desialylation of the molecule is required before cleavage can occur at some of these sites [32,40]. The sites at residues 30 and 31 are partial cleavage sites; 50% of native GPA molecules are cleaved at residue 31 and 10% at residue 30 [40]. When intact cells are treated with trypsin the N-terminal 39 amino acids of GPA are severed, resulting in loss of M antigen and GPA-borne N antigens, as well as any other

determinants located on this portion of the glycoprotein. Purified GPB may be cleaved by trypsin at amino acid residue 35 [45], but trypsin treatment of intact cells does not denature GPB. The blood group antigens S, s, and the 'N' antigen located at the N-terminus of GPB are, therefore, trypsin-resistant [95,96].

3.4.1.2 Chymotrypsin

Chymotrypsin, or more accurately α-chymotrypsin, normally hydrolyses the peptide bond on the carboxyl side of the aromatic amino acids phenylalanine, tryptophan, and tyrosine, as well as leucine, methionine, asparagine, and glutamine. Isolated GPA may be cleaved at residues 34, 64, 98, and 118 [32,40]. M and N antigens on intact cells are partially resistant to chymotrypsin treatment [95,96]; red cell membrane-bound GPA may be cut by chymotrypsin behind residue 34, but only in those molecules devoid of an *O*-glycan on Thr33 [40]. Treatment of red cells with sialidase followed by chymotrypsin results in abolition of all M and N activity. Treatment of red cells with chymotrypsin cleaves the N-terminal region of GPB at amino acid residue 34 [45,97], destroying S, s, and 'N' activity [95,96]. GPB.S is denatured by a lower concentration of chymotrypsin than GPB.s [96].

3.4.1.3 Papain, ficin, bromelin, pronase

The enzymes papain, ficin, and bromelin have a rather broad specificity and the preparations available are often crude compared with trypsin and chymotrypsin. Most GPA- and GPB-borne antigens are destroyed by treatment of red cells with these enzymes, only those situated close to the red cell membrane survive. Pronase, a bacterial enzyme, behaves in a similar way [96]. Whereas papain or ficin treatment of cells readily destroys M, N, 'N', and s antigens, S activity is less easily abolished [95,96,98].

3.4.2 Sialidase

GPA and GPB carry about 15 and 11 *O*-linked oligosaccharides, respectively, most of which contain two molecules of sialic acid. In addition, GPA has one *N*-glycan, which is also usually sialylated (see Figure 3.2). Sialidase (neuraminidase) treatment of red cells removes at least some of these sialic acid residues, altering the charge and possibly the shape of the molecules. Most human sera contain anti-T, which recognises desialylated *O*-linked oligosaccharides and consequently agglutinates sialidase-treated red cells (Section 3.17.2). High concentrations of sialidase are required to remove most of the sialic acid from GPA; α2→3 linked sialic acid is more easily removed from GPA by sialidase than α2 → 6 linked sialic acid [99].

M and N antibodies vary in their requirements for sialic acid in order to agglutinate red cells. Judd *et al.* [74] obtained the following results from testing human MN sera (adsorbed to remove anti-T) with sialidase-treated cells: 27 anti-M, reaction abolished with nine, unaffected with 16, and enhanced with two; seven anti-N, reaction abolished with three, weakened with two, and unaffected with two. Specific M and N antibodies produced by immunising rabbits with desialylated red cell glycoproteins only agglutinated sialidase-treated cells [100]. Most monoclonal anti-M and -N do not react, or react comparatively weakly, with desialylated red cells or isolated glycophorins (see Section 3.18.6). The effect of sialidase applies equally to N on GPA and 'N' on GPB. M and N activity may be restored to sialidase-treated red cells by resialylation catalysed by sialyltransferases [101].

S, s, and most other MNS system antibodies are not sialic acid-dependent.

3.5 The rare glycophorin A-deficient phenotypes En(a–) and M^K

The following section describes unusual MNS phenotypes caused by two very rare gene deletions. *En* (*GYPA*Null)*, a deletion of the coding region of *GYPA*, causes a deficiency of GPA, but not GPB. M^K (*GYPAB* Null*), a deletion of the coding regions of *GYPA* and *GYPB*, is responsible for deficiency of GPA and GPB. The multifarious antibodies detecting non-polymorphic determinants on GPA, collectively called anti-En^a, will also be described here. There are many other variant *MNS* genes that do not produce normal GPA, and many rare phenotypes in which part of GPA is missing and consequently anti-En^a (and/or anti-Wr^b) may be made. These are described in other sections, especially those on hybrid glycophorins.

3.5.1 En(a–)

When Darnborough *et al.* [102] described a new antibody to a high frequency red cell antigen, they noted that the red cells of the antibody maker, a pregnant English woman (MEP), and of several members of her family, gave a variety of unusual blood grouping reactions. These effects were deduced as being 'due to some factor affecting the red cell structure possibly by modifying the cell envelope'. The antibody was named anti-En^a (for envelope) and the rare red cell phenotype En(a–). A second

En(a–) propositus with anti-En^a was found in Finland [103] and two subsequent En(a–) propositi with anti-En^a, one found in Finland [104] and the other in the United States [105], are part of the same extended family. Two other En(a–) propositi with anti-En^a, a French Canadian [106] and a Pakistani [107], have been reported. Two En(a–) Japanese blood donors without anti-En^a were found by screening red cells from Japanese blood donors with monoclonal anti-En^a [108,109].

Anti-En^a represents an umbrella term, which describes antibodies to determinants on various parts of GPA. The En(a–) phenotype can arise in a number of ways. Typically, En(a–) represents homozygosity for a rare gene deletion (*GYPA*Null*) at the *GYPA* locus, resulting in no production of GPA, but normal production of GPB. The original En(a–) phenotype in an English family [102], however, did not arise in this way and probably represents heterozygosity for a complex *GYP(A–B)* hybrid gene [often called *En(UK)*] and an M^K gene [110–112]; this En(a–)UK phenotype will be discussed in more detail in Section 3.10.4. The Finnish, French Canadian, Pakistani, and Japanese En(a–) phenotypes [En(a–)Fin] appear to result from homozygosity for *GYPA*Null* [often called *En(Fin)*] [103–109].

Nine En(a–) individuals presumed to be homozygous for *GYPA*Null* are reported; five from the three branches of the Finnish family [103–105]. In serological MN testing of families, *En* behaves as a silent allele of *MN* (Figure 3.6) [113]. Parents or offspring of an En(a–) individual are M+ N– or M– N+; none are M+ N+.

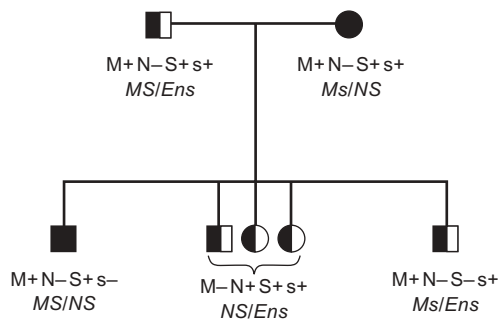


Figure 3.6 Family demonstrating how the presence of an *En* allele can explain an M+ N– father with three M– N+ children and associated red cell membrane modifications. Red cells of all family members are En(a+). The genotype of the mother is deduced from her parents and sibs (not shown). ■●, no modification of red cell membrane; ◧◐, modified red cell membrane, single dose of M or N. Redrawn from [113].

3.5.1.1 Serological characteristics of En(a–) cells

En(a–) cells do not react with alloanti-En^a in the sera of En(a–) propositi, with autoanti-En^a, or with monoclonal antibodies to epitopes restricted to GPA.

Typical En(a–) cells lack any M antigen or trypsin-sensitive N antigen; they do express trypsin-resistant N because of the 'N' antigen of GPB. En(a–)UK cells lack N and 'N', but have a trypsin-resistant 'M' antigen [110,114,115] for reasons that will be described in Section 3.10.4.

En(a–) cells have normal or enhanced expression of S and/or s.

En(a–) cells are Wr(a–b–) (DI:–3,–4). The significance of this is discussed below (Section 3.5.3.2) and in Chapter 10.

En(a–) cells have a number of other unusual serological characteristics, probably resulting from their reduced sialic acid content, which arises from absence of the major red cell surface sialic acid-rich glycoprotein. Most of these characteristics are seen, to a lesser extent, in red cells of individuals heterozygous for *En* and are also apparent in other MNS variants that result in a reduction of red cell membrane sialic acid content. En(a–) cells are not aggregated, or at least are aggregated only very weakly, by polybrene and protamine sulphate [105,106,116]. Saline suspensions of En(a–) cells are directly agglutinated by 'incomplete' anti-D and other Rh antibodies when the appropriate Rh antigens are present on the cells; these antibodies do not agglutinate En(a+) cells of the same Rh phenotype. En(a–) cells react more strongly with certain lectins than En(a+) cells [103,117]. Particularly useful for this purpose are extracts from the seeds of *Sophora japonica* (adsorbed with group AB cells to remove anti-A+B activity) and *Glycine soja*, although extracts from seeds of *Bauhinia purpurea* (anti-N), *Dolichos biflorus* (anti-A_1), *Phaseolus lunatus* (anti-A), and *Arachis hypogea* (anti-T) can all distinguish En(a–) cells from En(a+) cells. *Maclura aurantiaca* lectin, which binds to red cell sialoglycoproteins [114], reacts only weakly with En(a–) cells [117].

3.5.1.2 Frequency of En(a–) and the *En* allele

En is very rare: only five unrelated *En/En* individuals are known. Tests with anti-En^a on 12 500 English, 8800 Finnish, and 200 Estonian donors revealed no En(a–) individuals [102,103]; tests on 250 000 Japanese donors revealed one [108,109]. Three possible *En* heterozygotes were found by screening 6202 donors by direct agglutination of their red cells with 'incomplete' anti-D and anti-c (see above) [118]. An investigation of red cells from 1300

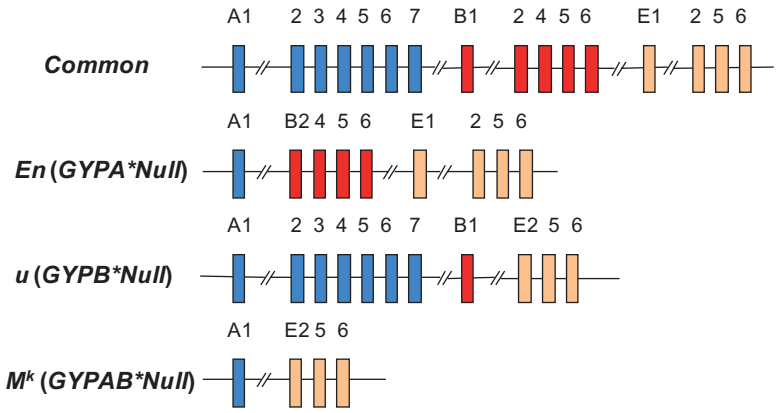


Figure 3.7 Diagram to show the extent of deletions of *GYPA*, *GYPB*, and *GYPE* responsible for En(a–) (*GYPA*Null* or *En*), U– (*GYPB*Null* or *u*), and M^K (*GYPAB*Null*) phenotypes [57,62]. In each case the deletion breakpoints occur within the very long first introns of these genes. *GYPA*Null* represents a deletion of exons A2–A7 and B1; *GYPB*Null*, a deletion of exons B2–B6 and E1; and *GYPAB*Null*, a deletion of exons A2–A7, B1–B6, and E1.

Scottish donors for aggregation in protamine sulphate revealed two probable *En* heterozygotes, one with *En(UK)* and the other with *En(Fin)* [116].

3.5.1.3 Biochemistry

Typical En(a–) red cells lack GPA; no GPA, or its dimer (GPA_2) and heterodimer (GPAB), is detected by SDS PAGE of En(a–) cells [22,23,39,105–107,109,114,115,118]. GPB of En(a–) cells has normal mobility on SDS PAGE. Band 3, the anion exchanger, has an elevated MW in En(a–) cells resulting from an increase in the length of its *N*-glycan [106,107,109,114,115,119,120]. GPA facilitates the movement of band 3 from internal membranes to the cell surface, so in GPA-deficient cells band 3 protein may remain longer in the Golgi network providing greater opportunity for elongation of the *N*-glycan [121] (see Section 3.23).

En(a–) red cells have reduced red cell electrophoretic mobility resulting from a low level of sialic acid [103]. En(a–) cells have about 40% of the sialic acid of normal cells and cells from *En* heterozygotes, about 70% of normal levels [103,106,119]. This reduction in sialic acid increases the agglutinability of red cells, explaining many of the unusual serological characteristics of GPA-deficient red cells.

3.5.1.4 Molecular genetics

Although results of Southern blotting of genomic DNA from two individuals with the En(a–) phenotype initially suggested a complete deletion of *GYPA* and normal *GYPB* [46,53,55], exon A1 and the upstream untranslated region of *GYPA* is not deleted and the deletion encompasses exons A2–A7 of *GYPA* and exon B1 of *GYPB* (Figure 3.7) [57,62]. As exon 1 of both genes codes for most of the leader sequence, but not for any of the mature protein, this would result in production of no GPA. It would, however, permit normal expression of GPB, which would be produced by a *GYP(A–B)* hybrid gene comprising the promoter sequences and exon A1 of *GYPA* and exons B2–B6 of *GYPB*.

3.5.2 M^K

The name M^K was coined for a new allele of *M* and *N* that appeared to produce neither M nor N [122]. A second family showed that not only did M^K appear to be a silent allele at the *MN* locus, it was also silent at the *Ss* locus [123]. The effect of the M^K gene was highlighted in this family by apparent maternal exclusions in three generations: an M+ N– S– s+ woman (presumed genotype Ms/M^K) had an M– N+ S– s+ (Ns/M^K) daughter, who married an M+ N– S+ s+ (*MS/Ms*) man and had one M+ N– S+ s– (MS/M^K) and two M+ N– S– s+ (Ms/M^K) daughters, one of whom had an M– N+ S– s+ (Ns/M^K) child.

The first M^K/M^K homozygotes were a Japanese blood donor and his brother [124]. Their red cells were M– N– S– s– U– En(a–) Wr(a–b–) and showed all the reactions characteristic of reduced sialic acid. This M^K phenotype has subsequently been found in two Japanese sisters [125], an African American child [126], and a Turkish woman and her brother [127].

Red cells of individuals with one M^K gene resemble cells of *En* heterozygotes regarding the unusual serological characteristics associated with reduced sialic acid levels [118,128–131] (see Section 3.5.1.1). Eight heterozygous M^K individuals were found in 10 097 Swiss donors, either by testing with 'incomplete' anti-D and anti-c by direct agglutination of untreated cells or by M and N dosage determination [118,132]. In one apparent MS/M^K heterozygote, a dysmorphic, mentally deficient child with part of the long arm of chromosome 2 translocated onto the long arm of chromosome 4, the rare gene was not present in either parent and appeared to result *de novo* from the effect of his chromosomal translocation [133,134]. (The MNS genes are on the long arm of chromosome 4.)

3.5.2.1 Biochemistry

M^K produces neither GPA nor GPB; red cells from M^K/M^K homozygotes are devoid of GPA and GPB [124–126]. Red cells of people heterozygous for M^K have about half the normal quantity of GPA and GPB [39, 135–137].

Band 3 of M^K cells, like that of En(a–) cells, shows an increase in MW resulting from increased glycosylation (Section 3.5.1.3). This amounted to an increase of about 3 kDa in band 3 in M^K homozygotes and heterozygotes [124,125,135]. M^K red cells have reduced sulphate transport activity owing to a lowered binding affinity of band 3 for sulphate ions [138]. M^k red cells also appear to have a reduction in size of the glucose transporter GLUT1 [138] and a 2 kDa increase in the cytoskeletal glycoprotein, band 4.1 [124]. Red cell sialic acid content is reduced by about 30% in cells of M^K heterozygotes [128,131,135,139] and 70% in cells from M^K/M^K homozygotes [124].

The M^K/M^K genotype has not been very informative about the functions of GPA and GPB. It had no obvious adverse effect on the health of five M^K individuals and no abnormal haematological effects were apparent [124].

3.5.2.2 Molecular genetics

Southern blot analysis revealed that genomic DNA from one of the Japanese M^K/M^K individuals lacked all fragments of *GYPA* and *GYPB* that encode mature GPA and GPB, suggesting a single deletion spanning both genes (*GYPAB*Null*) [53,55]. The deletion does not include exon A1 and the upstream promoter region of *GYPA*, but does include exon E1 of *GYPE*, to leave a hybrid *GYP(A–E)* gene (Figure 3.7) [57,62].

3.5.3 Anti-En^a, anti-Wr^b, and the determinants they define

3.5.3.1 Alloanti-En^a

The first three examples of anti-En^a, those from the English En(a–) propositus (MEP) and the first two Finnish En(a–) propositi (VB, GW), appeared to be antibodies of identical specificity, which reacted with all red cells save those of the En(a–) phenotype [102–104]. All three propositi had been transfused. None of the four En(a–) siblings of the three propositi had made anti-En^a; none had been transfused, but one had been pregnant five times. These three anti-En^a sera were later shown to contain at least two antibodies to high frequency antigens, anti-En^a and -Wr^b [103,140]. Neither antibody reacted with cells of the En(a–) Wr(a–b–) phenotype, but anti-En^a, unlike anti-Wr^b, did react with En(a+) Wr(a+b–) cells (described below). The other En(a–) propositus from the Finnish family (ERP), who had never been transfused but had been pregnant twice, made a similar mixture of antibodies [105]. The French-Canadian En(a–) propositus (RL), a man with no transfusion history, made anti-En^a and no anti-Wr^b; his anti-En^a differed from the other examples in that it defined a trypsin-sensitive antigen and could be inhibited by extracted M and N substances [106].

Adsorption and elution studies with red cells treated with different proteases (trypsin, papain, ficin) and with red cells of rare MNS phenotypes in which only part of GPA is present, have shown that anti-En^a is a collective term for antibodies to determinants at a variety of sites on the extracellular domain of GPA [103,141–143]. For convenience, Issitt *et al.* [144] defined three broad categories of anti-En^a according to the effect of proteases on the antigenic determinants they detect.

Anti-En^{a}TS recognises a **T**rypsin-**S**ensitive determinant and is typified by the antibody of the French-Canadian En(a–) propositus (RL) [106]. It does not react with En(a+) red cells treated with trypsin, ficin, or papain and can be inhibited by isolated GPA and reacts with a determinant around amino acid residues 31–39, but only on those GPA molecules that are not glycosylated at Thr33 [40]. GPA on intact cells is cleaved by trypsin at amino acid residue 39. Two other anti-En^{a}TS, one alloantibody and one autoantibody, had different binding sites on the N-terminus of GPA [40].

Anti-En^{a}FS represents those En^a antibodies that recognise a **F**icin-**S**ensitive (papain-sensitive), trypsin-resistant determinant. Anti-En^{a}FS is found as a separable component in the sera of some En(a–) propositi and may also be an autoantibody [145]. Anti-En^{a}FS is inhibited by

isolated GPA [141,142]. All of six anti-En^{a}FS were directed at a determinant around residues 46–56 of GPA and five of the antibodies required glycosylation at Thr50 for binding [40].

Anti-En^{a}FR represents those antibodies that react with a **F**icin-**R**esistant (papain-resistant), trypsin-resistant determinant. They differ from anti-Wr^b by reacting with En(a+) Wr(a+b–) cells. Anti-En^{a}FR have been isolated from the sera of some En(a–) individuals, which also contain anti-En^{a}FS. Anti-En^{a}FR is not easily inhibited with isolated GPA. En^{a}FR appears to represent a labile structure within amino acid residues 62–72 of GPA, requiring lipid for complete antigenic expression [146].

3.5.3.2 Anti-Wr^b and the Wr^b (DI4) antigen

The name anti-Wr^b was tentatively used by Adams *et al.* [147] in 1971 for an antibody detecting a public antigen in the serum of a woman whose Wr(a+) red cells had a double dose of Wr^a. The antibody reacted more strongly with Wr(a–) cells than with Wr(a+) cells. The association between Wr^b and MNS first became apparent when En(a–) cells were found to be Wr(a–b–) [148,149]. Subsequent immunochemical studies suggested that the Wr^b determinant is located on GPA [146,150], which presented an enigma as it had long been known that Wr^a is genetically independent of MNS [16].

Details of the nature of the relationship of Wr^b to the MNS system are provided in Chapter 10. The Wr^a/Wr^b dimorphism results from an amino acid substitution within band 3, but Wr^b can only be detected when band 3 is associated with GPA in the membrane. Hence, GPA-deficient red cells are Wr(b–). Whether Wr^a expression also requires GPA presence is unclear as no GPA-deficient individual with a *Wr^a* allele has been found.

3.5.3.3 Clinical significance of anti-En^a

The clinical outcome of transfusing En(a+) red cells to patients with anti-En^a is varied. A patient with anti-En^{a}TS and depressed red cell GPA expression died of an HTR [151] and an En(a–) patient with anti-En^a and anti-Wr^b suffered a mild delayed HTR after receiving six units of En(a+) blood [104]. Predominantly IgG1 anti-En^a with a lesser IgG3 component in a patient with M^K phenotype was responsible for severe HDFN [127]. Functional assays with anti-En^{a}FR/Wr^b provided further evidence that these antibodies are of clinical importance [152]. Ideally patients with alloanti-En^{a}FR/Wr^b should be transfused with compatible red cells.

3.5.3.4 Autoanti-En^a

Autoantibodies with En^a specificity have been identified [153], some in patients with severe and fatal AIHA [145,154,155]. These are usually of the anti-En^{a}FS type, though some may be anti-En^{a}FR [155]; pure anti-En^{a}FS occurs in 1.6% of warm autoantibody cases [156]. Anti-Wr^b is not uncommon as an autoantibody specificity (Chapter 10).

3.5.3.5 Antibodies produced by M^K individuals

Neither of two Japanese men with M^K phenotype had been transfused, yet both produced an antibody to a public antigen [124]. These antibodies did not react directly with En(a–) cells, but their reactivity with En(a+) cells was reduced by adsorption with En(a–) cells. The antibodies, which did not react with sialidase or pronase-treated cells and could be inhibited by sialoglycoprotein preparations, detect a Pr-like determinant common to GPA and GPB (see Section 3.5.4). Two M^K women made anti-En^a; both had been pregnant several times, but had not been transfused [125,127].

3.5.3.6 Monoclonal antibodies to non-polymorphic determinants on GPA

Many monoclonal antibodies to non-polymorphic epitopes on GPA have been described [22,25,81,157–160]. These antibodies can be loosely divided into four categories.

1 Antibodies to trypsin-, ficin- and papain-sensitive epitopes on GPA, but not GPB (anti-En^{a}TS). These epitopes are either on the N-terminal side of the trypsin cleavage site at Arg39 or overlap Arg39. They are mostly within the region of amino acid residues 30–45.

2 Antibodies to trypsin-resistant, but ficin- and papain-sensitive epitopes on GPA (anti-En^{a}FS). These epitopes are mostly in the region of amino acid residues 49–58.

3 Antibodies that detect epitopes, usually sialic acid-dependent, common to GPA and GPB. This epitope is generally situated within the N-terminal 26 amino acid acids, which are identical in GPA.N and GPB. Antibodies of this type react with En(a–) and S– s– U– cells, which lack GPA and GPB, respectively, but they do not react with M^K cells, which lack both GPA and GPB, or with trypsin-treated S– s– U– cells, which lack GPB plus the N-terminal 39 amino acids of GPA.

4 Antibodies to epitopes on the cytoplasmic, C-terminal domain of GPA. These antibodies do not react with intact red cells and are usually detected by immunoblotting.

One murine monoclonal antibody bound to ^{53}Pro-Pro-Glu-Glu-Glu^{57} of GPA (anti-En^{a}FS), but also reacted with ^{395}Pro-Pro-Glu-Gln^{398} of the cytoskeletal component, protein 4.1 [161]. Monoclonal antibodies directed at different epitopes on GPA have proved extremely valuable in the analysis of the many rare MNS variants described in this chapter.

3.5.4 Pr and Sa antigens and antibodies

The protease-labile Pr antigens [162] were originally named Sp_1 by Marsh and Jenkins [163] and HD by Roelcke [164] (Chapter 25). They are generally detected by cold-active IgM human monoclonal autoantibodies in cold haemagglutinin disease or post-infection [165]. Pr antigens have been subdivided into a number of subspecificities, Pr_1, Pr_2, and Pr_3, distinguished by chemical modification of sialic acid residues with periodate oxidation and carbodiimide treatment (reviewed in [162]). Anti-Sa cold agglutinins are similar to anti-Pr in detecting a sialic acid-dependent antigen, but anti-Sa react, albeit only weakly, with papain-treated cells [166].

Anti-Pr_1, -Pr_2, -Pr_3, and -Sa react with *O*-linked oligosaccharides on sialoglycoproteins [17,167–169]. Most anti-Pr and all anti-Sa recognise immunodominant α2,3-*N*-neuraminic acid groups linked to Gal, but a minority of anti-Pr may recognise α2,6-*N*-neuraminic acid groups [170]. It is probable that anti-$Pr_{1–3}$ detect the predominant form of *O*-glycan, the disialotetrasaccharide shown in Figure 3.2, and that anti-Sa detects incompletely sialylated glycoconjugates (monosialotetrasaccharides) found on the more internal parts of GPA [162]. GPA and GPB express $Pr_{1–3}$ [17,167,168]; GPA is also Sa-active [166,168]. Pr_2 and Sa are also detected on red cell gangliosides [169]. Pr antibodies agglutinate En(a–) cells very weakly and do not agglutinate M^K cells at all [16,106,171]. Unfortunately, no adsorption/elution studies were performed with M^K cells, which would be expected to carry some Pr determinants on other membrane components such as GPC and GPD.

In common with some other autoantibodies directed at determinants on GPA [172], anti-Pr has caused fatal or life-threatening AIHA, which is far more severe than would be predicted from the characteristics of the antibodies [172–176]. Brain *et al.* [176] have proposed a novel mechanism of immune destruction, independent of complement or macrophage classical processes, where antibodies to GPA damage a subpopulation of red cells by increased phosphatidylethanolamine exposure and membrane permeability, and formation of cation-permeable lipid pores.

3.6 U antigen and the GPB-deficient phenotypes S– s– U– and S– s– U+^{var}

3.6.1 U (MNS5) and anti-U

U was the name given by Wiener *et al.* [14,177] in 1953 to a high frequency blood group antigen present on the red cells of 977 of 989 African Americans and all of 1100 white Americans. When, in the following year, Greenwalt *et al.* [12] found a second example of anti-U, it became apparent that U was associated with the MNS system: both U– samples available were also S– s–, a phenotype not previously encountered. Adsorption and elution studies showed that anti-U was not a separable mixture of anti-S and -s [12,178].

U– red cells are almost always S– s–, but S– s– cells are often U+ [83,179–181]. S– s– U+ is often referred to as S– s– U+^{var}. Strength of U antigen expression on S– s– U+^{var} red cells is variable; adsorption/elution tests or sensitive agglutination tests with a particularly potent anti-U may be required for its detection [182]. Alternatively, molecular testing is very effective for distinguishing U– and U+^{var}. Like S– s– U–, the S– s– U+^{var} phenotype is virtually exclusive to people of African origin. About 50% of S– s– red cell samples are U+^{var} [83,181,183]. In this chapter the symbol *u* will represent the gene responsible for U– when it has not been defined by molecular genetic studies.

The precise serological definition of anti-U is unclear, but the term is traditionally used to describe antibodies produced by S– s– individuals to high frequency determinants on GPB. In a study of 17 'anti-U', Storry and Reid [181] found that five failed to react with all S– s– red cells. They called these antibodies anti-U. The other 12, which reacted with S– s– U+^{var} cells, but not S– s– U– cells, they called anti-U/GPB. By these definitions, S– s– U– cells are U–, U/GPB–, whereas S– s– U+^{var} cells are U–, U/GPB+. In this respect, anti-U and -U/GPB could be considered analogous to anti-En^{a}. S– s– U– cells are totally GPB-deficient, whereas S– s– U+^{var} cells have a variant GPB molecule that expresses neither S nor s. Following transfusion or pregnancy, anti-U may broaden in specificity to become anti-U/GPB and react with S– s– U+^{var} red cells that had previously been non-reactive with serum from the same patient [184,185]. Some individuals with S– s– U+^{var} red cells have made

anti-U or, at least, a U-like antibody [83,186]; one made anti-s [187].

S– s– U– and S– s– U+^{var} cells usually lack the trypsin-resistant 'N' antigen carried on GPB [179,188–190], although weak 'N' activity was detected on isolated sialoglycoprotein from two M+ N– S– s– U+^{var} individuals [191]. Consequently, apart from cells of certain very rare MNS variant phenotypes, M+ N– S– s– red cells are the only cells with no obvious expression of N. Immunised N– U– people are likely to make anti-U and/or potent anti-N, which reacts strongly with the N on both GPA and GPB [188].

He is a low frequency antigen expressed at the N-terminus of a GPB molecule that does not express 'N' (see Section 3.7.4). There is a strong correlation between expression of variant U antigen and He. Of 104 S– s– red cell samples, 51 (49%) reacted with anti-U/GPB, but not anti-U; of these 51 S– s– U+^{var} samples, 36 (71%) were He+ [83]. None of the S– s– U– red cells that were non-reactive with anti-U/GPB was He+.

U is generally resistant to denaturation by sialidase, trypsin, chymotrypsin, papain, and ficin. Unusual examples of anti-U, however, do not react with papain-treated cells and an antibody component to a papain-sensitive determinant (UPS) was identified in about 50% of sera containing anti-U [192]. U-like alloantibodies in two S– s– U+^{var} and two S– s– U– individuals were non-reactive with ficin-, pronase-, α-chymotrypsin-treated red cells, non- or weakly reactive with papain-treated cells, and reactive with trypsin-treated cells [186], resembling in this way some U-like autoantibodies [193].

S– s– U– red cells do not show most of the unusual serological reactions associated with reduced sialic acid that are characteristic of red cells deficient in GPA (Section 3.5.1.1), though *Glycine soja* lectin may agglutinate U-deficient cells [194].

Other rare phenotypes in which the red cells may be S– s– U– are the Rh-deficiency phenotypes (Section 5.16.5) and phenotypes arising from homozygosity for hybrid genes encoding the rare SAT and St^a antigens (Sections 3.10.3 and 3.14.2).

Further details of anti-U, including clinical significance and autoanti-U, can be found in Section 3.18.10. Anti-U^Z and anti-U^X are described in Section 3.18.11.

3.6.2 Biochemistry

S– s– U– red cells are deficient in GPB. This has been demonstrated by failure to inhibit anti-S, -s, or -U with SGPs isolated from S– s– U– cells, by SDS PAGE of red cell membranes or isolated SGPs, and by immunoblotting with antibodies and lectins directed at determinants on GPB [23,24,189–191,195–198,]. Red cells of individuals heterozygous for *u* have roughly half of the normal quantity of GPB [189,190,195]. Small quantities of GPB, about 2–3% of normal, were detected on S– s– U+^{var} cells [191].

GPB normally carries about 11 *O*-glycans and S– s– U– and S– s– U+^{var} red cells demonstrate a reduction in sialic acid by about 15% compared with normal cells [195,199]. Cells of individuals heterozygous for *u* have about a 9% sialic acid reduction [195]. Unlike the GPA-deficiency phenotypes, S– s– U– is not associated with any apparent alteration of band 3 [195].

U appears to be a labile structure requiring lipid for full expression [200]. In this respect it resembles En^{a}FR, which is located close to the membrane on GPA (Section 3.5.3). From the results of anti-U haemagglutination-inhibition tests with GPB extracts, in the presence of lipids, amino acid residues 33–39 of GPB appeared to be essential for U antigen expression [200], but U expression also appears to be dependent on an interaction between GPB and RhAG (Section 5.20). Unlike S and s, U, as defined by most anti-U, escapes denaturation by α-chymotrypsin treatment of intact cells, because the cleavage site for chymotrypsin is between residues 32 and 33. Some U-like antibodies, however, are non-reactive with α-chymotrypsin-treated U+ red cells, suggesting that their determinants are closely related to S and s [186].

3.6.3 Molecular genetics

The S– s– U– phenotype results from homozygosity for a deletion of *GYPB* (*GYPB*Null*) encompassing exons B2–B6 of *GYPB* and also including exon E1 of *GYPE* [53,57,83,197,201] (Figure 3.7). The deletion includes the whole of the sequence of *GYPB* encoding the mature protein.

At least four genes are responsible for S– s– U+^{var}, all of which are responsible for alternative splicing of all or part of exon B5 of *GYPB* and all of which have the S sequence encoding Met48 [83]. The most common (83% of samples) has g>t at position +5 of the donor splice site of intron 5, which causes skipping of exon B5 and loss of the region that usually constitutes the membrane-spanning domain of GPB (Figure 3.8). The reading frameshift abolishes the translation stop codon close to the 5′ end of exon B6 so that the C-terminus of the glycoprotein is elongated by a novel sequence of 41 amino acids. The most common form of this gene, *GYPHe(P2)* (*GYPB*03N.03*), has a *GYPA* insert within exon 2 responsible for He expression, whereas the less common form, *GYPB(P2)* (*GYPB*03N.04*), has the

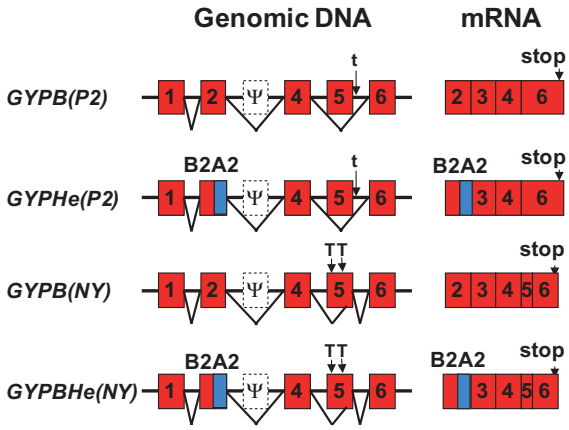


Figure 3.8 Four genes responsible for S– s– U+^{w} phenotype, showing genomic DNA with B2A2 hybrid exon 2 in genes encoding He and mis-splicing of exon 5 and resultant aberrant mRNA. There is no evidence that mis-spliced mRNA is represented as a protein in the red cell membrane [83].

normal 'N' sequence in exon B2 [83,202]. The abnormally spliced transcript encodes a variant protein of 81 amino acids, but this was not detected at the red cell surface [83].

Two other U+^{var} genes, *GYPHe(NY)* (*GYPB*03N.02*) and *GYPB(NY)* (*GYPB*03N.01*), have 208G>T and 230C>T changes in exon B5 that result in activation of a cryptic splice site at 251G causing partial skipping of exon B5 (Figure 3.8). *GYPHe(NY)* and *GYPB(NY)* have the He and 'N' sequences in exon 2, respectively. The coding sequence predicts a 43-amino acid protein, with no hydrophobic membrane-spanning domain, and which has not been detected by immunoblotting analysis [83].

The abnormal splice site sequences associated with the genes responsible for S– s– U+^{var} phenotypes suggest that skipping of exon B5 would not be absolute so that some normally spliced transcripts and low levels of normal GPB.He or GPB would be produced. This would explain the weak U and He expression detected by haemagglutination and the detection of weak bands representing a 24 kDa protein, the size of GPB, by immunoblotting with monoclonal anti-He or anti-GPA+GPB [83,183]. The low levels of GPB.He or GPB could result in conformational changes that are responsible for the absence of S and for the production of anti-U in a few individuals with S– s– U+^{var} red cells [83].

3.6.4 Frequency studies

Results of screening donors with anti-U are unreliable, because they vary according to the proportion of S– s– U+^{var} samples that give positive or negative results with the antibody reagent used, though the frequency in U– African Americans varies from 0.2 to 1.4% [188]. Table 3.2 shows M, N, S, and s phenotype frequencies in African Americans, together with genotypes in which the S– s– phenotype is assumed to have resulted from homozygosity for *u* at the *Ss* locus. The *MN* and *Ss* haplotype frequencies derived from studies of African American and African populations shown in Table 3.7 reflect a similar approach. Of 126 Pygmies from Congo, 35% were U– [203]. No S– s– U– individual was found among 1000 Bantu-speaking people of Natal [204], whereas three were found among 1000 black antenatal patients from the Eastern Cape [205].

PCR with allele-specific primers revealed that 94% of African Americans with the S– s– U+^{var} phenotype have an *He* allele of *GYPB*; the remainder have an *'N'* allele of *GYPB* [83]. Analysis of an *Eco*R1 site that is ablated by the intron 5 mutation in *GYPB(P2)* and *GYPHe(P2)* showed an allele frequency of 2.5% in African Americans [83]. From a molecular analysis of 267 African Americans, eight were heterozygous for *GYPB(P2)* or *GYPHe(P2)*, one was homozygous for *GYPB(NY)* or *GYPHe(NY)*, and in four *GYPB* was deleted [89].

Although extremely rare, the U– phenotype has been identified in people of non-African descent. S– s– U– members were found in a white family from France [196] and in a family originating from India [206]. Six of 324 Finnish Lapps [84] and two of 63 Central American Indians from Honduras [85] were S– s–.

3.7 M and N variants representing amino acid substitutions within the N-terminal region of GPA and GPB

M and N antigens are determined by the sequence and glycosylation of the N-terminal five amino acids of GPA and GPB (Table 3.5). Amino acid substitutions within this pentapeptide may affect expression of M or N and may create a new antigen. Three such variants are described in this section: M^{g} and M^{c} on GPA; He on GPB.

3.7.1 M^{g} (MNS11)

M^{g}, a very rare antigen first described in 1958 [207], is encoded by a gene that produces virtually no M or N antigen. Undetected, an *M^{g}* (*GYPA*Mg* or *GYPA*11*) allele in a family could result in apparent exclusion of parentage as an M+ N– (*M/M^{g}*) parent can have an M– N+ (*N/M^{g}*) child.

Tests with anti-M^g on over 100 000 English and American blood donors revealed no M^g+ sample [208]. In Swiss and Sicilians a much higher incidence of about one in 600 was found [208–210] (Table 3.9). Analysis of 21 Swiss families with the mating type M^g+ × M^g– and a total of 51 children confirmed that M^g behaves as an allele of *M* and *N* [208,209]. In two Bostonian families [207,251], a family from mainland Italy [210], and all of the Swiss families [208,209], M^g was aligned with *s*; in four families of Sicilian origin [210,252], the alignment was M^g with *S*. The M^g+ daughter of one of the Swiss propositi was found to have an M^g+ husband and an M– N– M^g+ child [208], the only reported person homozygous for M^g and the source of much of our serological and biochemical knowledge of M^g.

Red cells from the *GYPA.M*^{g} homozygote have a reduction in sialic acid level of about 12% from normal; heterozygotes have a 7% reduction [135]. They demonstrate many of the serological and physicochemical features characteristic of cells with reduced membrane sialic acid levels [128] (described in Section 3.5.1.1). Like M and N, M^g is denatured by treatment of the cells with trypsin, but not chymotrypsin [136,222,253,254]; unlike most anti-M and -N, anti-M^g generally react with sialidase-treated M^g+ cells [74,255,256].

M^g phenotype results from Thr4Asn (23) of GPA.N [253,256–258], the result of 68C>A in *GYPA.N* [50], possibly arising from a *GYPA.N* allele with a small *GYPB* insertion and untemplated mutations [18] (see Section 3.9). This asparagine residue is not glycosylated and the amino acid substitution also prevents, or at least grossly reduces, glycosylation of Ser2 and Thr3, a total reduction of three *O*-glycans responsible for a degree of sialic acid deficiency (Table 3.5).

Although Furthmayr *et al.* [256] detected no glycosylation of residues 2 and 3 of GPA.M^g, Dahr *et al.* [257] found them to be glycosylated in up to 25% of GPA.M^g molecules. Furthermore, 30% of GPA.M^g molecules lack the N-terminal leucine and up to 10% lack N-terminal leucine and serine, probably resulting from the *in vivo* action of amino-peptidases [79].

Anti-M^g is easily inhibited by the glycosylated N-terminal octapeptide cleaved from GPA.M^g, but not by that from GPA.N [256]. Haemagglutination-inhibition studies with various synthetic peptides and glycopeptides representing the N-terminal region of GPA showed that most anti-M^g primarily recognise a non-glycosylated structure with N-terminal leucine; only a minority are dependent on Asn4 [259,260]. Glycosylation of M^g-active peptides at positions 2, 3, or 4 abolishes M^g activity [259]. The epitope for one murine monoclonal anti-M^g is dependent on Glu5, but not Asn4; for another, Leu1 and Asn4 were the most essential components of the epitope [261]. One of six M^g antibodies reacted with a sialic acid-dependent antigen [74]. This antibody may detect a determinant on the minority glycosylated form of GPA. M^g [17].

Roughly half of the monoclonal anti-M tested reacted with cells of *M*^{g}*/N* or *M*^{g}*/M*^{g} individuals and, on immunoblots, bound to GPA.M^g [22,262–264]. The epitope detected by monoclonal anti-M that agglutinate M– M^g+ red cells is dependent on Val6 and Met8 of deglycosylated GPA (as occurs in GPA.M^g), but also requires Gly5 when the GPA is normally glycosylated [265]. Immunoblotting of M^g+ red cells with anti-M^g, polyclonal or monoclonal, revealed only GPA.M^g [222,264].

M^g+ red cells reacted with anti-DANE (-MNS32) and with the original anti-Mur (Murrell), but not with 14 other examples of anti-Mur [264]. Immunoblotting showed that the Murrell antibody was binding GPA.M^g. A possible explanation for these reactions is provided in Section 3.13.2.

3.7.1.1 Anti-M^g

M^g is extremely rare, yet anti-M^g is possibly the most common MNS antibody. In four separate searches for anti-M^g in sera of normal people the following frequencies were obtained: four from 500 sera (0.8%) in the United States [207]; 23 from 703 (3.3%) [16] and six from 340 (1.8%) [136] in England; 12 from 1614 (0.7%) in India [221]. In order to explain the high incidence of anti-M^g, Dahr *et al.* [257,260] speculated that people might be exposed to M^g-like structures by removal of carbohydrate from normal glycophorin during natural red cell destruction. Anti-M^g in 17.6% of sera from Liberia was attributed to the high level of parasitic infection [266].

Anti-M^g has been produced in rabbits [267] and as murine monoclonal antibodies [261].

3.7.2 M^c (MNS8)

Despite having an ISBT red cell antigen number, M^c cannot strictly be regarded as a blood group antigen as no anti-M^c exists. M^c is often considered to represent an intermediate between M and N [268]. M^c produces a determinant that reacts with the majority of anti-M and with the minority of anti-N (as demonstrated by the red cells of the *N/M*^{c} and *M/M*^{c} individuals, respectively) and M^c has subsequently been defined by a pattern of reactions with known anti-M and -N reagents. Several

Table 3.9 Incidence of MNS-associated low frequency antigens (in ISBT number order).

Antigen	Population	No. tested	No. positive	Antigen frequency (%)	References
He (MNS6)	African Americans	6997	207	2.958	[87,211–213]
	West Africans	1428	38	2.661	[214]
	Congolese	70	10	14.286	[213]
	South African Bantu	4000	247	6.175	[215]
	Pygmy Bush people	428	32	7.477	[216]
	African Bushmen	188	4	2.128	[217]
	Hottentots	201	21	10.448	[217]
	Papuans	33	3	9.091	[218]
	Europeans	1500	0		[214]
	White New Yorkers	500	4	0.800	[87]
	White South Africans	1000	0		[215]
Vw (MNS9)	White people	52635	30	0.057	[16]
	Grisons, SE Switzerland	1541	22	1.428	[16]
	Thais	2500	1	0.040	[219]
Mur (MNS10)	Thais	2500	1	9.640	[219]
	Minnan Chinese (Taiwan)	400	18	4.500	[220]
	Hakka Chinese (Taiwan)	100	3	3.000	[220]
	Ami Taiwanese	138	122	88.406	[220]
	Bunun Taiwanese	100	0		[220]
	White people	50101	6	0.012	[16]
M^g (MNS11)	Boston, USA	44000	0		[208]
	English	61128	0		[208]
	Swiss	6530	10	0.153	[208,209]
	Sicilians (in Belgium)	1889	3	0.159	[210]
	Italians (in Belgium, non-Sicilians)	4408	1	0.023	[210]
	Belgians	36683	0		[210]
	Bombay	9000	2	0.022	[221]
	African Americans	4254	0		[222]
Vr (MNS12)	Dutch	1200	3	0.250	[223]
Mt^a (MNS14)	White Americans	11907	28	0.235	[224]
	Swiss (Zürich)	1435	5	0.348	[16]
	African Americans	1007	1	0.099	[224]
	Thais	318	3	0.943	[225]
St^a (MNS15)	Chinese	490	8	1.633	[226,227]
	Japanese	220	14	6.364	[227]
	English	17013	20	0.118	[228]
Ri^a (MNS16)	Londoners	70501	1	0.001	[228,229]
Cl^a (MNS17)	Europeans	12541	0		[16,230]
Ny^a (MNS18)	Norwegians	9687	18	0.186	[231–233]
	Swiss	9395	1	0.010	[16]
	Germans	20000	0		[234]
	Americans	7400	0		[235]
	African Americans	350	0		[236]
	Japanese	3281	0		[236]
	Chinese	1032	0		[236]
Hut (MNS19)	White people	32591	21	0.064	[16]
	Thais	2500	1	0.040	[219]
M^v (MNS21)	English	2372	14	0.590	[237]
Far (MNS22)	Europeans	15373	0		[238,239]

(*Continued*)

Table 3.9 (*Continued*)

Antigen	Population	No. tested	No. positive	Antigen frequency (%)	References
s^D (MNS23)	White South Africans	1 000	1*	0.100	[240]
	Black South Africans	1 000	0		[240]
	Indian South Africans	500	0		[240]
	Mixed race South Africans	1 000	1	0.100	[240]
Mit (MNS24)	Canadians	3 311	4	0.121	[241]
	North Londoners	8 278	7	0.085	[242]
	Africans	662	0		[241]
Dantu (MNS25)	African Americans	3 200	16	0.500	[243]
	N. London (mostly white)	44 112	1†	0.002	[244]
Hop (MNS26)	Thais	2 500	17	0.680	[219]
Nob (MNS27)	English	4 929	3	0.061	[245]
Or (MNS31)	English	887	0		[16]
	African Americans	163	1	0.613	[16]
	Japanese	17 200	2	0. 012	[246]
DANE (MNS32)	Danes	467‡	2	0.428	[247]
SAT (MNS36)	Japanese	10 480	1	0.010	[248]
Os^a (MNS38)	Japanese	50 000	0		[249]
MNTD (MNS46)	Japanese	20 330	4	0.020	[250]

*Member of original family [240].
†Black/Indian/English/French donor from Mauritius.
‡Trypsin-treated cells screened with anti-M.

examples of M^c have been reported, all in people of European origin, and M^c exists as M^cs and M^cS [16,268,269]. Because anti-M^c does not exist, there is very little information on the frequency of M^c. Screening of red cells of 3895 Swiss with anti-M and -N reagents designed to disclose MN variants revealed one M^c/M individual [269].

The serological behaviour of M^c cells was explained in 1981 when the N-terminal amino acid sequence of GPA. M^c was determined [256,270]. At position 1 (20) is serine, characteristic of M, and at position 5 (24) is glutamic acid, characteristic of N (Table 3.5). Residues 2, 3, and 4 have normal glycosylation. *GYP*M*^{c} (*GYP*08*) represents *GYPA.N* with 59T>C, which could arise from a *GYPA.M* allele with a small *GYPB* insertion [18] (see Section 3.9).

3.7.3 Fine specificity of MN antibodies

M and N antigens differ at the first and fifth amino acids of the N-terminus of GPA (Table 3.5); anti-M detect either Ser1 or Gly5; anti-N either Leu1 or Glu5. Other factors, especially the presence of oligosaccharides, are usually also important to epitope integrity. Red cells with the rare M^c phenotype have been very useful in the elucidation of some of the fine specificities of MN antibodies, especially monoclonal antibodies, as have techniques for modification of the terminal amino acid residue by acetylation of the free amino group or by removal of the N-terminal amino acid by Edman degradation [22,81,271]. As a rough guide, most anti-M detect the presence of Ser1 and react with M^c/N cells but not with acetylated cells [262,271], whereas the minority detect Gly5 and do not react with M^c cells, but do react with acetylated cells and may bind to GPB.He, which has glycine at position 5 [272]. Some anti-M that react with a Gly5-dependent epitope cross-react with M^g [265]. The fine specificity of N antibodies is more difficult to determine. Most anti-N recognise Leu1 rather than Glu5 and do not react with M^c/M cells [271,273].

The fine specificity of MN antibodies has also been analysed by haemagglutination inhibition tests with acetone powders prepared from Chinese hamster ovary cells transfected with *GYPA* cDNA [274,275]. The cDNA either encoded GPA.M or GPA.N, or was modified by site-directed mutagenesis to encode the GPA.M^c sequence or a novel NM N-terminal sequence, Leu-Ser-Thr-Thr-Gly (see Table 3.5). One monoclonal anti-M required Gly5 and sialic acid for binding, three human alloanti-M

required Ser1 and not Gly5, and two monoclonal anti-N and *Vicia graminea* lectin required Leu1, but not Glu5.

3.7.4 He (MNS6) and M^e (MNS13)

3.7.4.1 He (MNS6)

The original anti-He was found in a rabbit serum containing anti-M [276]. Another example was made deliberately by immunising a rabbit with the red cells of Mr Henshaw from whom the antigen derived its name [214]. Subsequently, human alloanti-He have been identified [16,277,278] and many monoclonal anti-He produced [81,211].

He antigen is found in about 3% of African Americans and in various African populations with a similar or higher incidence (Table 3.9). He may be associated with *MS*, *Ms*, *NS* or *Ns*, predominantly with *NS* in black New Yorkers [212] and West Africans [214], with *MS* in Congolese [218], and with *Ns* in Papuans [218].

Serological evidence from an He+ woman with the likely genotype *MsHe/Mu*, who had made potent anti-N, and whose red cells lacked all expression of N antigen, including the 'N' antigen associated with GPB, suggested that the gene complex encoding He was producing no 'N' and that He is located on GPB [279]. Biochemical analysis of GPB from He+ red cells confirmed the association with GPB and explained the absence of 'N'. Three of the five N-terminal amino acid residues of GPB.He differ from those of normal N-active GPB: Leu1Trp; Thr4Ser; and Glu5Gly [280]. Glycosylation of this region is unchanged as Ser4 of GPB.He is *O*-glycosylated (Table 3.5). Immunoblotting with human and mouse anti-He confirmed the location of He antigen on GPB [211,281]. As would be expected of a determinant on GPB, He is resistant to trypsin treatment of the red cells, but weakened or abolished by chymotrypsin treatment [254,279,280]. The requirement for sialic acid is variable [74,280].

DNA analysis has shown that He is associated with *GYPB* (*GYPB*He or GYPB*06*) in which a small segment, including part of exon B2 and intron B2, have been replaced by the homologous segment from *GYPA*, the probable result of gene conversion [18,281] (see Section 3.9). A number of untemplated nucleotide changes would have occurred during the gene conversion, some of which produced the amino acid sequence characteristic of the He antigen. He-active glycophorins are produced by several other *GYP(B-A-B)* genes (see Figures 3.8 and 3.11) discussed elsewhere in this chapter: *GYP*He(P$_2$)* and *GYP*He(NY)* (Section 3.6.3); *GYP*He(GL)* (Section 3.11.2); *GYP*Cal* (Section 3.14.2.5).

Serological and immunochemical studies with monoclonal anti-He revealed a marked variation in He antigen strength: the strongest associated with S/s+ U+ phenotypes and the weakest with S–s– phenotypes [183,211] (see Section 3.6). Ninety percent of S– s– red cell samples that reacted with anti-U/GPB had the nucleotide sequence characteristic of He [83].

Of 38 He+ donors of African origin, all with the normal (strong) He antigen, 35 (92%) were S+ [211]. As about 30% S+ would be expected for the whole population, the *GYP(B-A-B)* gene encoding He usually produces S.

3.7.4.2 M^e

Anti-M^e was the name given to a rabbit anti-M that unexpectedly reacted with M– N+ He+ cells, as anti-M and -He activity could not be separated by adsorption and elution tests [282]. Human anti-M^e was found later [278]. Whereas the rabbit anti-M^e had reacted preferentially with M, the human antibody reacted equally strongly with M+ He– cells and M– He+ cells. Anti-M^e was found to be present in nine of 14 anti-M sera from M– N+ Israeli blood donors [283] and five of nine monoclonal 'anti-M' had anti-M^e activity [284]. Reactivity of anti-M^e with M+ He– cells is trypsin-sensitive; reactivity of anti-M^e with M– He+ cells is trypsin-resistant [283]. On immunoblots, monoclonal anti-M^e stains GPA on M+ He– membranes, GPB on M– He+ membranes, and both GPA and GPB on M+ He+ membranes.

The existence of anti-M^e is no surprise. Anti-M that are dependent on the presence of terminal leucine will not react with an He determinant on GPB, but anti-M that recognises Gly5 of GPA would be expected to react with GPA.M and GPB.He (Table 3.5).

3.8 The Miltenberger series

Miltenberger is a series of phenotypes that are rare in most populations and are associated with the MNS system. They are related to each other through the overlapping specificities of a number of low frequency alloantigens. The characteristics that place an MNS variant phenotype into the Miltenberger series, rather than just being considered as one of the many MNS variants, are purely serological and some of these serological connections between the categories are tenuous. It is no longer feasible to expand the Miltenberger series to accommodate new phenotypes, or to incorporate some existing

MNS variant phenotypes, such as M^g, which would become candidates for inclusion on the grounds of serological findings. Although the Miltenberger classification is now obsolete, it is mentioned here because it has appeared in the literature for many years and still continues to do so. Described below is a brief history of the Miltenberger series followed by an outline of an alternative notation proposed by Tippett *et al.* [285] and designed to encompass all variant MNS phenotypes. The Miltenberger classes, together with the new terminology, are listed in Table 3.10.

Cleghorn [290] initiated the Miltenberger series in 1966 in an attempt to bring some order to a complex pattern of reactions with several different antibodies to low incidence antigens. These antibodies were categorised into four type sera:

1 Verweyst (Vw) [288,291];
2 Miltenberger (Mi^a) [292];
3 Murrell (Mur) [293];
4 Hill (Hil) [16].

These four type sera defined four phenotypes: Class I to Class IV [290,294,295]. The original association with the MNS system originated from the observation that Vw appeared to be inherited with MNS [296]. Cleghorn [290] named the series Miltenberger after the type serum that reacted with red cells of all four classes. Six more classes have been added since (Table 3.10).

A fifth class was added to the series in 1970 [237]. Mi.V cells do not react with any of the antibodies found in Miltenberger type sera, but were included because, like Mi.III cells, they reacted with anti-Hil.

Three more classes were added following the identification of two specificities, anti-Hop and -Nob. Anti-Hop reacted with Mi.IV red cells and with cells of two of the new classes, Mi.VI and Mi.VIII, whereas anti-Nob reacted with Mi.VII and Mi.VIII cells [245,254,297,298]. This explanation is an over-simplification and some of the further complexities of Hop and Nob specificity are described by Tippett *et al.* [285].

Mi.IX was introduced for four propositi with Mur+ cells that also reacted with anti-DANE, a new antibody specific for Mi.IX [247]. Despite being Mur+, Mi.IX cells are MUT–. Mi.X is represented by red cells that are Hil+ and MUT+, yet Mur– and Hut– [299]. Mi.XI was added [300] for the phenotypes of two propositi on the basis of the reactions of their red cells with anti-TSEN and -MINY, antibodies that reacted with red cells of some other Miltenberger classes [301,302].

The antigens and phenotypes of the obsolete Miltenberger series will be described more fully in various sections according to their biochemical basis. For the convenience of readers still accustomed to the Miltenberger terminology, this will be provided in parentheses at regular intervals.

Table 3.10 Serological definition of the Miltenberger phenotypes and a replacement notation [285,286].

		Antigens										
Mi class	New notation	Mi^a	Vw	Mur	Hil	Hut*	MUT†	Hop	Nob	DANE	TSEN	MINY
I	GP.Vw	+	+	–	–	–	–	–	–	–	–	–
II	GP.Hut	+	–	–	–	+	+	–	–	–	–	–
III	GP.Mur	+	–	+	+	–	+	–	–	–	–	+
IV	GP.Hop	+	–	+	–	–	+	+	–	–	+	+
V	GP.Hil	–	–	–	+	–	–	–	–	–	–	+
VI	GP.Bun	+	–	+	+	–	+	+	–	–	–	+
VII	GP.Nob	–	–	–	–	–	–	–	+	–	–	–
VIII	GP.Joh	–	–	–	–	–	–	+	+	–	NT	–
IX	GP.Dane	–	–	+	–	–	–	–	–	+	–	–
X	GP.HF‡	+	–	–	+	–	+	–	–	–	–	+
XI	GP.JL	–	–	–	–	–	NT	–	–	–	+	+

*As defined by Giles and colleagues [254,287].
†Originally called Hut [16,288,289].
‡GP.HF previously named GP.Mor [285].
NT, not tested.

3.9 Hybrid glycophorins and the low frequency antigens associated with them

In 1979, Anstee *et al.* [198] looked to haemoglobin to provide an explanation for the unusual serological and biochemical characteristics observed with red cells of the GP.Hil (Mi.V) phenotype. The model illustrated in Figure 3.9 predicts that misalignment between *GYPA* and *GYPB*, followed by unequal crossing-over, results in the production of two new haplotypes. In one there is a loss of *GYPA* and *GYPB* and the formation of a novel fusion gene that produces a GP(A–B) hybrid molecule made up of the N-terminal region of GPA and the C-terminal region of GPB. This is often referred to as the Lepore type of hybrid glycophorin, after the analogous rare haemoglobin variant Lepore in which the non-α chain is a hybrid comprising a fusion of part δ-chain and part β-chain. In the opposite haplotype, formed at the same event (anti-Lepore), not only is a hybrid gene predicted that produces a GP(B–A) glycoprotein consisting of the N-terminus of GPB and the C-terminus of GPA, but also normal *GYPA* and *GYPB* flanking the hybrid gene.

Lepore-type hybrids may explain the unusual MNS phenotypes associated not only with GP.Hil, but also with several other variants including GP.En(UK) and GP.Sat. Anti-Lepore haplotypes are responsible for the unusual phenotypes associated with expression of Dantu and St^a antigens. It is likely that chromosomal misalignment, involving *GYPA* and *GYPB*, occurs as a result of the homology that occurs between some regions of those genes. Intron 3 of *GYPA* and the homologous intron of *GYPB* appear to be particular hotspots for recombination (review in [18]).

More complex GP(B–A–B) and GP(A–B–A) hybrids also exist, the former being a GPB molecule with a small GPA insert and the latter a GPA molecule with a GPB insert. The likelihood of two crossing-over events occurring in such close proximity is small, so gene conversion is a more likely explanation for these aberrant glycophorins [18]. Gene conversion is a non-reciprocal exchange of genetic material from one homologous gene to another resulting in a small segment of one gene being replaced by the equivalent segment of its homologue. A simplified model for gene conversion is illustrated in Figure 3.10. In some cases, the insertion of a functional splice site consensus sequence from *GYPA* into *GYPB* has led to the expression of the *GYPB*-pseudoexon.

The creation of novel amino acid sequences by the production of hybrid glycophorins often results in the expression of low frequency antigens. Some of these amino acid sequences and their associated antigenic determinants may arise by more than one genetic mechanism. The various hybrid glycophorin molecules and their associated low frequency antigens will be described in Sections 3.10 to 3.14.

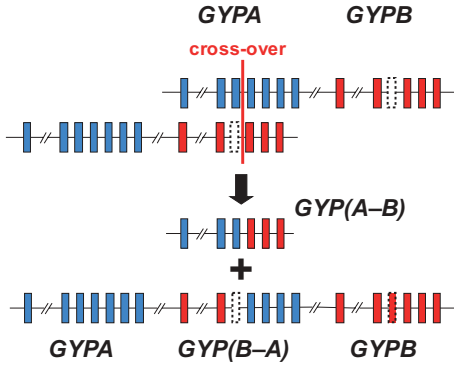


Figure 3.9 Development of hybrid genes involving *GYPA* and *GYPB* by chromosomal misalignment and unequal crossing-over. Two homologous genes become misaligned at meiosis and intergenic crossing-over occurs (red line). Result: one haplotype containing a *GYP(A–B)* fusion gene and another haplotype containing a *GYP(B–A)* fusion gene flanked by normal *GYPA* and *GYPB*. The two hybrid genes shown are typical of those encoding GP(A–B).Hil and GP(B–A).Sch.

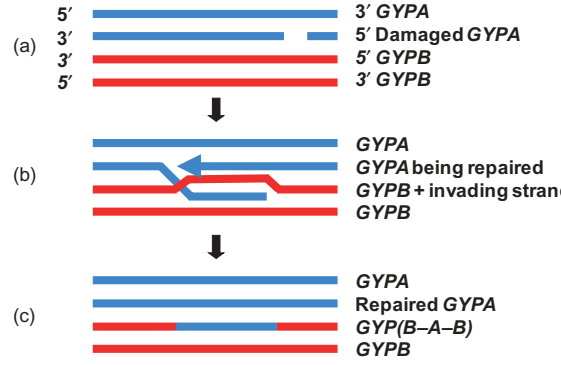


Figure 3.10 Simplified model for gene conversion occurring as the result of damage repair to *GYPA* and involving homologous regions of *GYPA* and *GYPB*. (a) *GYPA/GYPB* heteroduplex, resulting from chromosomal misalignment, with a nick in one *GYPA* strand. (b) An extra copy of one strand of the *GYPA* DNA is synthesised, displacing the original copy, which pairs with one strand of the homologous region of the *GYPB* DNA. The unpaired region of *GYPB* is then degraded. (c) Result: one *GYPB* gene contains a short segment of *GYPA* DNA.

Figure 3.11 shows the rare phenotypes resulting from hybrid glycophorins, the haplotypes that produce them, and a diagrammatic representation of the hybrid glycophorins. Often it is not possible to determine the precise location of recombination sites. In Figure 3.11 the smallest possible insert is assumed.

3.10 GP(A–B) variants

3.10.1 GP.Hil (Mi.V) and the Hil (MNS20) antigen

Red cells of a new phenotype reacted with anti-Hil, but, unlike GP.Mur (Mi.III) cells that also react with anti-Hil, they did not react with anti-Mur [237] (Table 3.10). Family studies have shown that the gene for GP.Hil may be inherited with weakened N or M and elevated expression of s [139,141,237,303,304].

Owing to the shortage of anti-Hil, no frequency studies have been reported. All the recorded GP.Hil individuals are probably of European origin.

Since Anstee *et al.* [198] suggested that the unusual glycophorins associated with GP.Hil (Mi.V) represented a Lepore type of hybrid glycophorin, its dimer, and its heterodimers with GPA and GPB, substantial serological and biochemical supportive evidence has followed [23,49,139,141,303,305,306]. This was facilitated by the finding of an M– N+ S– s+ Spanish-American woman homozygous for the GP.Hil gene [141] and of two individuals heterozygous for the GP.Hil gene and M^K [139,303]. Immunochemical studies revealed only two structures, the putative hybrid (apparent MW 40 kDa) and its dimer. Antibodies to the N-terminal region of GPA bound to the putative hybrid molecule; those to the C-terminal domain did not.

Genomic DNA analyses revealed that *GYP(A–B)*Hil* (*GYP*201.01*) comprises exons A1–A3 of *GYPA* fused to exons B4–B6 of *GYPB* [49,56,62,307] (Figure 3.11). The crossing-over point is located within intron 3 of *GYPA* and *GYPB* [56,307]. The primary structure of the polypeptide encoded by *GYP(A–B)*Hil*, therefore, comprises amino acid residues 1–58 (19–77) of GPA fused to residues 27–72 (46–91) of GPB.

Biochemical explanations can be provided for many of the unusual serological characteristics of GP.Hil red cells, especially those of *GYP(A–B)*Hil* homozygotes (and heterozygotes with M^K).

1 Reduced M or N expression; no 'N'. The N-terminus of the hybrid glycophorin carries M or N, although the gene produces less GP(A–B) than GPA produced by a normal gene [49,198,308]. These M or N antigens are trypsin-sensitive because of an intact trypsin cleavage site at amino acid residue 39 of GPA. There is no trypsin-resistant 'N' because no GPB is produced.

2 Elevated s expression. The hybrid contains Thr29 of GPB responsible for s expression. Although there is less GP(A–B) than normal GPA, there is substantially more than normal GPB. U antigen is also produced.

3 Presence of En^aTS and En^aFS; very weak expression of En^aFR; absence of Wr^b. The parts of GPA associated with trypsin-sensitive and ficin-sensitive determinants are retained in the hybrid, the parts associated with Wr^b and most of En^aFR are lost. En^aFR is detectable only by adsorption experiments [141]. The homozygous *GYP(A–B)*Hil* woman and those women heterozygous for *GYP(A-B)*Hil* and M^K were found because they had produced anti-Wr^b (and/or anti-En^aFR) [139,141,303].

4 Serological characteristics associated with reduced red cell surface sialic acid [118,139,141] (see Section 3.5.1.1). Red cells of *GYP(A–B)*Hil* homozygotes and heterozygotes have about 53% and 80% of normal sialic acid, respectively [131,141].

5 Hil antigen. Hil, which is trypsin-resistant, represents the unique amino acid sequence present at the point of fusion of GPA and GPB, but only when the third amino acid residue of the GPB-derived sequence is threonine (representing s). More details on the Hil antigen are provided in Section 3.13.1.

3.10.2 GP(A–B) hybrids associated with S antigen

An M+ N+ S+ s+ individual (JL) was heterozygous for *Ns* and a gene producing a hybrid glycophorin [309]. The red cells were Hil– and had unusual S; they reacted with only 14 of 19 anti-S. The hybrid glycophorin GP(A–B).JL is identical to GP(A–B).Hil apart from having methionine instead of threonine at position 61 (equivalent to position 29 of GPB), explaining the S activity (Figure 3.11). Genomic sequencing has shown that *GYP(A–B)*Hil* and *GYP(A–B)*JL* (*GYP*202.01*) differ in the location of the crossing-over sites within intron 3 [307]. GP.JL has also been referred to as Mi.XI [300] (Table 3.10).

Other examples of GP(A–B).JL have been described in people of European origin and in Chinese, some of whom were homozygous for the GP.JL gene (or heterozygous for GP.JL and M^k genes) and had produced anti-En^a and/or anti-Wr^b [142,143,152,301,310–312]. A similar phenotype was found in a Spanish-American woman (AG) who appeared to be homozygous for genes producing

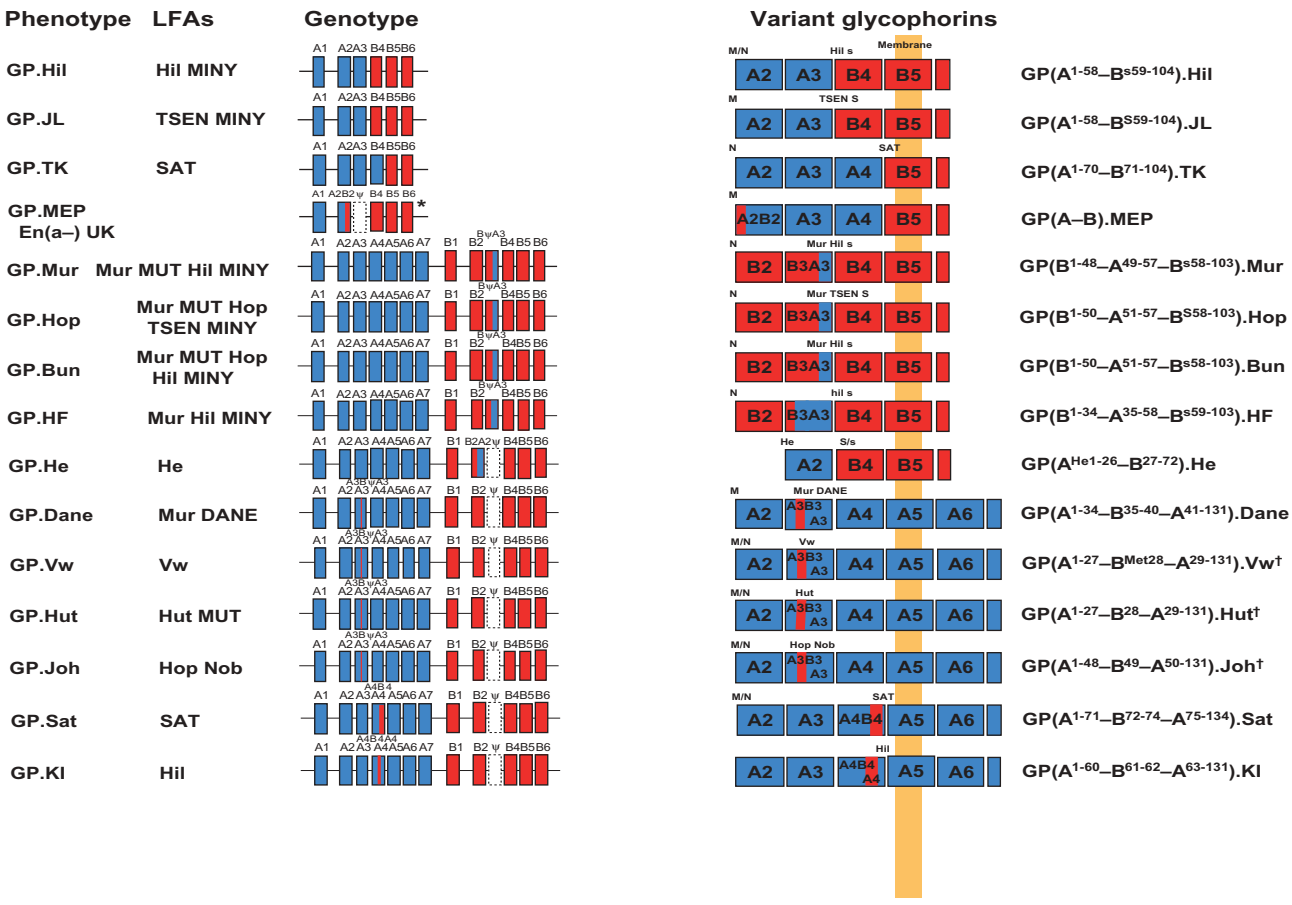


Figure 3.11 Rare MNS phenotypes associated with hybrid glycophorins. *Possible genotypes deduced from serological and biochemical evidence. †Alternatively could result from a point mutation.

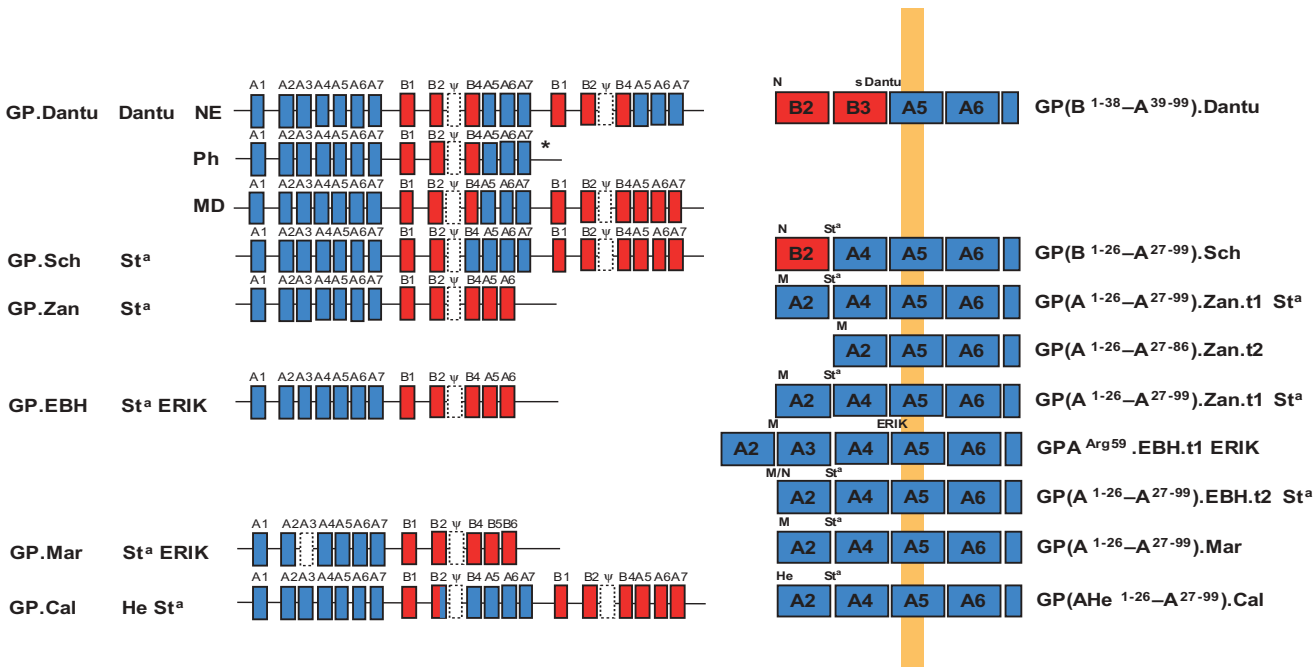


Figure 3.11 (*Continued*)

GP(A–B) hybrid glycophorins carrying M and S, but whose red cells were weakly Hil+ [313].

All these S-active GP(A–B) hybrids express TSEN (MNS33), whereas the s active GP(A–B).Hil molecule does not [301] (see Section 3.13.1).

3.10.3 SAT (MNS36)

A new low incidence antigen called SAT, found in two Japanese families, is described here because it is associated with a novel Lepore type of hybrid glycophorin in one of the families [248]. The second SAT+ propositus was found as a result of screening 10 480 Japanese blood donors (Table 3.9). Four examples of anti-SAT are known.

The red cells of one of the SAT+ propositi (TK), who had produced anti-Wr^b and/or anti-En^aFR, were M– N+ S– s– U– En^aTS+ En^aFS+ En^aFR– Wr(b–). The results of SDS PAGE and immunoblotting were consistent with the propositus being homozygous for a gene producing a GP(A–B) hybrid. All SAT+ members of his family had the same variant glycophorin; the SAT– members did not. Unlike all other GP(A–B) molecules described, GP(A–B).TK did not express S, s, or U [248]. Analysis of cDNA demonstrated that GP(A–B).TK is encoded by a gene (*GYP*203.01*) comprising exons A1–A4 of *GYPA* and B5 and B6 of *GYPB*, with a cross-over point within intron 4 [314] (Figure 3.11). This represents the reverse arrangement to that seen in GP(B–A).Dantu (Section 3.14.1). GP(A–B).TK is a 104-amino acid glycoprotein with the novel sequence Ser-Glu-Pro-Ala-Pro-Val produced by the junctions of exons A4 and B5 [314]. This sequence may represent the SAT antigen.

In another family with SAT+ members there was no sign of a hybrid molecule and SAT appeared to be associated with normal GPA and GPB, except that the GPA carried a very weak M antigen [248]. Of six more SAT+ propositi found in Japan, three had the GP(A–B) hybrid glycophorin and three apparently normal GPA and GPB [315]. Analysis of *GYPA* cDNA from the latter type revealed an insert, between exons A4 and A5, of nine nucleotide bases derived from the 5′ end of exon B5 of *GYPB*, encoding an insert of Ala-Pro-Val in a GPA molecule, creating the SAT specific sequence of Ser-Glu-Pro-Ala-Pro-Val in GP(A–B–A).Sat (Figure 3.11).

3.10.4 En(UK)

En(UK) is one of the genes responsible for the aberrant phenotype of the original En(a–) proposita (MEP) [102], who is heterozygous for *En(UK)* and M^K [110–112]. *En(UK)* produces a Lepore type of hybrid glycophorin of the same MW as GPB [112]. En(a–)UK cells lack the En^a, Wr^b, and C-terminal determinants associated with GPA. They have a weak, trypsin-resistant, M antigen, and no trypsin-resistant 'N' [15,39,110–112,115,308]. They also have enhanced expression of S. It is probable that En(UK) arose from the misalignment and unequal crossing-over between *GYPA.M* and *GYPB.S*, with the crossing-over occurring either within the homologous region encoding the first 26 amino acid residues of both molecules or within intron 1. Preliminary DNA analysis supported the hypothesis of a gene encoding a GP(A–B) hybrid [55].

Screening of red cells from 1300 British blood donors for reduced sialic acid by protamine sulphate aggregation revealed one donor who appeared to have *En(UK)* producing S and trypsin-resistant M [116]. Two individuals with *En(UK)* producing M and s [316], presumably represent a separate recombination event from that responsible for *En(UK)* in the other families studied [102,116]. Anti-M reagents that depend on Ser1 reacted with the M produced by *En(UK)*, whereas those that require Gly5 did not. This suggests that the original recombination may have occurred between the codons for amino acid residues 1 and 5, producing a molecule identical to GPB apart from a Leu1Ser substitution.

3.11 GP(B–A–B) variants

3.11.1 GP.Mur (Mi.III), GP.Hop (Mi.IV), GP.Bun (Mi.VI), and GP.HF (Mi.X)

3.11.1.1 Serology, frequency, and inheritance

GP.Mur and GP.Bun are similar phenotypes: the red cells are Mur+, Hil+, MUT+, and MINY+, but GP.Bun cells are Hop+ whereas GP.Mur cells are Hop– (Table 3.10). GP.Mur and GP.Bun are always inherited with s. In people of European origin GP.Mur may be inherited with *Ns* or with *Ms*, the former being more frequent than the latter [289]. In Thais and Chinese, GP.Mur is usually inherited with *Ms* [219,226]. GP.Bun is generally inherited with *Ms* [297]. GP.Mur and GP.Bun phenotypes are associated with an elevated expression of 'N', the trypsin-resistant N antigen carried on GPB [129,226,289,297,317,318]. The s antigen produced by GP.Mur differs qualitatively from normal s. GP.Mur red cells may fail to react with some potent anti-s sera [289] and one s+ woman with GP.Mur red cells made an anti-s, which did not react with her own cells.

Only two GP.Hop propositi are reported [289,319]. Like GP.Bun, GP.Hop red cells are also Mur+, MUT+, Hop+, and MINY+, but are Hil– and TSEN+ (Table 3.10). In the only family studied, GP.Hop is inherited with *NS*

[289]. Cells from individuals heterozygous for *Ms* and the GP.Hop gene reacted with only some anti-S sera [289,319] and failed to react with a monoclonal anti-GPB (MAb148) that usually reacts preferentially with S+ cells [320].

Tests on over 50 000 white people revealed only six Mur-positives [16]; five were GP.Mur (or possibly GP.Bun as anti-Hop was not used) and one was GP.Hop (Table 3.9). Mur is much more common in people of East Asia. About 10% of Thai blood donors were Mur+; of these, 93% were Hop– (GP.Mur) and 7% were Hop+ (GP.Bun) [219,297] (Table 3.9). In another study on Thais, molecular analysis on the 9% that were serologically Mi(a+) showed that 88% had the GP.Mur gene and 11% the GP.Bun gene [321]. GP.Mur has a frequency of around 6% and 7% in Hong Kong and Taiwan Chinese, respectively [220,322]. The frequency of GP.Mur reaches 88% in the Ami mountain people of Taiwan, but was not found in some other Taiwanese indigenous groups [220].

GP.HF (Mi.X) cells are unique in being MUT+, yet Mur– and Hut–; they are also Hil+, Hop–, TSEN–, and MINY+ (Table 3.10), and are M+ with elevated 'N' and S– with elevated s [285,299]. Several GP.HF propositi are known, all of Japanese ancestry.

Another phenotype, named GP.Kip, found in German and Australian propositi, is very similar to GP.Mur [323]. The red cells were Mur+, Hil+, MINY+, and MUT+, but despite being non-reactive with anti-Hop and -Nob, they did react with sera containing Hop+Nob specificities.

3.11.1.2 Biochemistry and molecular genetics

GP.Mur, GP.Hop, and GP.Bun are associated with replacement of normal GPB by a component resembling GPB, but of increased apparent MW (between 31 and 38 kDa). This abnormal component, which also exists in dimeric form and as heterodimers with GPA and GPB, has the same molecular weight in all three phenotypes and carries about twice as much sialic acid as normal GPB. Red cells from GP.Mur heterozygotes have about 13%, and those from homozygotes about 21%, more sialic acid than normal cells. In addition to the abnormal GPB molecule, the GP.Mur haplotype produces normal GPA, but no normal GPB [24,148,198,306]. GP.Mur red cells have enhanced expression of band 3 (see Chapter 10), possibly as the result of an additive effect of GPA and GP.Mur [324], and reduced expression of Rh and RhAG proteins (see Section 5.20) [325].

GP.Mur, GP.Hop, GP.Bun, and GP.HF arise from the replacement of a small segment of *GYPB* with a homologous segment from the 5′ end of exon A3 and the 3′ end of intron 3 of *GYPA*, probably the result of gene conversion [299,319,326–328] (see Section 3.9). This segment of *GYPA* replaces the non-functional donor splice site for the *GYPB* pseudoexon with the functional splice site sequence from *GYPA*, hence a new composite exon is now expressed consisting of the 5′ end of the pseudoexon of *GYPB* and the 3′ end of exon A3 of *GYPA*, resulting in an enlarged GPB molecule (Figure 3.12). This GP(B-A-B) molecule consists of the products of exons B1 and B2 of *GYPB* as its N-terminal domain (although exon 1 product is cleaved from the mature protein), followed by the composite exon comprising most of the activated *GYPB* pseudoexon and part of *GYPA* exon A3, followed by exons B4–B6 as its C-terminal domain (although most of exon B6 is untranslated) (Figure 3.11).

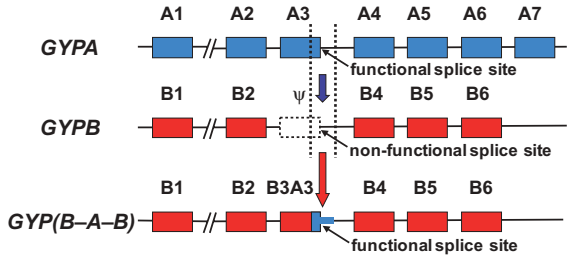


Figure 3.12 Diagram demonstrating the replacement of a small segment of *GYPB* by the homologous region from *GYPA*, including part of exon A3 and part of intron 3, to generate a novel *GYP(B–A–B)* gene; the result of non-reciprocal recombination by gene conversion (blue arrow). The mutated, non-functional splice site responsible for the *GYPB* pseudoexon (ψ) is replaced by a functional splice site from intron 3 of *GYPA*, and a composite exon comprising part of the *GYPB* pseudoexon and part of exon A3 is expressed. The resultant *GYP(B–A–B)* gene produces a GP(B–A–B) hybrid glycophorin typical of those present in GP.Mur, GP.Hop, GP.Bun, and GP.HF phenotypes.

The *GYP(B–A–B)* genes *GYP*Mur*, *GYP*Bun*, and *GYP*HF* have *GYPA* inserts of 55, 131, and 98 bp, respectively. The precise size of the *GYP*Hop* insert is not known. Only minimal differences exist between the encoded glycoproteins. GP(B–A–B).Mur and GP(B–A–B).Bun differ only at amino acid residue 48, arginine in the former and threonine in the latter. GP(B–A–B).Hop and GP(B–A–B).Bun have the same insert and differ only by Met60Thr (equivalent to position 29 in GPB), responsible for S and s expression. GP(B–A–B).Mur and GP(B–A–B).HF differ by five amino acid residues.

GP.Mur red cells have normal quantities of GPA and are Wr(b+), yet have about 22% higher expression of Wr^b than cells of common phenotype [329]. This probably results from higher band 3 levels in GP.Mur cells with increased formation of band 3-GPA complexes [324] (see Chapter 10).

3.11.1.3 Anti-Mur and other antibodies to GP.Mur red cells

Anti-Mur is a fairly common separable component of anti-'Mi^a' sera, though it also occurs alone [289,317, 318,330,331]. Antibodies to GP.Mur red cells (probably mainly anti-Mur, but often called anti-'Mi^a') have been responsible for immediate and delayed HTRs [332,333] and severe HDFN [333–335] (reviewed in [336]). Antibodies to GP.Mur cells are among the most common atypical alloantibodies detected in eastern Asia [219,220, 322,337,338]. They often have an IgM component; of those that contain IgG, it is almost always IgG1 and/or IgG3, and of those containing IgG, 69% were reactive in a monocyte monolayer functional assay [339]. The *DRB1*0901* allele frequency was significantly higher in patients with anti-'Mi^a' than in a control group [340]. It is important that in eastern Asia, GP.Mur red cells are included in antibody screening panels, particularly where abbreviated cross-match procedures are employed. Red cells resembling GP.Mur cells have been synthesised by embedding appropriate peptides attached to lipids in the membrane of red cells of common phenotype [337,341]. Complex PCR-based techniques make it possible to predict GP.Mur and related phenotypes from DNA [321,342].

Human IgM anti-Mur and murine anti-'Mi^a' monoclonal antibodies have been produced [160,343,344]. Murine monoclonal anti-NEV agglutinates red cells with glycophorins containing Asn-Glu-Val (NEV) and is specific for cells expressing Mur or DANE [160,345].

3.11.2 He (MNS6)

As mentioned in Section 3.7.4, a hybrid glycophorin is responsible for the He antigen. The gene encoding He (*GYP*He)* is *GYPB* in which a segment near the 5′ end is replaced by the homologous segment from *GYPA* [281]. A number of untemplated nucleotide changes, probably introduced during a gene conversion event, encode the abnormal amino acid sequence within the N-terminal pentapeptide of the hybrid glycophorin responsible for He antigen expression (Table 3.5). Although the gene is a *GYP(B–A–B)* hybrid, the *B–A* recombination site probably lies in the region of exon 2 encoding the leader peptide and the *A–B* site in intron 2, so the mature protein, after cleavage of the leader peptide, is a GP(A–B) hybrid (Figure 3.11).

Some variants of *GYP(B–A–B)*He* involve splice site mutations. These include *GYP*He(P2)* and *GYP*He(NY)* described in Section 3.7.4, in which partial splicing-out of exon B5 gives rise to a S– s– $U+^{var}$ phenotype. In another variant, *GYP*He(GL)*, there is a point mutation in exon B5 of the gene encoding the He-active glycophorin, which creates a new acceptor splice site, and another mutation in the exon B6 acceptor site in intron B5 [346]. These mutations affect splicing of exon B4 in a proportion of the mRNA transcripts, so that two glycoprotein isoforms are produced from the same gene: one virtually identical to GP(A–B).He; the other, with an apparent MW reduced by about 3 kDa resulting in absence of the product of exon B4, expresses He, but no S, s, or U. These two glycoproteins were easily detected by immunoblotting with anti-He, but the serological phenotype is not readily distinguished from common He+ phenotypes.

3.12 GP(A–B–A) variants

3.12.1 GP.Dane (Mi.IX); DANE (MNS32) and ENDA (MNS44)

The low frequency antigen DANE was associated with trypsin-resistant M and was inherited with *MS* in four Danish families [247]. Two of the four propositi were found by screening trypsin-treated red cells from 467 Danish blood donors with monoclonal anti-M (Table 3.9). An American woman of English ancestry had M+ N– S– s+ DANE+ red cells and was heterozygous for the GP.Dane gene and M^k [345]. Her red cells lacked ENDA, the high frequency antigen antithetical to DANE, and she had produced IgM anti-ENDA. One of her brothers was also ENDA–, as were En(a–) and M^k cells. DANE and ENDA are trypsin-sensitive. DANE+ cells are Mur+, but MUT– (Table 3.10).

Immunoblotting of DANE+ cells with antibodies to epitopes on the N- and C-terminal domains of GPA showed that DANE is associated with a GPA-like molecule with an apparent MW about 1 kDa less than that of normal GPA and which lacks the trypsin cleavage site at Arg39 and the determinants recognised by alloanti-En^aTS and by a number of monoclonal antibodies that detect epitopes between residues 26 and 39 of GPA [247].

The abnormal glycophorin associated with DANE is GPA with a small segment replaced by GPB [345, 347]. The whole *GYPB* insert is derived from the pseudoexon and replaces an internal segment of exon A3 of *GYPA*, creating two hybrid junctions within the exon (*GYP*Dane)*. The minimal amount of DNA transferred is 16 nucleotides. Amino acid residues 35-41 of GPA (-Ala-Ala-Thr-Pro-Arg-Ala-His-) are replaced by six residues from GPB (-Pro-Ala-His-Thr-Ala-Asn-). This results in the loss of the trypsin cleavage site at Arg39 of GPA and also the loss of one *O*-glycan, accounting for the reduced MW. The sequence derived from the *GYPB*-pseudoexon may represent the Mur determinant, although adjacent amino acid residues may also be involved (see Section 3.13.2). In one of the Danish families there is an additional untemplated point mutation, Ile46 of GPA to Asn45 of GP(A-B-A).Dane [347]. This amino acid substitution cannot be responsible for DANE antigen expression as it is not present in the American family [345].

Only one example of anti-DANE has been identified [247], made by a non-transfused man who is now dead. Very little of the antibody remains.

3.12.2 GP.Vw and GP.Hut; Vw (MNS9), ENEH (MNS40), and Hut (MNS19)

3.12.2.1 Serology, frequency, and inheritance of Vw and ENEH

Anti-Vw defines the phenotype GP.Vw (Mi.I) (Table 3.10). No aberrant expression of M or N antigens is associated with Vw.

The frequency of Vw in white people is about 0.06%, although in south-east Switzerland a frequency of 1.43% was found [16] (Table 3.9). Family studies have shown Vw to be associated with *Ns*, *NS*, *Ms*, and *MS*, in decreasing order of frequency [289]; Vw associated with M is very rare [285]. One person assumed to be homozygous for the gene producing Vw has been described, an M– N+ S– s+ multiparous woman with an antibody of the anti-En^{a}TS type, named anti-ENEH [348].

3.12.2.2 Serology, frequency, and inheritance of Hut

Anti-Hut (as defined by Giles [254,287]) determines the GP.Hut (Mi.II) phenotype. GP.Hut cells also react with anti-MUT (originally called anti-Hut [16]), which reacts with most Hut+ or Mur+ cells. Hut is not associated with aberrant expression of M or N.

Hut has a frequency in white people of about 0.06% [16], similar to that of Vw (Table 3.9). Hut has been shown to be aligned with *MS*, *Ns*, and *Ms* in decreasing order of frequency [289], but not with *NS*.

3.12.2.3 Biochemistry and molecular genetics of Vw and Hut

Vw and Hut are associated with the presence of abnormal GPA molecules, each with a decrease in apparent MW of about 3 kDa compared with normal GPA [23,171,306,349–351]. Sialic acid levels of Vw+ and Hut+ red cells appear normal. Manual amino acid sequencing revealed GPA with Thr28Met (47) in GPA.Vw and Thr28Lys in GPA.Hut [350]. Asn26 of GPA normally carries an *N*-glycan. The required amino acid sequence for *N*-glycosylation is Asn-Xaa-Thr/Ser (where Xaa represents any amino acid except proline). In normal GPA, which has Asn26 and Thr28, these criteria are fulfilled; in GPA.Vw and GPA.Hut Thr28 is substituted, so Asn26 is not *N*-glycosylated. This lack of *N*-glycosylation accounts for the 3 kDa decrease. Treatment with *N*-glycanase reduces the MW of GPA to that of GPA.Vw; similar treatment of GPA.Vw has no effect [352].

Vw and Hut are trypsin-sensitive and anti-Vw and -Hut could be inhibited by tryptic peptides comprising the N-terminal 30 or 39 amino acids of GPA from Vw+ and Hut+ cells, respectively [350]. GPA expressed by Chinese hamster ovary cells transfected with *GYPA* cDNA that has been altered, by site-directed mutagenesis, to encode GPA.Vw or GPA.Hut, lacked *N*-glycosylation and bound anti-Vw and -Hut, respectively [353]. Anti-Vw bound the abnormal GPA of Vw+ cells on immunoblots [354]. Anti-ENEH, the En^{a}TS antibody produced by a woman homozygous for *GYP*Vw*, might be specific for Thr28 of GPA, for GPA *N*-glycosylated at Asn26, or for both.

The codon for amino acid residue 28 of *GYPA* is ACG (Thr), that for *GYP*Vw* is ATG (Met) [352], an apparent point mutation. One of the codons for lysine is AAG, so point mutation could also account for *GYP*Hut*. Huang *et al.* [18,352] point out that AAG at the codon for amino acid residue 28 is identical to the equivalent codon within the unexpressed pseudoexon of *GYPB*. *GYP*Hut* could have arisen by gene conversion with the replacement of a small segment of *GYPA* with the homologous segment from *GYPB* (Figure 3.11). As the nucleotide substitution in *GYP*Vw* is at the same position as that for *GYP*Hut*, *GYP*Vw* could have arisen as a result of gene conversion during which an untemplated replacement of the mismatched nucleotide has occurred as a result of failure in heteroduplex repair [352]. The changed nucleotides lie between the two half sites of a direct repeat sequence that

has been implicated in recombination events responsible for the production of other hybrid glycophorins, though creation of the two rare genes by straightforward point mutations has not been ruled out.

3.12.2.4 Anti-Vw

Anti-Vw occurs in mixtures of antibodies to low frequency MNS antigens (as a component of anti-'Mi^{a}') [317] or by itself [288,296], where it has been responsible for severe HDFN [351,355–357] and for severe, acute HTRs [358,359] (although this is disputed in one case [360]) (reviewed in [336]). Anti-Vw is not uncommon in the sera of healthy individuals, with about 1% of normal sera containing anti-Vw [16,291,293]. It can be found regularly in sera of patients with AIHA [289]. Of eight anti-Vw sera, seven were IgG alone and one was IgG + IgM [361].

3.12.2.5 Anti-Hut and -MUT

Anti-Hut, an antibody specific for GP.Hut (Mi.II) cells, was first defined by Giles [254,287]. The original Hut antibodies, which would now be called anti-MUT, were isolated from 'anti-Mi^{a}' sera [289,295], but independent examples have also been identified and have caused severe HDFN [16,362]. Anti-MUT is not simply an antibody that cross-reacts with Mur and Hut. GP.Dane cells are Mur+, but MUT– [247]; GP.HF cells are Mur– Hut–, but MUT+ [285,299] (Table 3.10).

3.12.3 GP.Nob (Mi.VII) and GP.Joh (Mi.VIII); Hop (MNS26), Nob (MNS27), and ENKT (MNS29)

3.12.3.1 Serology, frequency, and inheritance

Anti-Nob defines two phenotypes, GP.Nob and GP.Joh [245,254,287,298] (Table 3.10). These phenotypes are distinguished by anti-Hop, which reacts with GP.Joh, but not with GP.Nob cells. Anti-Hop also reacts weakly with GP.Hop (Mi.IV) and GP.Bun (Mi.VI) phenotype cells (Section 3.11.1). This serological description is an oversimplification; anti-Hop sera may contain weak anti-Nob and *vice versa*, and these specificities may be inseparable.

Hop and Nob are trypsin-resistant, but papain- and ficin-sensitive [245,254,298,363].

Unusual expression of M, N, S, s, U, or 'N' antigens has not been reported for GP.Nob or GP.Joh phenotype cells. Red cells of a woman homozygous for the gene responsible for the GP.Nob phenotype lacked ENKT, a form of En^{a}FS [364].

GP.Nob was aligned with *MS* in three families and with *Ms* in one family [245,298]. GP.Joh was aligned with *Ns* in two families [298,365]. Hop has a frequency of about one in 150 Thais [319]; Nob has a frequency of about one in 1650 English blood donors [245] (Table 3.9).

3.12.3.2 Biochemistry and molecular genetics

GP.Nob and GP.Joh result from amino acid substitutions within GPA. Both have *O*-glycosylated Thr49 (68) instead of arginine, but GPA.Nob also has serine (which may be *O*-glycosylated) instead of Tyr52 (71) [366,367]. Both substitutions could be accounted for by point mutations or by the product of gene conversion [18]. Codons for Thr49 and Ser52 occur in the corresponding codons of the pseudoexon of a normal *GYPB*. Consequently insertion of *GYPB* segments of different sizes into *GYPA* by gene conversion, giving rise to *GYP*Nob* and *GYP*Joh*, could account for both amino acid substitutions in GPA. Nob and for the single amino acid substitution in GPA. Joh (Figure 3.11).

Inhibition assays showed that Hop and Nob antigens on GP(A–B–A).Nob and GP(A–B–A).Joh are located within amino acid residues 40–61 [366,367]. As Hop and Nob are both sialidase-sensitive, it seems likely that they are dependent on the glycosylation of Thr49 for binding to native GP(A–B–A).Nob and GP(A–B–A).Joh, yet binding of anti-Hop also appears to require Tyr52. The B–A junction in GP(B–A–B).Bun, but not GP(B–A–B). Mur, creates a Thr-Thr-Val-Tyr (TTVY) sequence that is also present in GP(A–B–A).Joh. It is probable that this sequence is required for the Hop determinant. In the GP.Nob phenotype, the Tyr residue is substituted by Ser and these cells are Hop–. A synthetic decapeptide (EISVTTVYPP) representing amino acid residues 44–53 of GP(B–A–B).Bun and 45-54 of GP(A–B–A).Joh and containing the Thr-Thr-Val-Tyr (TTVY) sequence, inhibited anti-Hop [368].

3.12.4 GP(A–B–A).KI

Red cells of a Czech blood donor and her sister had a novel phenotype: Hil+, yet they were MINY–, and no abnormal structure detected by immunoblotting with monoclonal antibodies to GPA and GPB [369]. Genomic sequencing revealed *GYPA* with two nucleotide changes encoding Arg61Thr (80) and Val62Gly (81) (*GYP*KI*) [370] (Figure 3.11). This creates PEEE**TG**ETGQL, a sequence recognised by anti-Hil (see Section 3.13.1 and Table 3.11). The abnormal GPA molecule is probably the

Table 3.11 Results of inhibition experiments with synthetic peptides representing amino acids encoded by the 3′ end of *GYPA* exon A3 and the 5′ end of *GYPB* exon B4 [301,302,368].

Peptides	Antibodies			
GPA-GPB	Hil	TSEN	MINY	S^{JL}
PEEET-GETGQLVHR s	+	–	+	–
PEEET-GEMGQLVHR S	–	+	+	+

+ Inhibition; – no inhibition.

product of a gene conversion event, with Thr61 and Gly62 encoded by a small *GYPB*-derived segment.

3.12.5 GP(A–B–A).Sat

Phenotypes in which the red cells react with anti-SAT occur as the result of at least two backgrounds. One involves a GP(A–B) hybrid and the other a GP(A–B–A) molecule with a small GPB insert [315]. Both are discussed in Section 3.10.3.

3.13 Further details on Hil, TSEN, MINY, Mur, and Mi^a; antigens associated with hybrid glycophorins

These antigens are considered together here because they are common to hybrid glycophorins of the GP(A–B) and GP(B–A–B) types, and Hil and Mur are also associated with a GP(A–B–A) molecule.

3.13.1 Hil (MNS20), TSEN (MNS33), and MINY (MNS34)

Hil, TSEN, and MINY are low frequency antigens associated with GP(A–B) and GP(B–A–B) hybrid glycophorins produced by genes with A–B junctions within intron 3; Hil is expressed when s is present, TSEN when S is present, and MINY when either s or S are present [301,302,368]. These hybrid glycophorins have the product of the 3′ end of exon A3 of *GYPA* (or of a *B–A* fusion exon) fused to the product of the 5′ end of exon B4 of *GYPB* (Figure 3.11, Table 3.11), which can be detected by altered *Rsa*I restriction sites [342]. GP(A–B).Hil, GP(B–A–B).Mur, GP(B–A–B).Bun, and GP(B–A–B).HF all have Thr29 of normal GPB and express an unusual s antigen; they are all Hil+ TSEN– MINY+. GP(A–B).JL, similar GP(A–B) hybrids, and GP(B–A–B).Hop express an unusual S antigen and presumably have the Met29 of normal GPB; they are Hil– TSEN+ MINY+.

A 14 amino acid synthetic peptide representing residues 54–67 of GP(A–B).Hil, including the Thr-Gly A–B junction and the threonine residue responsible for s activity, inhibited anti-Hil [368], but did not inhibit anti-TSEN [301] (Table 3.11). Another peptide, identical apart from the threonine residue replaced by methionine, inhibited anti-TSEN and those anti-S sera (S^{JL}) that react with red cells with the GP(A–B).JL hybrid glycophorin, but did not inhibit anti-Hil [301,368]. Both peptides inhibited anti-MINY [302]. The Hil determinant is probably smaller than that shown in Table 3.11, as the sequence of PEEETGETGQL is present in GP(A–B–A).KI (Section 3.12.4), which expresses Hil [370].

3.13.1.1 Anti-Hil, -TSEN, and -MINY

The original anti-Hil caused HDFN [16,289]. One other example has been reported [371] and a few more examples are now known. Five examples of anti-TSEN have been reported, four of them by screening sera from 80 000 donors [301,311].Only a single example anti-MINY is reported [302].

3.13.2 Mur (MNS10)

GP(B–A–B).Mur, GP(B–A–B).Hop, and GP(B–A–B).Bun include the product of the *GYPB*-pseudoexon activated by a *GYPA* insert, and all express the Mur antigen. Anti-Mur was inhibited by a 13 amino acid synthetic peptide (DTYPAHTANEVSE), representing a sequence encoded by the pseudoexon and by amino acid residues 32–44 of GP(B–A–B).Mur and GP(B–A–B).Bun [368]. Location of Mur on GP(B–A–B).Mur was confirmed by immunoblotting [354].

GP(A–B–A).Dane contains the sequence Pro-Ala-His-Thr-Ala-Asn (PAHTAN) originating from the *GYPB*-pseudoexon. DANE+ cells react with anti-Mur, so presumably this sequence represents at least part of the Mur determinant [347]. The original anti-Mur (Murrell) does not contain anti-M^g but reacts with M^g+ cells. The tripeptide Asn-Glu-Val (NEV) could represent the epitope of this atypical form of anti-Mur as it is present in the product of the *GYPB*-pseudoexon, in GP(B–A–B).Dane (last residue of GPB insert and following two residues), and in GPA.M^g (residues 4–6).

Clinical significance of anti-Mur is discussed in Section 3.11.1.3.

3.13.3 Mi^a (MNS7)

Although anti-Mi^a was the antibody that originally defined the phenotypes of the Miltenberger subsystem, it was subsequently considered to represent mixtures of antibodies to low frequency antigens, especially anti-Vw, -Mur, -Hut, and -MUT [120,285,317]. Production of two murine monoclonal anti-Mi^a, however, demonstrated that anti-Mi^a could exist as a separate entity [343,344]. Dahr [300] speculated that anti-Mi^a might detect the amino acid sequence QTND(M or K)HKRDTY. This sequence represents the junction of the 3′ end of *GYPA* exon 2 and the *GYPB*-pseudoexon, present in GP(B–A–B).Mur, GP(B–A–B).Hop, GP(B–A–B).Bun, and GP(B–A–B).HF, and is also present in the putative GP(B–A–B) molecules associated with GP.Vw and GP.Hut.

3.14 GP(B–A)-associated variants

3.14.1 Dantu (MNS25)

When Anstee *et al.* [198] postulated a GP(A–B) type of hybrid to account for the GP.Hil (Mi.V) MNS variant phenotype, the genetic mechanism proposed for the creation of the GP(A–B) molecule included the simultaneous production of a haplotype encoding a GP(B–A) type of hybrid glycophorin together with normal GPA and GPB (Figure 3.9). In 1980, Tanner *et al.* [372] proposed that a novel 32-kDa glycoprotein detected in an M+ N+ S– s+ black Zimbabwean (Ph) and his M+ N+ S– s– father, and which carried a trypsin-resistant N antigen, was a GP(B–A) hybrid. The gene producing this GP(B–A) molecule appeared to be inherited with a gene encoding normal GPA.M, but no *GYPB* (Figure 3.11). So it seemed that the initial recombination producing the unusual haplotype must have involved a U– gene, not uncommon in Africans, which produces no GPB. The putative GP(B–A) molecule was precipitated by a rabbit antibody to a determinant on the cytoplasmic (C-terminal) domain of GPA, but not by a monoclonal antibody to an epitope on the extracellular (N-terminal) domain of GPA [305]; the opposite result to that obtained with GP(A–B).Hil.

Four years later, Dantu, a new MNS-associated low frequency red cell antigen, was found in seven black propositi including the Zimbabwean blood donor (Ph) and an American woman (NE), who also appeared to have a GP(B-A) hybrid glycophorin [244]. In addition to the protease-resistant Dantu antigen, Dantu+ cells carry protease-resistant N and weak s (not denatured by trypsin, chymotrypsin, papain, ficin, or pronase).

The Dantu+ phenotype of Ph differs from that of NE, the latter having a substantially higher ratio of GP(B–A) molecules to GPA than the former [244,308,373]. NE is the usual variety of Dantu+ phenotype [343]; a second Dantu+ propositus of the Ph variety is yet to be found. One white Dantu+ propositus has been identified [374] and her phenotype represents a third variety.

The Dantu haplotype generally produces a normal M-active GPA and a variant glycophorin consisting of the N-terminal 39 amino acids of GPB.s fused to residues 72–131 of GPA [326,375,376]. The GYP(B–A) breakpoint resides in intron 4 [327] and, therefore, GP(B–A).Dantu is the reciprocal of GP(A–B).TK described in Section 3.10.3 [314]. GP(B–A).Dantu is protease-resistant [375, 377], explaining the trypsin- and papain-resistant N and s antigens [244,372,373]. The s antigen differs qualitatively from normal s. The Dantu haplotype produces little or no U [244,373] and GP(B–A).Dantu expresses no Wr^b [378]. The reason why a molecule containing the 39 N-terminal amino acids of GPB should have altered s and little or no U is not obvious, but may result from a conformational change in the molecule.

Dantu+ cells of the NE type have substantially more GP(B–A).Dantu (315 000 sites) than those of Ph (200 000) [308]. The gene producing GP(B–A).Dantu (*GYP*Dantu*) is duplicated and arranged in tandem (Figure 3.11), providing an explanation for the high level of GP(B–A) in Dantu+ cells of the NE type [326]. In contrast to En(a–), M^K, and other phenotypes with reduced GPA (Section 3.5.1.3), the apparent MW of band 3 is reduced by about 3 kDa, owing to shortening of the *N*-glycan [377].

Purified GP(B–A).Dantu inhibited activity of anti-N and -s, but only inhibited anti-Dantu in the presence of lipid [375]. Consequently, Dantu is probably a labile structure, like En^{a}FR and U, and might be located within residues 28–40 of GP(B–A).Dantu.

Dantu+ red cells are unusual in having a ficin-resistant N antigen. A simple way of searching for Dantu+ red cells is to screen ficin-treated red cells with *Vicia graminea* lectin [243,379]. Sixteen Dantu+ individuals were found by this method from testing 3200 African American blood donors (Table 3.9); all were of the NE type [243]. In South Africa, Dantu is rare in the black, white, and Asian populations, but relatively common (1.1%) in the people of mixed race, who have Khoi, Asian, Black, and European ethnic origin [380]. This suggests that *GYP*Dantu* originated from the Khoi people, an indigenous group of southern Africa.

Red cells of the only known Dantu+ white person (MD) contained a GP(B–A) hybrid that expressed N and

s and could not be distinguished from that of the NE and Ph types. The molar ratio of hybrid to GPA was only about 0.6:1, suggesting that there was no duplication of the hybrid gene in this individual [374,376]. The Dantu haplotype, in addition to producing GP(B–A).Dantu and normal GPA.M, also contained normal *GYPB*. Unlike the two types of Dantu found in Africans, Dantu of the MD type appears to have originated from an unequal crossing-over event involving active *GYPA* and *GYPB* – no surprise considering that the *GYPB* deletion gene (*GYPB*01N*) is extremely rare in white people.

In summary, three types of Dantu phenotype are known. In each type the Dantu haplotype probably produces an identical N- and s-active GP(B–A) hybrid glycophorin plus GPA.M. In the NE type the gene producing GP(B–A) is duplicated, in the Ph and MD types it is not. In the white, MD type, the gene encoding GP(B–A) is flanked by *GYPA* and *GYPB*; in the African NE and Ph types *GYPA* and *GYP(B–A)* are in tandem, but there is no *GYPB*.

3.14.1.1 Anti-Dantu

Several examples of anti-Dantu have been identified, all in sera containing other specificities, especially anti-Wr^a and other antibodies to private antigens, but also in some anti-S and -s reagents [244,381]. Most anti-Dantu are non-immune, although one immune IgG anti-Dantu was responsible for a positive DAT on neonatal red cells [244]. Screening of 1348 donor sera with Dantu+ red cells produced no anti-Dantu [244], but screening sera of western Canadian blood donors with Dantu+ red cells for 3 weeks revealed five sera containing anti-Dantu [381].

3.14.2 St^a (Stones, MNS15) and ERIK (MNS37)

The low incidence antigen St^a [228] is described in this section because it is usually associated with a GP(B–A) molecule. In a few individuals, however, St^a is encoded by *GYP(A–B–A)*, *GYP(A–E–A)*, and *GYP(B–A–B–A)* genes.

St^a is far more frequent in East Asian people than in people of European origin, with a frequency of over 6% in Japanese [227] compared with only about 0.1% in Europeans [118,228] (Table 3.9). Screening with anti-N *Vicia graminea* lectin against ficin-treated red cells revealed St^a frequencies of between 1.0 and 5.2% in different populations of Chinese in Taiwan [382], but no St(a+) in 100 African Americans [379]. Homozygosity for the St^a gene has been identified in a Japanese family [383].

3.14.2.1 GP.Sch

The GP(B–A) hybrid glycophorin associated with St^a [384], GP(B–A).Sch, binds antibodies directed at the cytoplasmic domain of GPA, but not antibodies to the extracellular domain of GPA [23,378,384,385]. GP(B–A).Sch usually carries N, St^a, and Wr^b, but neither S nor s [378,383,385–387]. GP(B–A).Sch is resistant to cleavage by trypsin and low concentrations of ficin [379,384–386,388], but is less protease-resistant than GP(B–A).Dantu [308].

GP(B–A).Sch comprises amino acids 1–26 (20–45) of GPB at its N-terminal region and 59–131 (78–150) of GPA at its C-terminal region [307,389,390] (Figure 3.11), the result of intergenic crossing-over between intron 3 of *GYPA* and the third intron of *GYPB* on the 3′ side of the pseudoexon. Like *GYPB* mRNA, the pseudoexon of *GYPB* is spliced out of *GYP*Sch* (*GYP*401)* mRNA. Asn26 is not glycosylated [386]. The product of the 3′ end of exon B2 of *GYPB* fused to the product of the 5′ end of exon A4 of *GYPA* results in a novel sequence, -Gln-Thr-Asn-Gly-Glu-Arg-Val-, which probably represents St^a.

There are several types of *GYP*Sch*, all producing identical hybrid glycophorins, but differing in their intronic recombination sites, the result of different events involving unequal crossing-over within the AT-rich recombination 'hot-spot' of intron 3 of *GYPA* and the homologous region of *GYPB*. Seven types were found in Japanese and two in African Americans [391,392], and one in a Polish family with NOR polyagglutination [393] (Section 4.5). One type of *GYP*Sch* has the same crossing-over site as the *GYP(A-B)* hybrid gene *GYP*Hil* (Mi.V), but in a reciprocal arrangement; these two variant genes could be derived from a single recombination event [307,391]. *GYP*Sch* is flanked by *GYPA* and *GYPB* [383–385,389,390] (Figure 3.11). Screening of 264 Taiwanese by a PCR-based test designed to recognise *GYP*Sch* revealed eight positives, one of whom was homozygous; a gene frequency of 0.017 [342].

3.14.2.2 GP.Zan

St(a+) red cells from members of one family reacted with an M-like antibody (no longer available), which did not react with other St(a+) samples [269]. Unlike the usual St^a phenotype red cells (GP.Sch) these variant St(a+) cells (GP.Zan) have trypsin-resistant M [292,394]. A variant glycophorin with the same amino acid sequence as that found in GP.Sch cells, except that the N-terminal pentapeptide had the M sequence, was isolated from the red cells of the only known GP.Zan propositus and his daughter [394].

The M-active variant glycophorin in GP.Zan cells is not a GP(B–A) hybrid, but a GPA molecule lacking amino acid residues 27–58 arising from a deletion of exon A3 of *GYPA* [395]. A GPA.M molecule lacking residues 27–58 would be identical to GP(B–A).Sch, apart from expressing M instead of N, because amino acid residues 1–26 of GPA.M and GPB differ only at positions 1 and 5. GP(A–A).Zan is the product of a *GYP(A–B–A)* hybrid gene (*GYP*Zan* or *GYP*101.01*), the result of gene conversion, in which the whole of exon A3 and the 5′ end of intron 3 of *GYPA* is replaced by the homologous segment from *GYPB*. This *GYPB* segment includes the pseudoexon and the defective splice site. Consequently, no product of exon 3 is expressed in the mature protein (Figure 3.11). Analysis of cDNA confirmed the skipping of exon 3, but also showed the presence of a minor transcript, a mRNA species in which both exon 3 and exon 4 are skipped. Immunoblotting revealed that both transcripts are represented as aberrant glycophorins at the red cell surface, one expressing M and St^a, the other only expressing M [395].

3.14.2.3 GP.EBH and ERIK (MNS37)

Another St^a variant is associated with the low frequency antigen ERIK [387]. In St(a+) ERIK+ red cells a variant glycophorin was detected with an apparent MW identical to that of GP(B–A).Sch. In two families (one of Italian origin, one Australian), St(a+) ERIK+ red cells had trypsin-resistant M and the variant glycophorin expressed St^a and M; in another two families (one Danish, one mixed race South African) no M antigen was detected and the variant glycophorin expressed St^a and N. Immunoblotting of red cell membranes from the Italian and Danish propositi revealed that ERIK was carried, not on the St^a-active variant glycophorin molecule, but on an apparently normal GPA.

The GP.EBH phenotype in the Danish and Italian families is caused by 232G>A in the 3′ terminal nucleotide of exon A3 of *GYPA* [396] (*GYP*EBH* or *GYP*101.02*) (Figure 3.11). This creates Gly59Arg (78) in an otherwise normal GPA molecule, presumably responsible for the ERIK antigen. As the mutation resides in the exonic part of the donor splice site consensus sequence for intron 3, partial disruption of RNA splicing occurs. At least four transcripts are produced: t1, a normally spliced transcript, which produces the ERIK-active GPA; t2, a transcript lacking exon A3, which produces a GPA molecule lacking amino acid residues 27–59 and, therefore, with the amino acid sequence characteristic of the St^a determinant, but no ERIK antigen; t3 and t4, two abnormally spliced transcripts in which exons A2 and A3 (t3), and A2, A3, and A4 (t4) have been removed. Protein products of transcripts t3 and t4 have not been detected, probably because of the loss of exon A2, which encodes part of the leader sequence involved in the incorporation of the glycoprotein into the red cell membrane.

3.14.2.4 GP.Mar, a molecule expressing St^a and ERIK derived from a *GYP(A–E–A)* gene

In the Australian family with St(a+) ERIK+ members, yet another genetic mechanism is involved [397]. Loss of the product of exon A3 to produce an St^a-active GP(A–A) molecule (like that in GP.Zan) resulted from the replacement of exon A3 and the active 5′ splice site of intron 3 with pseudoexon E3 and its inactive splice site in intron 3 from *GYPE*. Thus GP(A–A).Mar is encoded by a *GYP(A–E–A)* gene (*GYP*Mar* or *GYP*101.03*) (Figure 3.11). No explanation has been provided for ERIK expression on these cells.

3.14.2.5 GP.Cal, a molecule expressing St^a and He derived from a *GYP(B–A–B–A)* gene

Immunoblotting of membranes from red cells expressing St^a and He demonstrated that both antigens resided on the same molecule, an aberrant glycophorin resembling GP(B–A).Sch. This unusual glycophorin molecule is encoded by a *GYP(B–A–B–A)* gene (*GYP*Cal* or *GYP*101.04*), which probably arose from unequal crossing-over between *GYP(B–A–B)*He* and *GYPA* [281]. The first (5′) *GYPB* segment encodes the 5′ untranslated region and part of the leader sequence, the second *GYPB* segment is intronic and includes the *GYPB* pseudoexon; neither is expressed in the mature protein. The first *GYPA* segment represents exon A2 and encodes the N-terminal 26 amino acids of the mature protein including the sequence associated with He expression (see Section 3.7.4); the second *GYPA* segment represents exons A4–A7 of *GYPA* (Figure 3.11). The junction of the products of *GYPA* exons A2 and A4 creates the St^a antigen.

3.14.2.6 Anti-St^a and -ERIK

The original anti-St^a was found in a serum together with separable anti-Ri^a, -Wr^a, and -Sw^a [228]. Although other examples have been found since [227], anti-St^a is not a common specificity.

Anti-ERIK is present in the serum of the wife of the Danish St(a+) ERIK+ propositus and caused a positive DAT on the red cells of their baby [387]. Anti-ERIK is also present in two multispecific sera containing numerous antibodies to low frequency antigens [387].

3.15 Antigens associated with GPA amino acid substitutions proximal to the membrane and with abnormal expression of Wr^b

GPA is associated in the membrane with band 3, the red cell anion exchanger and Diego blood group antigen. This association is described further in Chapter 10 and in Section 3.23. The Wr^a/Wr^b (DI3/DI4) dimorphism is determined by a single amino acid substitution in band 3, but Wr^b is not expressed if GPA is not present or, more specifically, if the region around the junction of the extracellular and membrane-spanning domains of GPA is not present (Section 10.4.2). Described below are amino acid substitutions at positions 62, 63, and 65 of GPA that create low frequency and/or ablate high frequency MNS antigens and also affect Wr^b expression.

3.15.1 HAG (MNS41) and ENEP (MNS39)

A previously transfused man with an antibody to a high frequency determinant on GPA, anti-ENEP, was homozygous for 250G>C in exon A4 of *GYPA* (*GYPA*HAG* or *GYPA*41*), encoding Ala65Pro (84) in GPA [398]. This substitution, which appears to have created a new low frequency antigen HAG and abolished the high frequency antigen ENEP, also affected expression of Wr^b. Only eight of 15 monoclonal and polyclonal anti-Wr^b reacted with the red cells. The band 3 genes had the sequence for Wr^b homozygosity. Pro65 could disrupt the putative α-helix between GPA residues 56 and 70, and this may be responsible for the aberrant Wr^b expression. An unrelated HAG+ person, heterozygous for the Ala65Pro mutation, has been identified.

Anti-HAG was present in several sera containing multiple antibodies to low frequency antigens and in one monospecific serum.

3.15.2 MARS (MNS43) and ENAV (MNS42)

Concurrent absence of the high frequency MNS antigen ENAV and presence of the low frequency antigen MARS in a native American woman results from homozygosity for 244C>A in *GYPA* exon 4 (*GYPA*MARS* or *GYPA*43*) encoding Glu63Lys (82) in GPA. Her red cells also had weak expression of Wr^b, yet no abnormality was detected in her band 3 genes [399,400]. MARS appears to be unique to the Choctaw tribe of Native Americans, where it is aligned with *Ms*, with an incidence of about 15% [401]. All of 2437 Native American donors were ENAV+ [402].

Anti-MARS was found in sera containing multiple antibodies to low frequency red cell antigens. Three anti-ENAV are known. One patient with anti-ENAV was transfused with three incompatible red cell units with no adverse reactions [402].

3.15.3 ENEV (MNS45)

A patient whose parents were of Italian origin and first cousins produced an antibody to a high frequency, protease-resistant antigen, anti-ENEV, which reacted marginally weaker with ENEP– and ENAV– red cells than with normal cells [403]. Her red cells were M+ N– S+ s– ENEP– ENAV– and reacted weakly with anti-Wr^b. The patient was homozygous for 242T>G in *GYPA* (*GYPA*–45*), encoding Val62Gly in GPA.

Four units of packed red cells were eliminated from the peripheral blood of the patient within 10 days of transfusion [403] and another anti-ENEV was incriminated in a delayed HTR [404].

3.16 Other low frequency antigens of the MNS system

There are currently 31 low frequency antigens belonging to the MNS system (Table 3.1). Many of these have been described already; this section includes the remainder. All are inherited and some also accompany aberrant expression of MNSs antigens. Ten are associated with single amino changes: seven in GPA and three in GPB. The molecular bases for two remain unknown. The antigens will be mentioned in numerical order according to the ISBT nomenclature. Frequencies are shown in Table 3.9.

3.16.1 Vr (MNS12)

Aligned with *Ms* in three Dutch families and one Orcadian family (with a Dutch name) [233,405]; no unusual expression of MNSs antigens. Vr results from GPA Ser-47Tyr (66), encoded by 197C>A in exon 3 of *GYPA* (*GYPA*Vr* or *GYPA*12*) [406]. Tyr47 introduces an α-chymotrypsin cleavage site, explaining the chymotrypsin sensitivity of Vr despite being located on GPA [405].

The original anti-Vr producer had three Vr+ children, but none had HDFN [223]. Other examples of anti-Vr have been identified in anti-S sera and in multispecific sera [223].

3.16.2 Mt^a (Martin, MNS14)

Aligned with *Ns* in five families [224,407–409], Mt^a is destroyed by papain and ficin, but not by trypsin [254,408]. Eleven Mt(a+) individuals were heterozygous for 230C>T in exon A3 of *GYPA* (*GYPA*Mta* or *GYPA*14*), which encodes Thr58Ile (77) and destroys an *Msp*I restriction site [406].

Three anti-Mt^a have been identified in sera containing antibodies to other low frequency antigens [407]. No anti-Mt^a was found in 3500 donor sera [407]. In a case of HDFN caused by anti-Mt^a, the baby was jaundiced and required exchange transfusion [408]. Three Mt(a+) babies of a woman with anti-Mt^a were born with variable degrees of anaemia, jaundice, and hydrops, but none had DAT+ red cells [409].

3.16.3 Ri^a (Ridley, MNS16)

Ri^a is extremely rare: the original Ri(a+) propositus is the only one known [228]. The family showed that Ri^a is inherited with *MS* and that M and S are expressed normally [229]. Ri^a is trypsin-sensitive, but resistant to treatment of the cells with chymotrypsin, papain, or pronase [229], a pattern not usually associated with MNS antigens. Ri^a is associated with 220G>A in *GYPA* exon3 (*GYPA*Ria* or *GYPA*16*), encoding Glu55Lys (74) in GPA [410]. This amino acid change introduces a trypsin cleavage site and ablates a papain cleavage site.

Screening of 42 886 sera for anti-Ri^a revealed one example, in a woman with no history of transfusion or pregnancy [229]. Twelve other anti-Ri^a were found in sera containing other antibodies to low incidence antigens. Twelve of the 13 anti-Ri^a were IgM.

3.16.4 Cl^a (Caldwell, MNS17)

Aligned with *Ms* in two Scottish families (one originating from Ireland), with apparently normal expression of M and s [230]. Antigen destroyed by trypsin and papain.

Anti-Cl^a was found in 24 of 5326 (0.45%) donor sera. No anti-Cl^a was found in sera of five Cl(a–) women with Cl(a+) children.

3.16.5 Ny^a (Nyberg, MNS18)

Ny^a is present on the red cells of almost 0.2% of Norwegians (Table 3.9). In 20 families Ny^a was inherited with *Ns* [16,231–233]. The N and s antigens of Ny(a+) cells appear normal. Ny^a is denatured by trypsin, papain, and pronase treatment [254,232,233]. Two unrelated Ny(a+) individuals were heterozygous for 138T>A change in exon 3 of *GYPA* (*GYPA*Nya* or *GYPA*18*), encoding GPA Asp27Glu (46) [411]. Immunochemical analyses revealed no abnormality of GPA from Ny(a+) cells, so Asp27Glu does not appear to affect *N*-glycosylation of Asn26 [171,411].

Anti-Ny^a was found in about 0.1% of Norwegian and German blood donors [232,234]. Anti-Ny^a was not found in the sera of seven Ny(a–) women with Ny(a+) babies [232].

3.16.6 M^v (MNS21)

M^v is associated with a variant form of GPB. The original 'anti-M^v', which reacted with all N+ cells and with cells of about one in 400 M+N– white Americans [412], was later considered to be inseparable anti-NM^v [237]. A second example of anti-M^v, which lacked the anti-N activity, reacted with cells of about 0.6% of English blood donors [237] (Table 3.9). M^v was inherited with *Ms* in 14 families, with weakened expression of s, and with *MS* in two families, but with no obvious weakening of S [237,412]. In one African American donor, however, M^v was associated with a very weak S [413]. M^v is resistant to trypsin cleavage, but is destroyed by chymotrypsin, papain, ficin, and sialidase treatment [112,137,415].

Red cells of a woman heterozygous for GP.Hil (Mi.V) and M^v genes had no trypsin-resistant 'N' antigen and only about 25% of the normal quantity of GPB [112,137,415]. *GYP*Hil* produces no 'N' or GPB (Section 3.10.1). Expression of M^v and loss of 'N' from GPB is associated with 65C>G in *GYPB* exon 2 (*GYPB*Mv* or *GYPB*21*), encoding Thr3Ser (22) [416]. An analogy can be drawn between anti-NM^v (the original anti-M^v) and anti-M^e; the former cross-reacting with M^v on GPB and N on GPA, and the latter cross-reacting with He on GPB and M on GPA.

Anti-M^v may be red cell immune [412,414] or 'naturally occurring' [237]. IgG anti-M^v caused HDFN in two of the five M^v+ children of an M^v– woman with an M^v+ husband [414].

3.16.7 Far (MNS22)

The gene producing Far antigen appeared to be aligned with *Ns* in one family [238,417] and with *MS* in another [239,418], although neither family proves close linkage with *MNS*. Far is resistant to trypsin, papain, and ficin [239,254].

Anti-Far has been responsible for severe HDFN [417] and for an HTR [239]. Both Far antibodies are probably red cell immune. No example of anti-Far was found in 541 sera from normal donors [238].

3.16.8 s^D (Dreyer, MNS23)

Aligned with *Ms* in four generations of a white South African family with 41 s^D+ members [240]. Screening of red cells from 1000 white South Africans revealed one s^D-positive, subsequently shown to belong to the original family. One of 1000 mixed race donors was also s^D+ [240] (Table 3.9). *GYPB* exon 4 (*GYPB*sD* or *GYPB*23*) from two s^D+ individuals contained 173C>G encoding Pro39Arg in GPB.s [416]. Red cells of S+ s+ s^D+ individuals reacted weakly, or not at all, with several anti-s sera [240]. Anti-s^D caused HDFN [240].

Antibody to a high frequency antigen antithetical to s^D was found in an Australian patient apparently heterozygous for *GYPB*sD* and M^k [600].

3.16.9 Mit (Mitchell, MNS24)

Mit was inherited with *MS* in 13 families, with *NS* in one, and with *Ms* in one [241,242,419]. In S+ s+ Mit+ individuals S expression is often depressed [416,419], as was s in one family [242]. The extent of the S depression is variable and very dependent on the anti-S reagents used. In three Mit+ individuals 161G>A encoding Arg35His was present in *GYPB* exon 4 (*GYPB*Mit* or *GYPB*24*) [416]. This is consistent with GPB Arg35 being part of the S and s epitopes [97] (Section 3.3.2). Immunochemical techniques revealed no obvious reduction in GPB quantity in Mit+ red cells [242,416,419], although immunoblotting with anti-S clearly demonstrated a reduction in staining intensity of GPB with S+ s+ Mit+ cells [24]. Mit expression is reduced by pronase treatment of the cells, but not by trypsin or chymotrypsin treatment [242].

No example of anti-Mit was found in 500 antenatal sera or 660 donor sera [241]. The original anti-Mit was responsible for slight neonatal jaundice [241].

3.16.10 Or (Orriss, MNS31)

Or was transmitted with *Ms* in a white Australian family with seven Or+ members in three generations [420]. Or+ has also been found in two Japanese, an African American, and a Jamaican [16,246,421] (Table 3.9). Immunochemical analyses located Or on an apparently normal GPA [246,420] and 148C>T in *GYPA* exon 3 encoding Arg31Trp (50) (*GYPA*Or* or *GYPA*31*) was detected in cDNA from three unrelated Or+ individuals [246,421]. Or antigen is destroyed by pronase, ficin, and sialidase treatment of cells, is chymotrypsin-resistant, and, like M on Or+ cells, shows partial resistance to trypsin treatment [420,421]. Trypsin cleaves 50% of native GPA molecules at Arg31 [40]. Sialidase sensitivity suggests that glycosylation of Thr33 and Thr37 are involved in the Or epitope [421].

The original anti-Or was found in the serum of an AIHA patient [16]. Anti-Or has caused HDFN of moderate severity [421]. Twenty examples of anti-Or have been found in about 17 000 normal sera, and five in 50 sera containing antibodies to other low frequency antigens [16,246,420]. Two murine monoclonal antibodies are described as anti-Or and -Or-like [160].

3.16.11 Os^a (MNS38)

Os^a has been found in one Japanese family where it was associated with *Ms* [249]. No further Os(a+) was detected among 50 000 Japanese donors (Table 3.9). Os^a is trypsin-resistant, but destroyed by papain, ficin, and pronase. Os^a resides on a GPA molecule of normal electrophoretic mobility and sequencing *GYPA* exon 3 of an Os(a+) individual from the only family with Os^a revealed heterozygosity for 217C>T (*GYPA*Osa* or *GYPA*38*) encoding Pro54Ser (73) [411]. A synthetic peptide representing part of GPA with the Os^a mutation inhibited anti-Os^a, whereas the control peptide did not.

Anti-Os^a is present in several sera containing multispecific antibodies to low frequency antigens, but no example was found in testing 100 000 sera from Japanese donors [249].

3.16.12 MNTD (MNS46)

Four MNTD-positives were found by screening 20 330 Japanese blood donors with a human monoclonal IgM antibody produced from lymphocytes of an individual with anti-MNTD [250] (Table 3.9). MNTD is sensitive to red cell treatment with trypsin, chymotrypsin, papain, or ficin, but not sialidase. MNTD+ phenotype is associated with 107C>G in *GYPA* (*GYPA*MNTD* or *GYPA*46*) encoding Thr17Arg (36), and Arg17 in GPA was shown to be responsible for MNTD by expression of recombinant *GYPA* with 107G.

Sixteen sera containing anti-MNTD were found by screening 74 032 donors (0.02%) [250].

3.17 Antigens associated with atypical glycophorin glycosylation

3.17.1 Hu, M_1, Tm, Sj, and Can

Several antibodies have been identified that show a distinct preference for either M+ or N+ cells, but are not anti-M or -N. They react with red cells from a greater proportion of black than white people and demonstrate a

Table 3.12 Relative frequencies of antigens partially determined by *N*-acetylgalactosamine content of *O*-glycans on GPA, shown as a percentage of antigen-positive individuals in the whole ethnic group and in people of each MN phenotype.

	African Americans					White people					
		% Antigen positive					% Antigen positive				
Antigen	No. tested	Whole pop.	M+ N–	M+ N+	M– N+	No. tested	Whole pop.	M+ N–	M+ N+	M– N+	References
Hu	500	7	1	8	12	500	1	0	2	3	[87]
Sext	335	24	0	28	33	167	0	0	0	0	[422]
M_1[a]	822	24	46	26	0	500	4	10	1	0	[87,180]
M_1[b]	230	13	32	10	0	218	1*	1	0	0	[423]
Tm	500	31	3	27	64	900	25	2	24	61	[87]
Sj	500	4	0	3	9	500	2	0	3	3	[87]
Can	447	60	74	67	37	541	27	44	24	5	[424]

*One sample positive.
[a]Anti-M+M_1 used by condition in which only anti-M_1 reacts; [b]anti-M_1 used.

great deal of individual variation in antigen strength. These antibodies are not simply showing variation in the strength of M or N antigen; the M-related antibodies will often react more strongly with M+ N+ cells (with a single dose of M antigen) than with M+ N– cells (with a double dose). The same applies to the N-related antibodies with M+ N+ and M– N+ cells. Table 3.12 shows the frequencies of antigens detected by these antibodies, which form the 213, MN CHO, Collection of the ISBT terminology [425].

Binding of many examples of anti-M and -N is partially dependent on oligosaccharide moieties located on GPA and GPB. The polymorphism they detect, however, is determined primarily by the nucleotide sequence of the genes responsible for the amino acid sequence of the polypeptide chain of GPA and GPB. The antibodies described in this section appear to be recognising differences in the structures of the oligosaccharides around the N-terminus of GPA and possibly GPB, arising from inherited glycosyltransferase variation. Such heterogeneity in transferase specificity presumably derives from polymorphisms at a gene locus separate from *GYPA* and *GYPB*.

3.17.1.1 Serology and genetics

Hu (Hunter, 213001) and Sext (213005)

Hu is the oldest MNS antigen after M and N. In 1934 Landsteiner *et al.* [426] injected rabbits with the red cells of an African American, Mr Hunter, and the resulting antibody agglutinated the red cells of about 7% of African Americans [87,426]. Twenty-two percent of West Africans are Hu+ [214], but Hu is relatively rare in white people [87] (Table 3.12). All Hu+ samples, giving 'distinct, positive reactions' with anti-Hu, are N+, although many N+ red cells are Hu–. Anti-Hu has only been produced by immunising rabbits with Mr Hunter's red cells [426,427]; since these cells are no longer available, Hu specificity is close to extinction. Limited family data suggested that Hu is inherited in a Mendelian manner [214].

An antibody provisionally named anti-Sext may represent alloanti-Hu [422]. The antibody reacted with red cells of 24% of African Americans and no white people; all reactive cells were N+. Few red cells of known Hu type were available, but all 13 Hu+ samples reacted with anti-Sext; three Hu– samples did not.

M_1 (213002)

M_1 is only present on M+ red cells [428]. Early examples of anti-M_1 were found associated with anti-M in the sera of M– N+ individuals [16,180,351,428]. At the appropriate pH and dilution these sera behaved as anti-M_1 and, with these sera, 24% of African Americans were found to be M_1+ [87,188]. Two examples of anti-M_1 from M+ N+ individuals provided somewhat lower frequencies for M_1 antigen: 17% of black people and less than 1% of white people were M_1+ [423,429] (Table 3.12).

Tm (213 003), Sj (213 006), and Can (213 004)
Anti-Tm reacts preferentially with N+ cells [430]. Most M+ N+ Tm+ cells are also M_1+ [431]. Anti-Sj was identified as a second antibody in the serum containing the original anti-Tm [87]. Like Tm, Sj has a slightly higher incidence in black than white people (Table 3.12). Sj has only been detected on N+ cells.

The only example of anti-Can reacted with the red cells of 60% and 27% of black and white people, respectively, and showed a preference for M+ cells [424] (Table 3.12). Most M_1+ cells are also Can+ [431].

3.17.1.2 Biochemistry

When tested with desialylated red cells, anti-Can and -Tm (adsorbed free of anti-T) behaved as anti-M and -N, respectively [424,432]. One of the major factors determining Hu, Sext, M_1, Tm, Sj, and Can activity appears to be the GlcNAc content of the *O*-glycans attached to amino acids 2–4 of GPA and GPB [431]. The predominant *O*-glycan on GPA is the disialotetrasaccharide shown in Figure 3.2. An alternative oligosaccharide, in which one of the sialic acid residues is replaced by GlcNAc, also occurs, more commonly in black than white people [258,433]. Dahr *et al.* [431] have suggested that anti-Hu, -Sext, -M_1, -Tm, -Sj, and -Can react with GPA molecules with these variant *O*-glycans when present on the appropriate M or N peptide backbone. If a high enough level of the variant *O*-glycan is present, then some of these antibodies will react with the red cell regardless of MN type. Weakening of N antigen on M_1+ M+ N+ cells compared with M_1– M+ N+ cells [16] could result from anti-N binding less effectively to GPA.N with a high proportion of oligosaccharides containing GlcNAc.

There can be little doubt that the series of antibodies described in this section are distinguishing not only a *GYPA* polymorphism, but also polymorphisms of genes producing the glycosyltransferases responsible for the biosynthesis of the *O*-glycans of the N-terminal region of GPA. Limited family studies have implied that Hu, M_1, and Tm have a regular mode of inheritance [214,434], although one family study suggests anomalous inheritance of M_1 [435].

3.17.2 T, Tn, and Cad

T, Tn and Cad represent alterations of the *O*-linked oligosaccharides of glycophorins. Although studied predominantly on GPA, these determinants are not found exclusively on red cell sialoglycoproteins and may be detected on other red cell components as well as on other cells. They will be considered only briefly here.

T and Tn are cryptantigens; that is, they are not normally detectable. Most human sera contain anti-T and -Tn, so red cells expressing these antigens are polyagglutinable (agglutinated by most human sera) and are described in detail in Chapter 33. Red cells become T-active when they are desialylated, either by sialidase treatment *in vitro* or by the action of bacterial sialidase *in vivo*, resulting in the cleavage of the sialic acid residues from the *O*-linked tetrasaccharides, revealing the T-active structure Galβ1→3GalNAc. Desialylated En(a–) cells have depressed T expression [102,103,106]. The Tn determinant is GalNAc linked to serine or threonine; the *O*-glycans of Tn-active cells consist of this monosaccharide or of a sialylated disaccharide. Tn-active red cells lack β1,3-D-galactosyltransferase (T-synthetase) as a result of somatic mutation in a gene encoding a molecular chaperone required for effective T-synthetase function. Consequently, Gal cannot be added to the *O*-linked GalNAc of glycophorins and other structures. T- and Tn-active red cells have depressed expression of M and N.

In the Sd(a++) phenotype (described in Chapter 31) some of the *O*-linked oligosaccharides of glycophorins have an additional GalNAc residue linked to Gal, producing a disialopentasaccharide.

3.18 M, N, S, s, and U antibodies

3.18.1 Human anti-*M*

Anti-M is a relatively common 'naturally occurring' antibody. With a low-ionic strength-polybrene Auto-Analyser and M+ N+ screening cells, 64 anti-M in 22 500 (0.3%) were identified in donor sera, 62 from M– and two from M+ donors [436]. Most anti-M are only reactive at temperatures below 37°C, with an optimum temperature of 4°C, but occasional examples will agglutinate red cells at body temperature. Although generally considered 'naturally occurring', there is evidence that anti-M can be stimulated by transfusion [437,438] or by bacterial infection in children [439]. Many examples of anti-M show a pronounced dosage effect, reacting more strongly with M+ N– than with M+ N+ cells. An incidence of anti-M of one in 2500 donor sera was found by agglutination of M+ N– cells at room temperature, but when M+N+ cells were used for screening an incidence of only one in 5000 sera was found [440]. Anti-M is more common in infants than in adults [441].

Most human anti-M contain an IgM component, though 78% were found to be at least partially IgG and

these IgG antibodies could agglutinate saline suspensions of M+ red cells [442]. Anti-M bind very little or no complement [438,440,443].

MN antibodies are often pH dependent and this topic will be discussed in more detail in Section 3.18.6. By acidifying sera from 1000 M– N+ donors, 21 examples of anti-M dependent on low pH were found [444]. These IgM anti-M had a pH optimum of 6.5 and were mostly inactive at pH 7.5; below pH 6.5 they became non-specific.

M-like alloantibodies, which do not react with the antibody maker's own cells, have occasionally been identified in the sera of M+ individuals [445–447]. In one case, the patient's M-like alloantibody did not react with the cells of his four M+ N+ children who had inherited his M [445]; in another example, the M-like antibody did not react with the M+ N+ red cells of the patient's sister [447].

3.18.2 Human anti-N

Any discussion on anti-N and the N antigen is complicated by the presence of N determinant, not only on GPA of individuals with an *N* allele, but also on GPB of most people. Consequently, most *M/M* people (often denoted M+ N–) do have N on their red cells (usually designated 'N') and only very rarely make anti-N. When they do it is generally weakly reactive. These antibodies, which often agglutinate M+ N– cells at low temperatures and can be removed from the serum by adsorption with M+ N– cells [179,448,449], are not strictly alloantibodies.

Red cells of individuals with the rare M+ N– S– s– (U– or U+^{var}) phenotypes lack 'N' and may produce a potent alloanti-N, which will agglutinate all cells carrying an N determinant, whether on GPA or GPB [180,449–451]. These antibodies have been referred to as anti-'N', -N'N', or -NU; misleading terminologies that suggest they differ in specificity from the anti-N produced by M+ N– S+/s+ people.

Anti-N is relatively rare compared with anti-M [16,436]. Most anti-N are 'naturally occurring', IgM, and inactive above 25°C [440]. Immune anti-N resulting from multiple transfusions do occur [451], usually in people of African origin with M+ N– S– s– U– red cells. A pH-dependent anti-N in the serum of an M+ N– S– s+ man demonstrated optimum reactivity at a pH below 7 [452]. Anti-N often show a pronounced dosage effect.

A few healthy M+ N+ people have produced N-like antibodies, which did not agglutinate autologous cells [453–457].

3.18.3 Clinical significance of anti-*M* and -N

3.18.3.1 Alloantibodies

Most anti-M and anti-N are not active at 37°C and are not clinically significant. They can generally be ignored in transfusion practice and, if room temperature incubation is eliminated from compatibility testing and screening for antibodies, will not be detected. When M or N antibodies active at 37°C are encountered, crossmatch-compatible blood should be provided.

Anti-M and -N have been implicated as the cause of immediate and delayed HTRs [441,451,458–461], though Issitt and Anstee [188] cast doubt on the validity of some of these claims. The suggestion that anti-M and -N can have haemolytic activity was supported by the results of ^{51}Cr survival tests and monocyte phagocytosis assays [451,461].

HDFN caused by anti-M is rare, although anti-M is responsible for over 40% of cases of HDFN in Japan [462]. Anti-M HDFN is often severe, leading to hydrops and fetal death or requiring treatment by exchange transfusion, and is often associated with the absence of a positive DAT [462–470]. One high-titre IgG plus IgM anti-M was responsible for neonatal pure red cell aplasia and caused a substantial reduction in proliferation of erythroid cells in culture [469]. Therefore, like anti-K (Section 7.3.5.2), anti-M may cause HDFN primarily by destroying erythroid progenitors rather than mature erythrocytes. No serious case of HDFN caused by anti-N is recorded, but anti-N in a woman of phenotype M+ N– S– s– U+^{var} caused mild HDFN in her M+ N+ baby [450].

3.18.3.2 Autoantibodies

Of 15 cases of patients with autoanti-M, 11 of the autoantibodies were considered innocuous, whereas the other four gave some symptoms of cold haemagglutinin disease [471]. Where anaemia was reported, it was mild and easily controlled [472,473]. Autoanti-M responsible for warm AIHA has not been reported [155]. A few cases of warm AIHA caused by autoanti-N have been described [155], one of which had a fatal outcome [474].

3.18.4 Anti-N and renal dialysis

In 1972, Howell and Perkins [475] identified 12 examples of apparent anti-N from the sera of 416 prospective kidney transplant patients maintained on chronic haemodialysis. The antibodies disappeared after transplantation. Production of these N-like antibodies (anti-Nf) arose from immunisation of the patients by small numbers of residual red cells on which N determinants

had been altered by the formaldehyde used in sterilisation of the dialysis membranes. Between 21 and 27% of dialysis patients using formaldehyde-sterilised membranes had anti-Nf, regardless of their MN phenotype [476–478]. Anti-Nf is now seldom encountered as formaldehyde is rarely used in reprocessing dialysis units or is used in lower concentrations.

Dahr and Moulds [479] showed that formaldehyde treatment greatly increased the ability of glycophorin to inhibit haemagglutination by anti-Nf, but only if there had been no prior blocking of N-terminal amino groups. They concluded that anti-Nf recognises N determinants on GPA and GPB in which the free amino group of N-terminal leucine is modified by reacting with formaldehyde. Sialic acid residues on the second, third, and fourth amino acids may also be involved in the binding site.

3.18.5 Glucose-dependent antibodies

Some antibodies that only react with red cells previously exposed to glucose have M or N specificity, probably because glucose binds to the amino group of the N-terminal amino acid residues of GPA and GPB, altering the steric configuration of the M or N determinant [480–482]. They were identified because of the presence of glucose in red cell preservative solutions used for antibody identification panels. Incubation in 1–2% glucose solutions at neutral or alkaline pH, for a few hours at 37°C or days at 4°C, rendered red cells agglutinable by these glucose-dependent antibodies. With some of the antibodies other sugars, such as Gal, mannose, or GlcNAc, had the same effect [480,482]. One glucose-specific anti-M, produced in an M– N+ diabetic, agglutinated M+ red cells from six of seven patients with diabetes mellitus without prior incubation of the cells in glucose, presumably as a result of non-enzymatic glycosylation of proteins resulting from elevated serum-glucose levels [481].

3.18.6 Monoclonal and recombinant anti-M and -N

Numerous monoclonal antibodies to M and N antigens have been produced and many examples have been analysed in international workshops [22,81,160]. Most are IgG, although some IgM and IgA anti-M and -N have been generated.

Monoclonal antibodies are usually more sensitive to variations in pH than are the polyclonal antibodies in human and animal sera, which are cocktails of antibody molecules to different epitopes on the same antigenic determinant, all with different pH optima. A number of charged groups exist in the region of the M and N determinants, including the free amino group of the terminal amino acid, the carboxyl groups of sialic acid on amino acid residues 2, 3, and 4, and the glutamic acid at position 5 in N. Variations in pH affect the charge on these groups leading to conformational changes in the region of the M and N determinants, altering the binding affinity with various monoclonal antibodies [483].

Most MN monoclonal antibodies do not react with, or show greatly reduced avidity for, sialic acid-depleted red cells or glycophorins. There are, however, a few monoclonal anti-M and -N that detect sialic acid-independent epitopes [22,81].

F(ab) fragments of murine monoclonal anti-M and -N displayed on the surface of bacteriophages transformed with cDNA representing the light chain variable region had similar immunological properties to those of their parental hybridoma antibodies [484,485]. The affinity of soluble, recombinant anti-N F(ab)-fragment, derived from murine cDNA, was enhanced 100-fold by shuffling of Fd fragments with library-derived light-chains [486].

Stable dimers of F(ab) fragments with anti-M and -N specificities directly agglutinated red cells at concentrations similar to those of corresponding IgG antibodies [487]. Comparison of high- and low-affinity recombinant F(ab) fragments with N specificity and site-directed mutagenesis experiments demonstrated that L-chain amino acid sequences, and particularly Gly91 in complementarity-determining region 3 (CDR3), were important for determining high affinity [488]. Crystallographic analysis has provided a model to explain diminished antigen binding resulting from Gly91Ser substitution in L-chain CDR3 involving steric clashes with H-chain CDR3 [489].

3.18.7 Lectins

A seed extract from *Iberis amara* was found to have M specificity [490], but no seed lectin has proved satisfactory as an anti-M blood grouping reagent.

One of the most useful lectins in blood group serology comes from seeds of a Brazilian plant, *Vicia graminea* [491]. This lectin binds GPA and GPB from M– N+ and M+ N+ cells, but only to GPB from M+ N– cells [492,493]. At the appropriate dilution *V. graminea* lectin behaves as anti-N and is a useful blood grouping reagent because M– N+ cells bind approximately 20 times more molecules of the lectin than M+ N– cells [492,494].

Trypsin treatment enhances the ability of *V. graminea* lectin to bind to 'N'. The lectin agglutinates all trypsin-treated red cells apart from those of the S– s– U– and S– s– $U+^{var}$ phenotypes, and those of other rare phenotypes in which 'N' is not present [495]. *V. graminea*

lectin binds sialidase-treated cells more strongly than untreated cells [492]. The determinant recognised by *V. graminea* lectin is often referred to as N_{Vg} to distinguish it from N.

The minimum binding requirement for *V. graminea* lectin is the disaccharide Galβ1→3GalNAc [95,496], present in the *O*-glycosidically linked tetrasaccharides located around the N-terminus of GPA and GPB. For most efficient binding, N-terminal leucine, which probably affects the steric arrangement of neighbouring *O*-glycans, is required, hence the binding preference for N-active glycophorins. The lectin does not bind GPA.M^c [497], which, like N, has Glu5, but, unlike N, has Ser1 (see Table 3.5). Edman degradation of GPA^N, which removes the N-terminal amino acid residue, results in failure of the molecule to combine with *V. graminea* lectin [498].

Some other lectins are potentially useful as anti-N reagents, especially seed extracts from *Bauhinia purpurea* [499] and *B. variegata* [500], and the extract from leaves of *Vicia unijuga* [501]. Lectins prepared from the seeds of *Mollucella laevis* [502] and *Bandeiraea simplicifolia* [503] have A+N activity; they agglutinate all group A cells and also N+ group O and B cells.

A number of other lectins that have proved useful are those that indicate rare variants of the MNS system by detecting deficiency of normal GPA and/or GPB. Lectin from the seeds of *Maclura aurantiaca* is specific for the disaccharide Galβ1→3GalNAc, but, unlike *V. graminea* lectin, does not distinguish between M and N [504]. Haemagglutination by this lectin is depressed in En(a–) cells compared with En(a+) cells [117]. *Phaseolus vulgaris* lectin binds to the *N*-linked oligosaccharide present on GPA [114]. Like *M. aurantiaca* lectin, radioiodinated *P. vulgaris* lectin has been useful for visualising GPA in gels after electrophoresis [114]. Some lectins, such as *Sophora japonica* (after adsorption with A_1B cells) and *Glycine soja*, preferentially agglutinate sialic acid-deficient red cells [103,117]. These lectins have been utilised in screening for MNS variants with deficiency or alteration of GPA and/or GPB.

3.18.8 Anti-S

Anti-S are usually immune, although 'naturally occurring' examples are known [505,506]. Anti-S, -s, and -U are generally non-complement binding IgG antibodies [440], although IgM anti-S has been reported [507]. S, s, and U antibodies usually react at 37°C, but most are optimally reactive at temperatures between 10°C and 22°C by manual antiglobulin tests under normal ionic conditions [508,509].

Anti-S do not react with S+ red cells that have been exposed to low levels (0.5 mg/l) of sodium hypochlorite (chlorine bleach), probably as result of oxidation of GPB Met29 to methionine sulphoxide; s is not similarly affected [510,511].Sodium hypochlorite contamination of commercial saline has been responsible for false-negative typing for S [511].

Anti-S reagents are notorious for containing antibodies to private antigens: of nine single donor anti-S sera tested, four contained one antibody to a low frequency antigen, one contained two such antibodies, and two were polyspecific with fifteen antibodies to low frequency antigens detected in each [512]. Sera containing alloanti-S are more likely to contain autoantibodies than are sera containing alloantibodies of other specificities [513].

Anti-S has been implicated in HTRs [514,515] and has caused severe and fatal HDFN [516,517]. S– red cells should be selected for transfusion to patients with anti-S.

Autoanti-S has been responsible for AIHA [518,519]. An autoanti-S appeared in the serum of a S+ patient two months after treatment for AIHA caused by an apparently 'non-specific' autoantibody [520]. Autoantibodies that are probably detecting non-polymorphic determinants on GPB may 'mimic' anti-S because of the greater quantity of GPB molecules on S+ cells than on S– cells [521,522] (see Section 3.2.3).

Three human IgM monoclonal anti-S directly agglutinated S+ red cells, but differed in their fine epitope specificity [81,82]. Two reacted with sialidase-treated red cells, one did not. No murine monoclonal anti-S is reported, but some antibodies to GPB react more strongly with S+ than S– cells and behave as anti-S under certain conditions [82,320,523].

3.18.9 Anti-s

Anti-s is rare. It may be IgM or IgG; four of five anti-s consisted of IgG3 alone [524]. No 'naturally occurring' anti-s is reported. Anti-s are usually optimally reactive at 22°C or below [508,509]. Anti-s has been responsible for severe and fatal HDFN [10,525,526] and for delayed HTRs [515,527]. Red cells of s– phenotype should be selected for transfusion to patients with anti-s.

Five murine monoclonal IgG anti-s of reagent quality were produced by immunising mice with a GPB.s peptide [523].

3.18.10 Anti-U

Many of the serological complexities of anti-U are given in Section 3.6.1. Described here are details about the antibodies themselves and their clinical significance.

Anti-U are generally non-complement-binding IgG antibodies containing an IgG1 component [524,528]; no 'naturally occurring' anti-U has been reported. Like anti-S and -s, U antibodies may have greater reactivity at temperatures below 22°C than at body temperature [508,509].

The first anti-U was responsible for a fatal HTR [177] and several examples of delayed HTRs caused by anti-U are documented [184,529–532]. Monocyte monolayer assays on all of three anti-U gave high scores suggesting potential for clinical significance [528]. In one case the transfused cells responsible for the reaction were S– s– $U+^{var}$, the U antigen being too weak to be detected during compatibility testing [184]. Several examples of anti-U causing HDFN are reported, including one resulting in stillbirth [533].

Autoanti-U, either alone or associated with other autoantibodies, has been implicated in AIHA [534–539]. An IgG2 autoanti-U was responsible for severe AIHA with apparent intravascular haemolysis and bone marrow dyserythropoiesis [539]. Autoanti-U has also been involved in alpha-methyldopa-induced haemolytic anaemia [540]. Nine of 28 (32%) hospitalised patients with AIDS had autoanti-U, detectable in their serum by enzyme tests only [541]. Some autoanti-U only react at low pH and low temperature [535,542]. Whereas makers of alloanti-U are almost invariably black, most patients with autoanti-U are white.

In accord with the concept that the anti-U represents any antibody detecting a protease-resistant determinant on GPB, the epitope of one murine monoclonal antibody defined serologically as anti-U was identified as ^{21}Ile-Ser-Ser-Gln-Thr^{25} [523], separate from the region of amino acids 35–40 considered to represent the determinant of alloanti-U [200].

3.18.11 Anti-U^Z and -U^X

Anti-U^Z and -U^X were originally detected in the sera of Melanesians [543,544]. Anti-U^Z reacts with the red cells of 36% of Melanesians and 61% of Caucasians. Most S+ samples are U^Z+, although the phenotypes S+ U^Z– and S– U^Z+ do exist. Anti-U^X is a similar antibody. It is likely that U^Z and U^X represent determinants on GPB, the apparent association with S resulting from greater quantity of GPB on S+ cells than S– cells (Section 3.2.3).

U-like autoantibodies, similar to anti-U^Z, are quite common in black people of S– s+ U+ phenotype [193] and similar antibodies have also been found in S– s– U– and S– s– $U+^{var}$ black people [186]. An antibody closely resembling anti-U^Z was detected in the serum of a S– s+ U+ $He+^{w}$ Hispanic woman and in an eluate from the red cells of her newborn fourth child [545].

3.19 GYPA mutation assay

The proportion of a small minority of M– N+ or M+ N– red cells in M+ N+ individuals can be determined by flow cytometry with monoclonal anti-M and -N. This has been exploited to estimate the frequency of somatic mutation in erythroid cells [546,547]. Significant increases in apparent mutation were found in cancer patients after exposure to mutagenic chemotherapy drugs [546], in Hiroshima atomic bomb survivors [548], and in Chernobyl accident victims exposed to ionising radiation [549]. The technique has also been used to diagnose 'DNA repair' diseases, ataxia telangiectasia, Fanconi anaemia, and Bloom syndrome [547]. In M+ N+ chemical industry workers exposed to benzene, the presence of M– N+ red cells with a double dose of N (*NN*), but not with a single dose of N (*NØ*), suggested that benzene is responsible for gene-duplicating mutations rather than gene-inactivating mutations [550].

3.20 Association with Rh

The first signs of an association between antigens of the MNS and Rh systems came with the recognition that Rh_{null} cells, which lack all Rh antigens, often have reduced expression of S, s, and U antigens [16,551]. Depression of U expression is generally more manifest than that of S or s. Rh_{null} (regulator and amorph type) and Rh_{mod} cells have between 60 and 70% reduction in GPB compared with normal cells [552,553]. Red cells of individuals heterozygous for the regulator (RhAG-null) alleles have about a 30% decrease in GPB content.

Anti-Duclos (-RHAG1) and -DSLK (-RHAG3) are alloantibodies to high frequency antigens that react with red cells expressing either Rh antigens or U antigen, but not with Rh_{null} U– cells [554,555]. Red cells of the antibody maker had normal Rh antigens and slightly depressed U. Duclos– and DSLK– phenotypes result from separate mutations in *RHAG*, encoding amino acid substitutions in the Rh-associated glycoprotein (RhAG) [555] (see Section 5.20.1). Trimers of RhAG and the Rh proteins are part of a protein macrocomplex in red cell membrane, which includes band 3, GPA, and GPB, plus other membrane proteins and is linked to the cytoskeleton (see Section 10.7 and Figure 10.2).

Two alloantibodies provide serological support for an association between RhD protein and GPB in the red cell membrane. One, an antibody in a multiply transfused D+ S– s+ patient that reacted only with cells bearing both D and S antigens [556]. The other, an apparent anti-D in a

D– S+ s+ U+ patient, did not react with D+ U– red cells or with D+ cells treated with papain or chymotrypsin [557].

3.21 Glycophorins as receptors for pathogens

3.21.1 Glycophorins and malaria

Of the four species of malarial protozoa that parasitise humans, *Plasmodium falciparum* is responsible for the most severe and prevalent form of malaria. An essential stage in the life cycle of malarial parasites is the invasion of host red cells by merozoites. This invasion involves an interaction between receptors on the parasite and ligands on the surface of the red cell. Unlike *P. vivax*, which exploits only one red cell receptor, the Duffy glycoprotein (Section 8.8), *P. falciparum* utilises multiple ligand-receptor interactions, with redundancies in each pathway. Basically, there are two types of pathways: sialic acid-dependent, involving glycophorin A, B, and C as receptors, and sialic acid-independent, involving receptors that include band 3 (Section 10.7), CR1 (Section 20.7), and basigin (Section 22.5).

The first suggestion that human glycophorin may be involved in this interaction came from the observations that GPA-deficient, En(a–) red cells are more resistant to invasion than normal cells [558,559] (Table 3.13). The minority of merozoites that do succeed in entering the En(a–) cells develop normally. S– s– cells, which lack GPB, are less susceptible to invasion than S+/s+ cells, but substantially less resistant than En(a–) cells [560,562]. GPC- and GPD-deficient red cells also demonstrated a degree of resistance to invasion (Section 18.8). Trypsin treatment of red cells, which removes N-terminal segments of GPA, GPC, and GPD, makes them relatively resistant to invasion, and the degree of resistance is greater in trypsin-treated S– s– cells [558,560,562]. The results shown in Table 3.13 are those of Pasvol and his colleagues. Other workers, often using different strains of the parasite, have obtained different data, but the trends are much the same. Surprisingly none of the glycophorin-deficiency phenotypes is common, even in regions where malaria is endemic, apart from in the Efe pygmies of Congo, who are 36% S– s– U– [203].

Sialic acid clustered on the *O*-linked oligosaccharides of sialoglycoproteins is critical to the invasion process of some strains of *P. falciparum*. Tn red cells, which lack sialic acid and Gal from their *O*-linked oligosaccharides, are virtually refractory to invasion by some strains [560,563,564] (Table 3.13). Sd(a++) (Cad) cells, which have a normal level of sialic acid but have an additional GalNAc residue attached to most of their *O*-glycans (Chapter 31) are relatively resistant to *P. falciparum* invasion, possibly because the additional GalNAc residue prevents access of the parasite to its sialic acid ligand [563].

The *P. falciparum* ligand for GPA is the Duffy-binding-like (DBL) protein EBA-175, with both sialic acid and the peptide backbone of GPA essential for binding [565]. GPA dimers bind dimers of EBA-175 that contain six glycan binding sites [566]. Attempts to evade EBA-175, the product of a rapidly evolving gene, could explain why the glycophorin genes are among the fastest evolving in the human genome [567,568]. The ligand for GPB is another *P. falciparum* DBL protein EBL-1, which bound to normal red cells, but not to S– s– U– cells [569]. The ligand for GPC is EBA-140 (Section 18.8).

Table 3.13 Invasion of red cells of various phenotypes with *Plasmodium falciparum* merozoites [559–561].

Phenotype	Deficient structure	Invasion (% of normal)
Normal		100
En(a–) GW	GPA	8
En(a–) RL	GPA	14
S– s– U–	GPB	72
Ge:–2,–3,–4 Leach	GPC	57
Tn*	Gal+sialic acid	8
Trypsin-treated normal	GPA-T1, GPC-T1	38
Trypsin-treated S– s– U–	GPA-T1, GPC-T1, GPB	5

*Approximately 90% Tn and 10% normal cells.
GPA-T1 and GPC-T1, N-terminal glycopeptides of GPA and GPB.

3.21.2 Other pathogens

GPA, especially GPA.M, acts as a receptor for some bacteria. The uropathogenic *Escherichia coli* strain 1H11165 specifically agglutinates red cells carrying an M antigen [570]. This agglutination is not affected by sialidase treatment of the cells. Binding of the bacteria to GPA.M could be inhibited by the glycosylated N-terminal octapeptide of GPA.M. A haemagglutinating adhesin isolated from *E. coli* F41 agglutinated M+ red cells more effectively than M– cells [571]. Glycophorins appear to act as receptors for bacterial toxins that lyse red cells. Coating of red cells with antibodies to GPA and GPB protects the cells from lysis by haemolysins from *E. coli* and *Vibrio cholerae*, respectively [572,573].

GPA is used as a receptor by influenza virus [574] and certain other viruses [567]. Purified GPA, GPB, GPC, and GPD inhibited haemagglutination by influenza viruses A and B [575].

3.22 Development and distribution of MNS antigens

M, N, S, s, U, and most of the other MNS system antigens are well developed at birth, and some have been shown to be present on red cells quite early in fetal life. GPA is present on proerythroblasts, the earliest morphologically recognisable red cell precursor [576–580]. The degree of *O*-glycosylation increases as the erythroid precursor cells differentiate [578], hence M and N antigens are only detectable at a later stage in erythroid development [581].

GPA is restricted to blood cells of erythroid lineage [582] and is often used as an erythroid marker. It is not present on lymphocytes, granulocytes, megakaryocytes, or platelets [17,576,583]. GPA and GPB, and M and N antigens, are present on the erythroleukaemia cell line K562 [576,583,584].

M, N, and certain other GPA-borne antigens are expressed on endothelial cells of human kidney, but only those anti-M and -N detecting sialic acid-independent determinants reacted with kidney tissue, suggesting that the GPA in renal endothelium is incompletely sialylated [585,586].

3.23 Function and evolution of glycophorins

All glycophorins have a long, heavily glycosylated extracellular domain, which carries a lot of sialic acid and, therefore, a substantial negative charge. Consequently, a prime function could be to keep red cells apart and prevent spontaneous aggregation. They also contribute to the glycocalyx or cell coat, an extracellular matrix of carbohydrate that protects the cell from mechanical damage and microbial attack [587]. Phenotypes in which red cells are totally deficient in GPA and GPB (M^K) or GPC and GPD (Leach) are rare and are not associated with ill health. In GPA-deficiency phenotypes, in which the most abundant glycophorin is absent, sialic acid deficiency is partially compensated by increased glycosylation of band 3. M^K red cells have only a 20% reduction in sialic acid content, compared with a predicted 60% reduction if there were no increased glycosylation of band 3 [588].

GPA, which is closely associated in the membrane with the anion exchanger band 3 (see Section 10.4.2), has two major functions relating to band 3. The C-terminal cytoplasmic tail of GPA, including the region close to the membrane-spanning domain, enhances trafficking of band 3 to the cell surface, whereas the extracellular residues 68–70 (87–89) are important for the efficient anion transport activity of band 3 [30,589,590]. In GPA-deficient red cells, band 3 may remain in the Golgi complex longer, resulting in increased extension of the oligosaccharide chains of the *N*-glycan on band 3, but GPA deficiency does not affect the levels of band 3 at the red cell surface [590]. Red cells of band 3 knockout mice do not express GPA at their cell surface, despite the presence of *GYPA* mRNA [591]. In human cells, however, GPA can be expressed in almost complete absence of band 3. GPA-deficient red cells and red cells with the GP.Hil (Mi.V) phenotype, with a GP(A–B) molecule lacking residues 59–131 of GPA, had about 60% of normal levels of sulphate and chloride transport [590]. Bruce *et al.* [590] suggest that when GPA is absent, there is increased flexibility of the membrane domain of band 3 that is associated with reduced anion transport.

GPA may function as a complement regulator, providing limited protection to red cells from complement-induced reactive lysis by inhibiting the formation or binding of C5b–C7 [592]. GPA inserted into K562 cells by electropulsation increased their resistance to natural killer cell attack [593].

GPA is an important factor for the invasion of red cells by malarial parasites (Section 3.21.1). GPA-deficiency phenotypes should, therefore, have a strong selective advantage in areas where *P. falciparum* is endemic, particularly as no pathology has been associated with these phenotypes. Yet GPA-deficiency phenotypes are extremely rare, suggesting that GPA has an important function or,

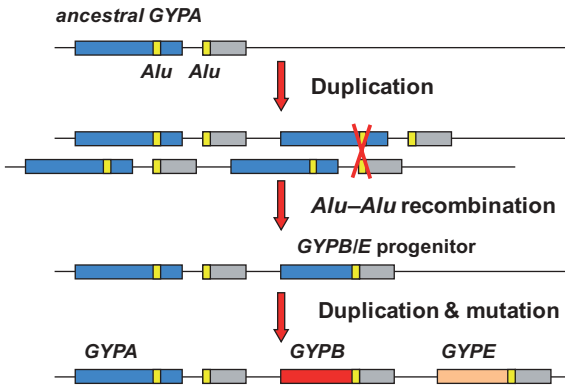


Figure 3.13 Model to explain the evolution of the three glycophorin genes on chromosome 4. Duplication of an ancestral *GYPA* was followed by chromosomal misalignment and unequal crossing-over occurring at an *Alu* sequence within intron A5 of the duplicated *GYPA* ancestral gene and another *Alu* sequence downstream of that gene. Duplication of the resulting hybrid *GYPB/E* progenitor then produced ancestral *GYPB* and *GYPE*. All three genes have been further modified by insertion and deletion. Redrawn with permission from [61], Copyright (1993) National Academy of Sciences, U.S.A.

at least, had one until recent evolutionary history. Glycophorin genes are among the most rapidly evolving genes in humans and analysis of non-synonymous mutations in the *GYPA* gene of primates suggests strongly positive selection in favour of GPA [567,568]. GPA acts as receptor for numerous viruses that are unable to infest erythroid cells (Section 3.21.2). It has been suggested that glycophorins could function as decoy or sink receptors, the red cells 'sopping-up' glycan-binding viruses that can only replicate in nucleated cells and providing the advantage responsible for GPA selection [567,568].

The three glycophorin genes on human chromosome 4 show marked homology from their 5′ flanking sequences to an *Alu* sequence approximately 1 kb downstream of exon 5, the exon encoding the transmembrane domains [48,54]. Figure 3.13 outlines the probable series of events that led to the formation of the three-gene cluster [61]. Duplication of ancestral *GYPA*N* was followed by unequal crossing-over between the *Alu* sequence within intron A5 of duplicated *GYPA* gene and another *Alu* sequence downstream of that gene. This produced a precursor *GYPB/E* gene lacking the 3′ exons of *GYPA*, but acquiring a new sequence from the region downstream of the ancestral *GYPA*. Duplication of this *GYPB/E* gene, followed by divergence, produced ancestral *GYPB* and *GYPE*. *GYPE* subsequently appeared to acquire a segment of *GYPA*M*, including exon 2, by gene conversion [594,595]. This would explain why GPB has the N sequence, but GPE has the M sequence.

GYPA has been detected in all primate species tested; *GYPB* is present in chimpanzee, pygmy chimpanzee, and gorilla, but absent from orangutan and gibbon; and *GYPE* is present in all species with *GYPB*, but only seven of 16 gorillas had *GYPE* [596]. *GYPB* and *GYPE* probably arose from the ancestral *GYPA* prior to gorilla divergence. Chimpanzee and gorilla GPB is larger than human GPB, because of expression of the exon B3, which has become the *GYPB*-pseudoexon in humans. *GYPE* has acquired mutation much more rapidly than *GYPB* or *GYPA*, suggesting that *GYPE* is non-functional, or less functional than the other glycophorin genes [596,597].

Chimpanzee red cells express an M-like antigen. This is probably due to terminal serine on chimpanzee GPA, which has an N-terminal pentapeptide sequence identical to that of the human M^c sequence (see Table 3.5) [598]. N-like activity in red cells of some chimpanzees probably derives from chimpanzee GPB [596,598]. He activity in some gorillas may arise from N-terminal Trp-Ser-Trp on GPA, GPB, and, possibly, GPE [597]. For a review on the expression of MNS antigens on the red cells of non-human primates see [599].

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4 P1PK, Globoside, and FORS Blood Group Systems, plus Some Other Related Blood Groups

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4.1 Introduction

The antigens described in this chapter are classified into three blood group systems and a collection (Table 4.1).

Whilst looking for new polymorphisms by injecting rabbits with human red cells, Landsteiner and Levine [1] discovered the P (now P1PK) blood group system in a series of experiments that also revealed the MN groups. After removing anti-species agglutinins, the immune sera were tested for antibodies that reacted differently with red cells from different people. One such antibody, which could not be explained by ABO or MN, defined two types of blood, now called P1+ (or P_1 phenotype) and P1– (or P_2 phenotype). Human alloantibodies of the same specificity were soon found.

The P system was expanded in 1955 by Sanger [2], who observed that red cells of the very rare phenotype Tj(a–) were always P1– and Tj(a–) was renamed p. Recognition in 1959 of another rare phenotype, P^k, created further complexity [3]. P^k red cells have strong expression of P^k antigen and lack a high frequency antigen, now called P, which is strongly expressed on all other red cells except those of the p phenotype (Table 4.2). P^k red cells may be P_1 or P_2. The Luke antigen (LKE) is another associated antigen of relatively high incidence lacking from p cells [4]. The reactions of antibodies defining these phenotypes are shown in Table 4.2.

The first biochemical steps were taken by Morgan and Watkins [5], who isolated a P1-active glycoprotein from hydatid cyst fluid (HCF). The P1 determinant was identified as a trisaccharide [6]. The identification of the P1, P, and P^k red cell antigens as glycosphingolipids (GSL) followed the work of Naiki and Marcus [7] in identifying the P antigen as the most abundant red cell glycosphingolipid, globoside (Gb4).

A single transferase encoded by a single gene (*A4GALT*) catalyses synthesis of P1 and P^k from different substrates [8]. Consequently, P1 (P1PK1) and P^k (P1PK3) belong to the P1PK blood group system. The product of another gene (*B3GALNT1*) catalyses synthesis of P from P^k, so P (GLOB1) is the only antigen of the globoside system. LKE (209002) and PX2 (209003) are classified in the 209 collection because their genetic backgrounds remain unclear. The rare FORS1 antigen, the Forssman glycolipid, is biochemically related to P [9].

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Table 4.1 Numerical notation for PIPK, globoside, and FORS systems, and for collection 209.

P1PK (System 3)		Globoside (System 28)		FORS (System 31)	Collection 209	
P1PK1	P1	GLOB1	P	FORS1	209003	LKE
P1PK3	P^k				209004	PX2
P1PK4*	NOR					

Obsolete: P1PK2, previously P; 209001, previously P; 209002, previously P^k.
*Provisional assignment.

Table 4.2 The P blood groups: phenotypes and antibodies.

Phenotype	Frequency in white people	Anti-P1	Anti-P	Anti-P^k	Anti-LKE	Anti-PP1P^k
P_1	75%	+	+	–*	+	+
P_2	25%	–	+	–*	+	+
p	Very rare	–	–/w	–	–	–
P_1^k	Very rare	+	–	+	–	+
P_2^k	Very rare	–	–	+	–	+
LKE+	98%	+ or –	+	–	+	+
LKE–	2%	+ or –	+	+†	–	+
Source of alloantibodies		P_2 people	P^k people	Anti-PP1P^k adsorbed with P_1 cells	LKE– people	p people

*Very weak P^k on these cells cannot be detected by agglutination tests with anti-P^k separated from anti-PP1P^k by adsorption with P_1 cells.
†P^k expression on LKE– cells less strong than P^k expression on P_1^k and P_2^k cells.
w, weak positive reaction.

4.2 Biochemistry, biosynthesis, and genetics

P antigenic determinants on red cells reside in the carbohydrate residues of glycosphingolipids, oligosaccharide chains attached to ceramide that form an important part of lipid raft microdomains [10]. Biosynthesis of the P antigens, like the ABH antigens, occurs by the sequential addition of monosaccharides to a precursor substrate, catalysed by glycosyltransferases. Two biosynthetic pathways are involved in production of these antigens, the globoside series and the paragloboside series, with a common precursor lactosylceramide (Gb2) (Table 4.3 and Figure 4.1). Reviews on P biochemistry include [11,12].

The early biochemical studies showed a close relationship between P1 and P^k, but gave no clue to the structure of P antigen. Using purified glycolipids to inhibit anti-PP1P^k, Naiki and Marcus [7] made the observation that globoside and ceramide trihexoside (Gb3), two very well-characterised glycolipids, constituted red cell P and P^k antigens, respectively. Characterisation of these antigens demonstrated that P^k was the direct precursor of P. Paragloboside (lacto-*N*-neotetraosylceramide) is a precursor of Type 2 ABH antigens, of some gangliosides, and of P1.

4.3 P1 (P1PK1) and anti-P1

4.3.1 Frequency and inheritance

The frequency of P1 varies in different populations. About 80% of white people are P_1. The frequency of P1 is much higher in some African and South American

Table 4.3 Structures of some glycosphingolipids associated with P antigens. P1 trisaccharide shown in red; NOR-active trisaccharide shown in blue.

Antigen	Structure	
	Lactosylceramide (Gb2)	Galβ1→4Glc-Cer
	Paragloboside series	
	Paragloboside	Galβ1→4GlcNAcβ1→3Galβ1→4Glc-Cer
P1	Galactosylparagloboside	Galα1→4Galβ1→4GlcNAcβ1→3Galβ1→4Glc-Cer
	Sialosylparagloboside	NeuAcα2→3Galβ1→4GlcNAcβ1→3Galβ1→4Glc-Cer
PX2		GalNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glc-Cer
	Globoside series	
P^k	Globotriosylceramide, Gb3	Galα1→4Galβ1→4Glc-Cer
P	Globoside (globotetraosylceramide) Gb4	GalNAcβ1→3Galα1→4Galβ1→4Glc-Cer
	Galactosylgloboside, Gb5	Galβ1→3GalNAcβ1→3Galα1→4Galβ1→4Glc-Cer
LKE, SSEA-4	Sialosylgalactosylgloboside, MSGb5	NeuAcα2→3Galβ1→3GalNAcβ1→3Galα1→4Galβ1→4Glc-Cer
	Disialosylgalactosylgloboside, DSGb5	NeuAcα2→3Galβ1→3(NeuAcα2→6)GalNAcβ1→3Galα1→4Galβ1→4Glc-Cer
H	Globo-H (Type 4 H)	Fucα1→2Galβ1→3GalNAcβ1→3Galα1→4Galβ1→4Glc-Cer
FORS1	Forssman (Gb5)	GalNAcα1→3GalNAcβ1→3Galα1→4Galβ1→4Glc-Cer
NOR	NOR1	Galα1→4GalNAcβ1→3Galα1→4Galβ1→4Glc–Cer
	NOR_{int}	GalNAcβ1→3Galα1→4GalNAcβ1→3Galα1→4Galβ1→4Glc–Cer
NOR	NOR2	Galα1→4GalNAcβ1→3Galα1→4GalNAcβ1→3Galα1→4Galβ1→4Glc–Cer

peoples and very much lower in some Asian populations, as low as 30% in Japanese. For details of frequencies for many populations see [13]. In a survey of 2345 Scandinavians, 78.85% were P_1, providing the following gene and genotype frequencies:

P^1	0.5401	P^1/P^1	0.2917
P^2	0.4599	P^1/P^2	0.4968
		P^2/P^2	0.2115

Landsteiner and Levine [1] showed that P1 was inherited and behaved as a Mendelian dominant character. This is supported by all subsequent work.

4.3.2 Variation in strength

The strength of P1 on red cells shows individual variation and appears to be under genetic control [14–16]. Dosage contributes to this variation in strength, as confirmed by molecular genetic testing (Section 4.3.5). Fisher [17] analysed Henningsen's data and calculated that 66% of individuals with strong P1 were homozygous P^1/P^1 and all individuals with weak P1 were heterozygous P^1/P^2.

In(Lu), the rare dominant inhibitor of Lutheran and other red cell antigens that represents *EKLF* mutations, inhibits P1 expression [18,19] and has been responsible for P_2 parents with a P_1 child [19,20] (see Section 6.8).

4.3.3 Development and distribution

P1 is considerably weaker in children than in adults and the frequency of P_2 is substantially higher in newborn babies than in adults [14]. Complete development of P1 is not reached until seven years of age or older [21]. Despite this weak expression at birth, P1 is strongly expressed on fetal red cells. Fetal P1 expression is weaker than adult P1, but the strength of P1 decreases with increasing age of the fetus; P1 was more strongly, and more frequently, expressed by 12 week fetuses than by 28 week fetuses [22].

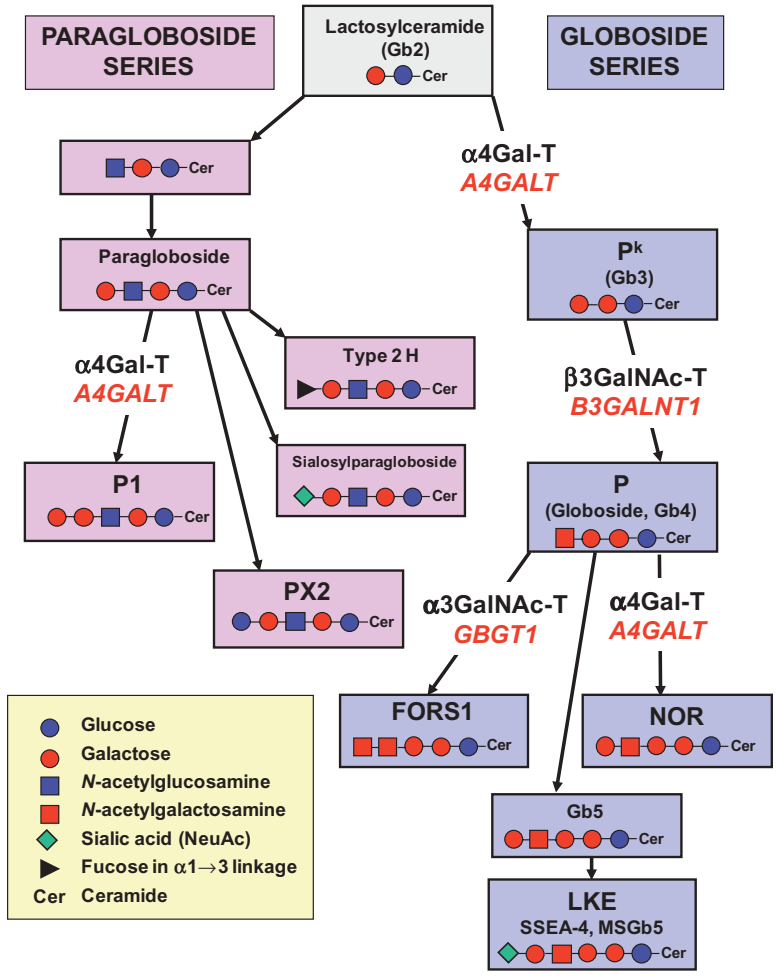


Figure 4.1 Biosynthetic pathways for formation of P and related antigens from a common precursor, lactosylceramide. Glycosyltransferases responsible for production of P1, P^k, P, and Forssman antigens and the genes that encode them are shown.

Flow cytometry with alloanti-P1 revealed that P1 is expressed on lymphocytes, granulocytes, and monocytes [23].

4.3.4 Other sources of P1 substance

Helminths (tapeworms and flukes) are sources of P1-active substances. Fluid from hydatid cysts of sheep livers inhibits anti-P1, but only if the fluid contains scolices [24]. The frequency and avidity of anti-P1 is increased in P_2 patients infested with certain helminths [24–27]. Annelid and nematode worms are also sources of P1 substance; extracts of *Lumbricus terrestris* (earthworm) and *Ascaris suum* inhibit anti-P1 [28].

Some other sources of P1 substance are avian in origin. Red cells, plasma, and excrement of pigeons and turtle doves, and ovomucoid of turtle dove egg white, all contain P1 substance [29–31]. Anti-P1 is more commonly found in P_2 pigeon-fanciers (34%) than in P_2 donors (6%) [29]. Substances like turtle dove ovomucoid and the hydatid cyst wall and protoscolices of helminths, which inhibit anti-P1 and can be used to stimulate anti-P1 production, have branching structures with the P1-trisaccharide (Table 4.3) [32,33].

4.3.5 Biochemistry and biosynthesis

The first information on the biochemical nature of P1 was derived from agglutination-inhibition tests, which

indicated the involvement of α-D-galactose in P1 specificity [34]. Morgan and Watkins [5] applied techniques previously used for extracting A, B, and H substances from body fluids to isolate a P1-active glycoprotein from HCF of sheep, which inhibits anti-P1. The products of partial acid hydrolysis of this glycoprotein led to characterisation of a trisaccharide, Galα1→4Galβ1→4GlcNAc as the P1 determinant [6].

P1 on red cells is a GSL [35–37]. After extensive purification, the structure of the active GSL was identified as the ceramide pentasaccharide shown in Table 4.3 [38,39], with the terminal trisaccharide identical to that isolated from the hydatid fluid P1 glycoprotein [6]. This structure is paragloboside with an additional non-reducing α-galactosyl residue.

P1-trisaccharide is very efficient at inhibiting monoclonal anti-P1 [40,41]. Synthetic glycoproteins containing the P1-trisaccharide have been used to immunise mice in the production of monoclonal anti-P1 [42].

The structure of P1 suggested that an α1,4-galactosyltransferase is responsible for the synthesis of P1 from paragloboside (Figure 4.1). The possibility that P_2 might reflect the lack of the precursor of P1 was eliminated by the observation that normal amounts of paragloboside were found in P_2 cells [43,44]. Red cells of the p phenotype lack P^k (Gb3), P (globoside), and P1 (Table 4.2). The p phenotype is associated with homozygosity for inactivating mutations in the P^k synthetase gene, *A4GALT*, explaining the absence of P^k (Section 4.10.2), but not the absence of P1.

Although P^k synthetase (α4Gal-T1) did not catalyse the synthesis of P1 from paragloboside *in vitro* [45] and no polymorphism correlating to P_1/P_2 phenotypes was detected in the coding region of *A4GALT* [45,46], incomplete associations between polymorphisms in the promoter region of *A4GALT* and the P_1/P_2 polymorphism suggested that the product of either *A4GALT* or of a closely linked gene was responsible for P1 synthesis [47–49]. In 2011, Thuresson *et al.* [8] confirmed that *A4GALT* is responsible for the P_1/P_2 polymorphism. *A4GALT* mRNA analysis revealed novel transcripts containing only the non-coding exon 1 and a 289/290-bp sequence (exon 2a) from intron 1. These transcripts contained three polymorphisms, one of which, 42C>T (counting from the first residue in exon 2a) was completely associated with P_1/P_2 phenotype. All P_2 samples were 42T/T, whereas P_1 samples were 42C/C or 42C/T. Nucleotide 42T introduces a putative start codon in P^2 alleles, potentially opening a short reading frame encoding 28 amino acids. *A4GALT* transcript levels were about 30 times higher in P_1 samples relative to P_2 samples. Comparison of P_1/P_2 phenotype with genotype confirmed that zygosity provided at least a partial explanation for variability in P1 antigen strength between P_1 individuals.

It is feasible to speculate that a genomic sequence, transcript, or peptide derived from the P^2 allele downregulates transcription at the *A4GALT* locus so that less enzyme is produced. As lactosylceramide is the favoured substrate, P^k is still synthesised at the expense of P1 synthesis from paragloboside, resulting in P_2 phenotype [8].

Identification of a SNP in exon 2a of *A4GALT* associated with the P_1/P_2 polymorphism makes it possible to predict P1 phenotype from genomic DNA [8].

4.3.6 Anti-P1

4.3.6.1 Alloanti-P1

Alloanti-P1 is a common specificity, usually a weak agglutinin active only at low temperature. Rarely has anti-P1 been attributed to stimulation by transfusion of red cells [50–53].

Most examples of anti-P1 do not agglutinate red cells at 25°C or above and these cold-reactive antibodies should not be considered clinically significant. There are two reports of immediate HTRs caused by anti-P1 that agglutinate red cells at 37°C; one had a fatal outcome [54,55]. Some examples have been reported to have caused delayed HTRs, although no anti-P1 was detected in the pretransfusion sample and, in one case, the antibody had disappeared within four months of the reaction [52,56]. Anti-P1 active at 37°C rapidly eliminated 50% of injected radiolabelled P1 cells; the rest were eliminated slowly [57]. Anti-P1 responsible for an immediate HTR gave a strongly positive result in an indirect monocyte monolayer assay with P1 red cells [55]. Patients with anti-P1 should be transfused with red cells compatible by IAT at 37°C. Anti-P1 has not been implicated in HDFN.

Anti-P1 has been found as a separable specificity in the serum of some p people by adsorption with P_2 cells, but anti-P1 has not been reported in any P_2^k individual. Alloanti-P1 in a P_1 pigeon breeder led to the suggestion that the antibody might be directed at a determinant absent from the patient's own P1 antigen [58].

4.3.6.2 Animal anti-P1

The first anti-P1 resulted from immunisation of rabbits with human red cells [1]. Since then, anti-P1 has been found as a 'naturally-occurring' antibody in rabbits and

other animals. Anti-P1 reagents have been made by injecting rabbits or goats with tanned P_2 cells that had been exposed to HCF [59], with partially purified P1 substance from sheep HCF coupled with a protein from *Shigella shigae* [34], with extracts of earthworms [28], or with soluble ovomucoid from turtle dove eggs [31].

4.3.6.3 Monoclonal anti-P1

Monoclonal antibodies with P1 specificity have been produced by immunising mice with turtle dove ovomucoid [40], with synthetic glycoproteins containing the P1-trisaccharide (Galα1→4Galβ1→4GlcNAc) [42], or with human red cells expressing strong P1 [60]. Agglutination of P1 red cells by monoclonal anti-P1 was inhibited by the P1 trisaccharide and by the disaccharide (Galα1→4Gal), the former being 200 times more efficient than the latter [40,41]. P1 monoclonal antibodies produced by immunisation with P1-trisaccharide bound equally well to the P1-trisaccharide and the P^k-trisaccharide (Galα1→4Galβ1→4Glc) [42].

4.4 P^k phenotype, P^k (P1PK3) antigen, and anti-P^k

4.4.1 P^k phenotype

Red cells of most people express P^k very weakly and P strongly, the P^k phenotype refers to those red cells that express P^k strongly and lack P. The expression of P^k on red cells of P^k people is uniformly strong regardless of P_1 or P_2 status; the variation in strength of P1 antigen is similar to that of P+ people. All P^k individuals have 'naturally occurring' anti-P in their serum, which reacts equally strongly with P_1 and P_2 cells. Most sera from P^k people react weakly with p cells, probably as a result of an additional antibody to the PX2 antigen [61] (Section 4.9).

All P^k propositi have been ascertained through anti-P in their sera. No random P^k individual has been reported despite the testing of 28 677 Finnish and 39 939 English donors [62]. P^k appears less uncommon in Finland and Japan than in other populations.

The red cells of parents of P^k propositi are not agglutinated by anti-P^k separated from anti-PP1P^k by adsorption with P1 cells, suggestive of recessive inheritance for the P^k phenotype, and a recessive mode of inheritance was supported by family studies [62–64]. P^k phenotype has a recessive mode of inheritance because it is the precursor of P antigen and is only detected by conventional serological methods on red cells of individuals homozygous for the gene responsible for inactive P synthetase (Section 4.4.2 and Figure 4.1).

4.4.2 P^k antigen, biochemistry, and biosynthesis

Initially red cells of people other than those with the rare P^k phenotype were thought to lack P^k antigen. Red cells of parents and children of P^k propositi were not agglutinated by anti-P^k (separated from anti-P1PP^k by adsorption with P_1 cells) and adsorption tests appeared to confirm this lack of P^k [65]. However, P^k was present on the fibroblasts of P_1 and P_2 individuals, and only absent from those of p people [66]. The following findings demonstrated that P^k is present on P+ red cells, though weakly expressed: the glycolipid Gb3 isolated from membranes of red cells of common phenotype inhibited anti-P^k [7], anti-P1P^k made by addition of globoside to anti-P1PP^k (to inhibit anti-P) agglutinated P_2 cells [67], and a monoclonal anti-P^k of high titre reacted weakly with P_1 and P_2 cells.

Red cells of P_1 LKE– and P_2 LKE– people have stronger expression of P^k antigen than those of individuals with the common P_1 LKE+ and P_2 LKE+ phenotypes, but weaker P^k expression than cells of $P_1{}^k$ and $P_2{}^k$ phenotypes, which are always LKE– [68,69] (Section 4.8).

The involvement of α-D-galactose in P^k specificity, first postulated by Voak *et al.* [70], was subsequently confirmed [71,72]. Anti-P^k, like anti-P1, is inhibited by HCF [3]. Partial acid hydrolysis of the P1P^k glycoprotein, isolated from HCF, yielded the P1-trisaccharide, which inhibited anti-P^k and -P1, and a disaccharide Galα1→4Gal, which inhibited anti-P^k, but not anti-P1 [72]. Other α-galactosyl-terminal oligosaccharides also inhibited anti-P^k [72], confirming the immunodominance of α-galactose in P^k expression.

P^k antigen is Gb3, which has the expected terminal Gal residue (Table 4.3) [7]. Gb3 is absent from p red cells and increased in P^k red cells [73,74]. Monoclonal anti-P^k react with Gb3 [75]. Several monoclonal anti-P^k were derived from mice immunised with synthetic glycoproteins containing the P^k-trisaccharide (Galα1→4Galβ1→4Glc) [42].

As the gene governing the P_1/P_2 polymorphism had been mapped to chromosome 22q13.2 [76,77], Steffenson *et al.* [45] carried out a database search within this region for a sequence homologous to that encoding an α-*N*-acetylglucosaminyltransferase in the hope of finding the gene for the P1 α1,4-galactosyltransferase. They identified a cDNA that encodes an α1,4-galactosyltransferase

(named α4Gal-T1), which catalyses the transfer of Gal from UDP-Gal to lactosylceramide (Gb2) to produce Gb3, the P^k antigen (Figure 4.1). It did not, however, convert paragloboside to P1 antigen, *in vitro*. When transfected with an α4Gal-T1 cDNA construct, Namalawa human lymphoblastoid cells, which have no endogenous α1,4-galactosyltransferase activity, strongly expressed P^k. The same gene, *A4GALT*, was identified by two other groups in the same year [46,77]. It comprises four exons [78], with the whole coding region in the fourth exon, and is located at chromosome 22q13.2 [45]. The GC-rich 5′-flanking region of *A4GALT* contains two silencer elements and a promoter element containing three Sp1 binding sites [79].

Homozygosity for a variety of mutations in *A4GALT* are responsible for the p phenotype by inactivating α4Gal-T1, preventing production of P^k and consequently P, and also of P1. The p phenotype is described in Section 4.10. The role played by *A4GALT* in P1 synthesis is described in Section 4.3.5.

Fabry disease is an X-linked, multisystemic, lysosymal-storage disease resulting from a *GLA* genetic defect leading to a deficiency of α-galactosidase A, which is responsible for the degradation of Gb3 [80]. Although there is an accumulation of Gb3 in capillary endothelial cells, testing of red cells for enhanced P^k expression has not been reported.

4.4.3 P^k on other cells

P^k (also known as CD77) has been detected on lymphocytes, granulocytes, monocytes, platelets, smooth muscle of the digestive track and urogenital system, and in other tissues [81]. P^k is also expressed on malignant cells and cell lines derived from them [75,82,83] and is a useful marker for Burkitt's lymphoma (BL) [83]. Of 40 different types of cells, BL cells showed the highest expression of *A4GALT* [84]. P^k (CD77) is a B cell differentiation antigen, with its expression largely restricted to germinal centre cells. Ligation of P^k to CD19, a B cell-restricted antigen, and their subsequent internalisation, appears to be involved in germinal centre B cell apoptosis [85]. Binding of monoclonal antibodies or verotoxin-1 (Section 4.12.1) to P^k induces apoptosis in BL cells through different pathways [86].

4.4.4 Anti-P^k

4.4.4.1 Alloanti-P^k

Alloanti-P^k is found, together with anti-P and -P1, in sera of p people. It can be separated from some of these sera by adsorption with P_1 cells [62]. These anti-P^k react equally strongly with P_1^k and P_2^k cells [62,87].

Anti-P^k is completely inhibited by HCF [3,65]. By inhibition of anti-P^k with fractions of HCF prepared by partial acid hydrolysis, and with oligosaccharides of known structure, Watkins and Morgan [72] concluded that anti-P^k was less demanding in its specificity than anti-P1. They found that the disaccharide Galα1→4Gal purified from the P_1^k glycoprotein of HCF inhibited anti-P^k, as did the P1 trisaccharide (Galα1→4Galβ1→4GlcNAc) and other oligosaccharides with terminal Galα1→4Gal. Anti-P1P^k isolated by addition of globoside to anti-PP1P^k was completely inhibited by P^k GSL (Gb3) [67].

4.4.4.2 Autoanti-P^k

Four examples of autoanti-P^k are recorded: two in patients with AIHA and two in patients with biliary cirrhosis [62].

4.4.4.3 Monoclonal anti-P^k

A rat monoclonal antibody (38.13) raised to a human Burkitt's lymphoma cell line (Daudi) [83], was shown to define Gb3 [75]. Tests against red cells demonstrated the expected anti-P^k specificity. Some other monoclonal anti-P^k resulted from immunising mice with synthetic glycoproteins containing the P^k trisaccharide (Galα1→4Galβ1→4Glc) [42] or with liposomes containing Gb3 glycolipid [88]. *A4GalT*-knockout mice were much more efficient at generating antibodies to Gb3 than conventional mice [88].

4.5 NOR (PIPK4) antigen and polyagglutination

NOR is a form of polyagglutination found in only two families, American and Polish, that appears to be inherited in a dominant manner [89,90]. Red cells of a total of nine individuals from two generations of each of the families were agglutinated by IgM antibody in 71–75% of ABO-compatible adult sera, but were not agglutinated by cord sera. The reaction of NOR cells with human sera was enhanced by papain and sialidase, but reduced by α-galactosidase treatment of the cells. NOR polyagglutination was completely inhibited by HCF and avian P1 substance, but NOR red cells had normal expression of P1 and P antigens.

Thin-layer chromatographs stained with a lectin specific for Galα1→3Gal (*Griffonia simpicifolia* IB4) revealed that NOR red cell membranes contained at least

two unique neutral glycosphingolipids (NOR1 and NOR2, Table 4.3) [90,91]. NOR antibodies (both mouse monoclonal and from human sera) are inhibited by the trisaccharide Galα1→4GalNAcβ1→3Gal (NOR-tri) and, to a much lesser extent, the disaccharide Galα1→4GalNAc (NOR-di) [92]. NOR1 is produced by extension of globoside (Gb_4Cer) by an α4-glactosyltransferase; NOR2 is produced by further extension of NOR1 by a β3-*N*-acetylgalactosaminyltransferase to NOR_{int}, which does not have NOR expression, and then by an α4-glactosyltransferase to NOR2 (Table 4.3, Figure 4.1) [93]. Weak cross-reactivity with Galα1→4Gal explains why the NOR polyagglutination is inhibited by P1 antigen [91]. Fourteen individuals with the NOR phenotype, from both families, were heterozygous for 631C>G in *A4GALT* (the P1PK gene), encoding Gln211Glu [94,95]. Transfection of 2102Ep cells with *A4GALT* led to expression of P^k on the cells; transfection with *A4GALT* containing the Gln211Glu mutation resulted in P^k and NOR expression [95]. It is likely, therefore, that Gln211Glu effects a change in enzyme activity, permitting transfer of Gal to GalNAc instead of, or in addition to, Gal. NOR antigen expression is, therefore, controlled by *A4GALT* and NOR antigen (P1PK4) belongs to the P1PK system.

4.6 P (GLOB1) antigen and anti-P

4.6.1 P antigen

P is found on all red cells except those of the rare phenotypes p and P^k (Table 4.2 and Sections 4.4 and 4.10). P is well developed at birth but, although P is expressed equally on cells from P_1 and P_2 adults [3], P_2 cord cells have a weaker expression of P than P_1 cord cells [96].

P was detected by flow cytometry with human alloanti-P on lymphocytes, granulocytes, and monocytes [23], although other antibodies failed to detect P on granulocytes, most peripheral blood lymphocytes, or fibroblasts [97,98]. P antigen is found on malignant cells and cell lines derived from them [82,97–99] and has also been detected on fetal liver, fetal heart, and placenta [12].

4.6.2 Biochemistry and biosynthesis

P antigen is globoside (Gb4) [7], which is lacking from P^k and p red cells, (or possibly present in trace amounts in p red cells) [72,73]. Globoside is the most abundant red cell membrane GSL with about 14×10^6 molecules per red cell [43,100] and represents Gb3 (P^k) with an additional non-reducing GalNAc residue (Table 4.3). Monoclonal anti-P was inhibited by the terminal trisaccharide of globoside, GalNAcβ1→3Galα1→4Gal, and by Galβ1→3GalNAcβ1→3Gal [101].

Okajima *et al.* [102] used an eukaryotic cell expression cloning system to isolate the cDNA encoding globoside synthetase, a β1,3-*N*-acetylgalactosaminyltransferase (β3GalNAc-T1). The cloned cDNA encodes β3GalNAc-T1, an enzyme that was previously considered to be a galactosyltransferase (β3Gal-T3) [103]. β3GalNAc-T1 synthesises P antigen from P^k by catalysing the transfer of GalNAc from UDP-GalNAc to Gb3 (Figure 4.1). The gene, *B3GALNT1*, is located at chromosome 3q25 [103] and consists of 5 exons, with only exon 5 encoding the enzyme [104]. It is widely expressed, with strong expression in brain and heart, moderate expression in lung, placenta, and testis, and low expression in kidney, liver, spleen, and stomach [102,103].

The P_1^k and P_2^k phenotypes, in which P^k is not converted to P, result from homozygosity for at least eight different mutations in exon 5 of *B3GALNT1*, including single nucleotide insertions, nonsense mutations converting a codon for an amino acid to a stop codon, and missense mutations encoding Asp150Gly, Arg216Ser, Glu266Ala, and Gly271Arg [104–108]. P^1/P^2 genotyping (Section 4.3.5) generally predicts P_1^k and P_2^k phenotypes, although there may be exceptions [106].

4.6.3 Anti-P

4.6.3.1 Alloanti-P

Anti-P is found in the serum of all P^k individuals and can be separated from serum of p individuals by adsorption with P_1^k or P_2^k cells [3,87], or by inhibition with HCF [72]. When complement is present, anti-P will haemolyse P_1 or P_2 cells. P antibodies are IgM and often also IgG, are usually reactive at 37°C, and can cause severe intravascular HTRs [109].

4.6.3.2 Autoanti-P and paroxysmal cold haemoglobinuria (PCH)

PCH is a form of AIHA occurring predominantly in young children following viral infections [110]. Sera from patients with PCH usually give a positive Donath-Landsteiner (DL) test; that is, the antibody binds in the presence of complement at 0°C and haemolyses the cells when subsequently warmed (reviewed in [111]). These biphasic haemolysins, or DL antibodies, generally have P specificity [112–114]. Sera from PCH patients react with P_1 and P_2 red cells, but not with p or P^k cells. Anti-P DL antibody is always IgG. Often the DL test is very weak in PCH and papain-treated red cells or acidified sera may be required before a positive result is obtained [115,116].

Very rarely the specificity of DL antibodies may anti-I, -i, -Pr [115] or 'anti-p' [96,117]. A few cases of AIHA, one with fatal consequences, were caused by IgG monophasic anti-P, haemolytic at temperatures between 20°C and 32°C [118–120]. P autoantibodies only detected in low ionic-strength solution (LISS) at room temperature did not give a positive DL reaction [121,122].

4.7 FORS1 and the Forssman glycolipid

Forssman (Fs) is a glycolipid, named after its eponymous discoverer [123], with a structure representing globoside (P) with an additional non-reducing GalNAc [124] (Table 4.3). Fs glycolipid is present on red cells of a variety of mammals, such as dog, sheep, horse, guinea pig, and mouse [125], but not usually on the red cells of humans and other primates. The gene encoding Fs-synthetase, which catalyses the addition of GalNAc to globoside (Figure 4.1), was initially cloned from a canine cDNA library [126]. A human orthologue, *GBGT1*, has seven exons and is located at chromosome 9q343 [127]. It is part of the GT6 glycosyltransferase family, which includes *ABO*, but transfection of *GBGT1* cDNA into COS-1 produced no Fs-synthetase activity [127] and *GBGT1* has been considered a pseudogene (Section 2.3.2.4).

A putative blood group A variant, named A_{pae}, was found in 1987 in three UK families [128]. A_{pae} red cells were agglutinated by 3 of 18 anti-A and 8 of 18 anti-A,B polyclonal reagents, but no monoclonal reagents, and by *Helix pomatia* lectin (see Table 2.21). The reaction with the lectin was inhibited by GalNAc. An A_{pae} propositus secreted H, but no A. Genotyping, however, revealed that A_{pae} individuals were homozygous for common *O* alleles [9].

In 2011, Hult *et al.* [9] showed that the A_{pae} determinant was not an A antigen, or related to ABO, but was Fs glycolipid. Consequently, Fs became FORS1, the sole antigen of the FORS blood group system. Monoclonal anti-Fs stained a distinct band in thin-layer chromatography of A_{pae} cell membranes, but not of membranes from other group O cells. Further structural analyses and characterisation of naturally occurring anti-Fs in donor sera confirmed that Fs was responsible for the A_{pae} phenotype.

Individuals with A_{pae}, that is FORS1-positive, red cells have 887G>A in *GBGT1*, encoding Arg296Gln. As glutamine is the residue at position 296 of the active animal Fs-synthetase, it is likely that Arg296 is responsible for inactivating Fs-synthetase in most humans and that Arg296Gln is responsible for activating the enzyme in A_{pae} individuals. Three-dimensional modelling of Fs-synthetase based on the crystal structure of the homologous ABO transferase, suggested that residue 296 of Fs-synthetase corresponds to His301 of the ABO transferase, which is important for catalytic activity [9].

4.8 LKE and anti-LKE

The P story was made more complex in 1965 when Tippett *et al.* [4] reported an agglutinin to a high frequency antigen in the serum of Mr Luke P, which behaved like anti-P because it did not react with p and P^k cells, but unlike anti-P because it also failed to react with the cells of about 2% of P_1 and P_2 people. In 1985, the monoclonal antibody 813-70, which defines the murine stage-specific embryonic antigen SSEA-4 [101], was shown to recognise the same red cell antigen as that detected by the antibody in the Luke serum [129]. The red cell antigen was given the symbol LKE.

4.8.1 Frequency and inheritance of LKE

The frequency of LKE– was about 2% in tests with the original Luke serum [4]. Tests on 950 English donors with MAb 813-70 gave the phenotype frequencies, LKE+ 98.84% and LKE– 1.16% [129]. From these the following gene and genotype frequencies were calculated:

LKE^+	0.8923	LKE^+/LKE^+	0.7962
LKE^-	0.1077	LKE^+/LKE^-	0.1922
		LKE^-/LKE^-	0.0116.

Similar frequencies of LKE– were found in Denmark (0.7%) with a human serum [130] and in the United States (1.2%) with 813-70 [69]; only four LKE– individuals were found among 2400 Scottish blood donors [131], an incidence for LKE– of 0.0017. LKE appears to be inherited as a Mendelian dominant character, though data from family studies are too few to be conclusive.

4.8.2 Biochemistry and biosynthesis

Recognition that a monoclonal antibody detecting SSEA-4, a murine stage-specific embryonic antigen [101], defined the red cell antigen LKE, demonstrated that

LKE is a globoseries antigen: monosialosylgalactosylgloboside (MSGb5), a globoside molecule with additional Gal and sialic acid residues [129] (Table 4.3). An LKE-active GSL was identified by high-performance thin-layer chromatography in the ganglioside fraction from LKE strongly positive red cells [69]. The molecular basis for the P+ LKE– phenotype is not known, but there are two prime candidate genes for biosynthesis of LKE from P: *B3GALT5*, which encodes a β1,3-galactosyltransferase (β3Gal-T5) that catalyses the synthesis of Gb5 from globoside (P) [132]; and *ST3GAL2*, which encodes an α2,3-sialyltransferase (ST3Gal-2) that catalyses the synthesis of MSGb5 from Gb5 [133].

4.8.3 Variation in strength of LKE

Variation in strength of reaction of LKE+ cells, classified as LKE+, LKEw, and LKE–, was observed with the Luke serum [4]. LKEw was more common in P_2 than in P_1, and more common in A_1 and A_1B than in O, A_2, A_2B, and B. Variation in the strength of LKE+ cells was also observed with the monoclonal antibody 813-70, but no effect of P_1 or A_1 was demonstrated [69,129], though in one study with the monoclonal antibody LKEw was more common in groups B and AB than in O, A_1, and A_2. The second human anti-LKE did not show any effect of P_1 or ABO groups on the strength of LKE+ reactions.

4.8.4 Development and distribution

Cord red cell samples react well with anti-LKE [129,131]. Monoclonal antibody 813-70 defines a mouse embryonic antigen, SSEA-4, which is also found on human teratoma cell lines [101], and is a marker for human embryonic stem cells [134] and mesenchymal stem cells [135]. LKE-active structures were detected in gangliosides isolated from platelets [136].

4.8.5 Involvement of other P antigens

LKE– individuals may be P_1 or P_2. Parallel testing with anti-P from P^k people and with monoclonal anti-P demonstrated that the strength of P on LKE– cells is the same as that on LKE+ cells [4]. P^k is more strongly expressed on P+ LKE– red cells than on P+ LKE+ red cells [68,69], with LKE– red cell membranes containing almost twice the quantity of Gb3 than LKE+ cells [69]. LKE– red cells have increased binding of verotoxins [69] (Section 4.12.1). Unlike P_1^k and P_2^k cells, which express P^k equally strongly, P_1 LKE– red cells have stronger P^k expression than P_2 LKE– red cells [131].

4.8.6 Anti-LKE

Six examples of human alloanti-LKE are known. The first was found in the serum of a black patient who had never been transfused [4]. The antibody was an agglutinin; the strength of reaction was increased by incubation at low temperature and by enzyme (trypsin, papain, or ficin) treatment of cells. When fresh, the Luke serum lysed papain-treated LKE+ red cells. The agglutinin was not inhibited by saliva or HCF. Five other examples of alloanti-LKE have been found, one present together with anti-P1 [130,131,137,138]. LKE+ babies of mothers with anti-LKE had no symptoms of HDFN [131,137], but one antibody, which was non-reactive in pre-warmed tests but haemolysed red cells in the presence of fresh human serum, was associated with post-transfusion haemolysis [138].

4.9 Sialosylparagloboside and PX2 antigen

An antibody reacting preferentially with p cells was specifically inhibited by sialosylparagloboside [139] (Section 4.11.1), paragloboside with a terminal sialic acid residue (Table 4.3). Sialosylparagloboside levels may be increased in p cells because a blockage in the synthesis of both P^k and P1 results in increased quantities of precursor glycolipids for other biosynthetic pathways (Figure 4.1).

PX2 represents paragloboside with an additional β1→3GalNAc residue [140,141] (Table 4.3). Considerably enhanced quantities of PX2 and its sialylated derivative are present on p phenotype red cells. It is possible that the product of *B3GALNT1*, the same enzyme as that responsible for synthesis of P, catalyses synthesis of PX2 in p cells, where its usual substrate, P^k is absent [141]. Weak reactions with p red cells by most antibodies produced by individuals with the P^k phenotype are probably explained by the presence of anti-PX2 in addition to anti-P [61].

4.10 p Phenotype and anti-PP1P^k

In 1951, Levine *et al.* [142] described an antibody in the serum of a woman with gastric carcinoma, which reacted with all cells except for her own and those of her sister. The antibody was called anti-Tj^a (T for tumour, J for the patient's name). Sanger [2] proposed that Tj(a–) be

called p following her observation that six unrelated Tj(a–) individuals were P_2. Red cells of the p phenotype lack P1, P^k, P, and LKE antigens, although they express enhanced levels of PX2.

4.10.1 Frequency and inheritance of p phenotype

The p phenotype is very rare. Race and Sanger [62] calculated a frequency of 0.0024 for the *p* gene, giving p a phenotype frequency of 5.8 per million people of European origin. The p phenotype is more common in Japan, but screening of over 1 million Hong Kong Chinese revealed no example of p [143]. In the Vasterbotten country of northern Sweden, eight p individuals were found from screening 40 149 donors with anti-$PP1P^k$, a phenotype frequency of about 141 per million [144].

Information from many families with p propositi supports recessive inheritance of *p* [62,145–148], as does the high consanguinity rate.

4.10.2 Molecular genetics of p phenotype

The p phenotype results from homozygosity (or compound heterozygosity) for various missense mutations and nonsense mutations in *A4GALT*, the gene that encodes the enzyme responsible for converting lactosylceramide to P^k (Gb3) and paragloboside to P1 (Figure 4.1) [45,46,104,106,108,149–153]. Most p Swedes are homozygous for 548T>A, Met183Lys or 560G>A, Gly187Asp, with the former as the predominant allele [45,46,104,150]. The most commonly encountered mutations in p Japanese appear to be 752C>T, Pro251Leu [46,104], a triple nucleotide deletion (241_243delTTC) resulting in deletion of Phe81 [104,149,150], and a single nucleotide insertion (1026_1029insC) resulting in a frameshift, disruption of the stop codon, and an additional 92 amino acids [104,149,152]. The latest published list of mutations is in Hellberg *et al.* [153] and they are also listed in dbRBC [108]. Transfection experiments for many of these mutations have shown that they resulted in either no enzymatic activity or only marginal activity, *in vitro* [46,152,153]. P^1/P^2 genotyping (Section 4.3.5) demonstrated the expected linkage between the *p* mutation in *A4GALT* and the P^1/P^2 polymorphism in the same gene: of 20 distinct mutations in 22 different alleles, 11 were associated with P^1 and 11 with P^2 [106]. In all Amish p samples, for example, *A4GALT* 299C>T (Ser100Leu) was linked to P^1.

P synthetase was present in cultured fibroblasts and B-lymphocytes from p individuals [154,155], but could not synthesise globoside in the absence of its acceptor substrate, P^k, explaining the absence of P.

4.10.3 Biochemical effects of p phenotype

Despite the lack of two abundant GSLs, Gb3 and globoside, p red cells appear normal in behaviour and in morphology. Red cells with p phenotype have increased quantities of lactosylceramide and other complex glycolipids [44,73,154], including the PX2 antigen and its derivatives [140,141]. Kidney contains high levels of extended globoseries compounds. Kidney obtained at autopsy from a group A, p phenotype individual, had enhanced levels of lactosylceramide, no Gb3 or globoside, and no Type 4 A (globo-A) chain structure [156] (Section 2.2.2).

4.10.4 Antibodies in serum of p individuals

All p people have antibody in their serum, generally called anti-$PP1P^k$, which agglutinates and/or haemolyses all red cells except those of the p phenotype. Adsorption of p serum with P_2 cells to remove anti-P leaves activity against P_1, but not P_2, cells [2]. Adsorption with P_1^k cells removes anti-P1 and -P^k leaving anti-P [3]; surprisingly, adsorption with P_2^k cells has the same effect [87]. Specific anti-P^k can be made from only some anti-$PP1P^k$ sera. Tippett [157] adsorbed sera from 47 p people with P_1 cells, but only succeeded in making anti-P^k from less than half of these sera, and with those sera continued adsorption with P_1 cells removed or weakened the anti-P^k. Inhibition tests on four p sera with various GSLs indicated that, after inhibition of anti-P with globoside, most of the remaining antibody is cross-reacting anti-$P1P^k$ [67]. This offers an explanation for the inability to isolate anti-P1 from anti-$PP1P^k$ by adsorption with P_2^k cells. Anti-$P1P^k$ was mostly IgG [67], in contrast to the anti-P1 of P_2 people, which is usually IgM. The anti-P component in the sera of two p individuals was IgM and cross-reacted with Forssman antigen; the rest was IgG and specific for globoside [158]. Most of the anti-P^k in these sera was IgG.

IgG and IgA activity to P, P1, and P^k carbohydrate structures, but IgM activity to only P1 and P^k structures, was detected in p sera by radioimmunoassay. All but one of 13 p sera contained IgG3 antibodies to P, P1, and P^k oligosaccharides; some also contained IgG1 and/or IgG2 antibodies, but none contained IgG4 [159].

Anti-$PP1P^k$ is capable of causing rapid removal of transfused cells and severe HTRs [109]. Injection of the original p individual with 25 ml of incompatible red cells resulted in a severe HTR [142]. Anti-$PP1P^k$ as a

potential cause of early abortion and HDFN is discussed in Section 4.13.

4.10.5 p Phenotype and cancer

The original p phenotype was in a woman with gastric carcinoma [142]. She was treated by subtotal gastrectomy, which was a complete success and in the 22 years until her death from unrelated causes there was no evidence of tumour recurrence or metastasis [160]. Unlike her red cells, the tumour expressed P system antigens [161], which led Levine [160] to propose his theory of 'illegitimate' antigens, antigens present on tumours contrary to the genetic constitution of the patient. Moreover, Levine suggested that her anti-PP1P^k had prevented further growth of the tumour.

4.11 Other P antibodies

4.11.1 'Anti-p'

Several alloantibodies have been described that react strongly with p cells and much more weakly, or not at all, with P_1, P_2, and P^k cells [96,117,162]. These antibodies differed slightly in their serological characteristics. One of them was an agglutinin and biphasic haemolysin, which reacted very strongly with p cells, less strongly with P_2 and $P_2{}^k$ cells, and much less strongly with P_1 and $P_1{}^k$ cells [162]. The red cell antigen recognised by this antibody was destroyed by sialidase treatment and was identified as sialosylparagloboside [139]. P2X antigen is also responsible for enhanced reactivity of some antibodies with p red cells [61,140,141] (Section 4.9, Table 4.3).

4.11.2 Anti-IP1, -I^{T}P1, -I^{T}P, and -IP

Anti-IP1 behave as anti-P1 except that they are non-reactive with P1 cord or P1 adult i cells [163]. Anti-I^{T}P1 was identified in a Melanesian [164]. Bithermic anti-I^{T}P behaved as anti-I^T, apart from its failure to agglutinate p cells [165]. Anti-IP, together with anti-IP1, was found in a patient with unusual P and I antigens [166].

4.12 P antigens as receptors for pathogenic micro-organisms

4.12.1 Pathogenic bacteria and their toxins

Escherichia coli is responsible for most recurrent urinary tract infections. Uropathogenic *E. coli* attach to uroepithelial cells before they invade them. Adherence is achieved by lectin-like structures called adhesins, encoded by *pap* genes and located on P fimbriae on the bacterial surface. Isolates of uropathogenic *E. coli* expressing *pap*-encoded adhesins bind to globoseries glycoconjugates containing the disaccharide Galα1→4Gal, including P^k, P1, P, LKE (MSGb5), DSGb5, and globo-A (Type 4 A) (Table 4.3) (reviews in [167,168]). Red cells of the p phenotype are not agglutinated by pyelonephritogenic *E. coli* fimbriae and the bacteria have impaired adhesion to uroepithelial cells from p individuals [169]. Uroepithelial cells from ABH non-secretor women have enhanced adherence to uropathogenic *E. coli* compared to those from secretors [170]. *E. coli* R45 binds to MSGb5 (LKE) and DSGb5 (Table 4.3), structures that are selectively expressed by epithelial cells of non-secretors, presumably as a result of sialylation of the galactosylgloboside precursor, which is fucosylated to globo-H (Type 4 H) in secretors [170].

Some strains of enterohaemorrhagic *E. coli* produce enterotoxins, called verotoxins, which are highly homologous to the Shiga toxin produced by *Shigella dysenteriae*. These verotoxins are associated with diarrhoeal illness and other diseases including haemolytic uraemic syndrome (HUS) [168,171]. P^k antigen is a ligand for VT1 and VT2 [171,172]. Chinese hamster ovary cells that do not express P^k and are resistant to Shiga verotoxin become susceptible to the toxin following transfection with P^k synthetase cDNA [78] and P^k synthetase knockout mice are resistant to doses of verotoxins 100 times higher than those required to kill wild-type mice [173]. Verotoxins induce apoptosis through binding to P^k on megakaryoblasts, which could be a cause of thrombocytopenia in HUS [84], and on Burkitt's lymphoma cells and other malignant cells [86,172,174], which might have therapeutic potential. Verotoxin-induced cytotoxicity and transmembrane signalling require that Gb3 (P^k) is situated within a lipid raft [171].

4.12.2 Parvovirus B19

P antigen is a cellular receptor for parvovirus B19 [175], a human pathogen that is highly tropic to bone marrow and only replicates in erythroid progenitor cells. B19 is the cause of fifth disease, a common childhood illness, and occasionally more severe disorders of erythropoiesis, particularly in immunocompromised patients [176]. B19 empty capsids agglutinate P_1 and P_2 red cells, but not P^k or p cells. The cytotoxic effect of B19 on erythroid colony formation in culture is prevented by sensitising the cells with monoclonal anti-P, but not with anti-P1 or -P^k; there is no cytotoxicity when cells are derived from a p marrow

[175,177]. P antigen is necessary, but not sufficient, for attachment and entry of B19 in erythroid progenitors [178]. Mature red cells express large quantities of P antigen, yet are not invaded by the virus. Activated α5β1 integrin [179] and the Ku80 autoantigen [180] have both been proposed as co-receptors for B19. Individuals with the p phenotype appear to be naturally resistant to B19 infection [177].

4.12.3 HIV-1

GSLs within cholesterol-rich lipid rafts play a vital part in the infection of host cells by HIV-1. Interactions between GSLs, CD4, and the HIV envelope glycoprotein gp120 may facilitate the migration of the virus to its co-receptors CCR5 or CXCR4 and to membrane fusion (reviews [171,181]). Peripheral blood mononuclear cells (PBMC) from patients with Fabry disease, which have enhanced P^k expression (Section 4.4.2) are resistant to infection by the R5 strain of HIV-1 [182]. PBMCs from individuals with the P_1^k phenotype, where P^k is heavily expressed, are highly resistant to R5 and X4 HIV-1 infection, whereas PBMCs from p individuals are up to 1000 times more susceptible to HIV-1 infection [183]. A competitive inhibitor of α-galactosidase A induced P^k accumulation in HIV-infectable cell lines and decreased susceptibility to HIV-1 infection, whereas a glucosylceramide synthase inhibitor, which depletes cells of P^k, substantially increased susceptibility [184]. P^k, therefore, appears to afford protection against HIV-1 infection, possibly through disruption of the organisation of the lipid rafts. A synthetic P^k mimic (FSL-Gb3), which is non-toxic and completely soluble in aqueous solution, prevents HIV-1 infection by direct inhibition of virus and inhibition of viral entry, and so might provide a therapeutic approach for HIV/AIDS [185].

4.13 The association of P antibodies with early abortion

The incidence of habitual spontaneous abortion is significantly higher in women with the p phenotype than in most of the population. Many women with the p phenotype have been ascertained through habitual abortion, though other p women have several live children. Abortions occur characteristically in the first trimester; embryos that survive this critical period usually develop to healthy babies. Most P_1 or P_2 babies of p mothers have no sign of HDFN, although there are a few reports of mild HDFN [145,186].

It is almost certain that anti-$PP1P^k$ in the sera of p women is the cause of the abortions [187], and the anti-P component is the most likely culprit. Habitual spontaneous abortion has also been reported in women with the P^k phenotype: a P_2^k Japanese woman and a P_1^k Kuwaiti woman suffered four and 13 early abortions, respectively [188,189]. Neither had any live children, but in both a procedure of therapeutic plasmapheresis begun at the fifth or sixth week of pregnancy was rewarded by a live birth. Neither baby required any treatment other than phototherapy. In the Japanese case, autologous plasma was returned to the mother after *ex vivo* removal of anti-P by adsorption with donor red cells [188]. Plasmapheresis procedures have subsequently been used successfully for p women with a history of multiple abortions and no live children [159,190–193]. Other children born to P^k mothers have been reported to have no sign of HDFN or only mild HDFN [63,194].

Glycosphingolipid fractions prepared from 12- and 17-week-old fetuses obtained following spontaneous abortions in two p women had only trace amounts of P and P^k antigen activity, whereas the placental fractions had high P and P^k activity. IgG3 antibodies from the serum of one of the p mothers bound strongly to placental glycolipids, but not to glycolipid fractions from the fetus [195]. IgM, IgG (mostly IgG3), and IgA antibodies, strongly reactive with globoside (P antigen) isolated from placenta, were present in the serum of the Kuwaiti P_1^k woman [196]. The primary target for antibodies in p and P^k aborters appears, therefore, to be the placenta and not the fetus.

An unusual antibody was reported in the serum of 'habitual aborters' (pregnant women who threatened to abort for at least a second time) in Perth, Western Australia [197]. This antibody haemolysed, but did not agglutinate, all P_1 and P_2 red cells, but did not haemolyse or agglutinate p cells. The patients were of normal P1 groups. The haemolysin was only present at the time of the threatened abortion [197]. The haemolytic activity did not appear to be complement dependent [198]. Vos [197–200] exhaustively studied these puzzling patients, looking for an environmental or immunological cause for the phenomenon, but no explanation was forthcoming.

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5 Rh and RHAG Blood Group Systems

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5.1 Introduction

Rh is the most complex of the blood group systems, comprising 54 antigens numbered RH1 to RH61 with seven numbers obsolete (Table 5.1). The Rh antigens are encoded by two homologous, closely linked genes on the short arm of chromosome 1: *RHD*, producing the D antigen, and *RHCE*, producing the Cc and Ee antigens. *RHD* and *RHCE* encode RhD (CD240D) and RhCcEe (CD240CE), highly hydrophobic, non-glycosylated proteins, which span the red cell membrane 12 times. Rh antigens are very dependent on the conformation of the Rh proteins in the membrane and may involve interactions between two or more extracellular loops.

The first discovered and clinically most important antigen is D (RH1). D is often referred to as the Rh or rhesus antigen, because it was initially thought to be the same as the antigen, now called LW, defined by antibodies produced in rabbits immunised with rhesus monkey red cells (Section 5.2). D is present on red cells of about 85% of white people and is more common in Africans and Asians. Before the introduction of anti-D immunoglobulin prophylaxis, anti-D was a common cause of severe haemolytic disease of the fetus and newborn (HDFN) (Section 5.18.1.4). Although most people are either D+ or D–, variants of D exist resulting in weak or partial antigen expression (Section 5.6). D– phenotype occurs from the absence of the RhD protein from the red cell membrane. In white people, D– usually results from homozygosity for a deletion of *RHD*, but in D– Africans inactive *RHD* is common.

C and c, E and e represent two pairs of antithetical antigens; polymorphisms controlled by *RHCE*. As no recombination between D, Cc, and Ee has been disclosed, the alleles are inherited as haplotypes denoted *DCe*, *DcE*, *dce*, etc., where *d* represents an *RHD* deletion or any inactive *RHD* gene. From serological results it is often impossible to determine the true genotype of an individual and phenotypes are often symbolised as the most probable genotype deduced from known haplotype frequencies (Section 5.4).

The term 'haplotype' is used throughout this chapter to represent the haploid complement of Rh genes, even though most D– 'haplotypes' will comprise a single Rh gene.

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Table 5.1 Antigens of the Rh system.

No.	Symbol	Comments
RH1	D	Polymorphic; no antithetical antigen
RH2	C	Polymorphic; antithetical to c
RH3	E	Polymorphic; antithetical to e
RH4	c	Polymorphic; antithetical to C
RH5	e	Polymorphic; antithetical to E
RH6	ce, f	Polymorphic; c and e encoded by the same gene
RH7	Ce, rh_i	Polymorphic; C and e encoded by the same gene
RH8	C^w	Polymorphic; antithetical to C^x and MAR
RH9	C^x	LFA; antithetical to C^w and MAR
RH10	V	Associated with ce^s VS+, but not (C)ce^s VS+
RH11	E^w	LFA associated with E variant
RH12	G	Polymorphic; expressed when either C or D present
RH17	Hr_o	HFA; absent from Rh_{null}, D−−
RH18	Hr	HFA; absent from E− e+ hr^S−, D−−, Rh_{null}
RH19	hr^S	e variant
RH20	VS	Associated with ce^s V+ and (C)ce^s V−
RH21	C^G	C-like antigen of r^G
RH22	CE	LFA; C and E encoded by the same gene
RH23	D^w	LFA; associated with DV
RH26	c-like	Variant of c
RH27	cE	Polymorphic; c and E encoded by the same gene
RH28	hr^H	Variant of VS
RH29	total Rh	HFA; only absent from Rh_{null}
RH30	Go^a	LFA; associated with DIVa, DAU-4
RH31	hr^B	e variant
RH32		LFA; associated with D(C)(e) R^N and DBT
RH33		LFA; associated with DHAR
RH34	Hr^B	HFA; absent from E− e+ hr^B−, D−−, Rh_{null}
RH35		LFA; associated with D(C)(e)
RH36	Be^a	LFA; associated with d(c)(e)
RH37	Evans	LFA; associated with D·· and DIVb
RH39		Anti-C-like autoantibody
RH40	Tar	LFA; associated with DVII
RH41		Ce-like
RH42	Cce^s	Associated with (C)ce^s VS+ V−
RH43	Crawford	LFA; associated with ceCF, d(C)(e)
RH44	Nou	HFA; on DIV(C)− and common phenotypes
RH45	Riv	LFA; associated with DIV(C)−
RH46	Sec	HFA; absent from R^N, D−−, Rh_{null} cells
RH47	Dav	HFA; on D·· and common phenotypes
RH48	JAL	LFA; associated with D(C)(e) and D(c)(e)
RH49	STEM	LFA; associated with some hr^S− and hr^B−
RH50	FPTT	LFA; associated with DFR and DHAR
RH51	MAR	HFA; antithetical to C^w and C^x
RH52	BARC	LFA; associated with DVI-3, -4
RH53	JAHK	LFA; associated with r^G
RH54	DAK	LFA; associated with DIIIa, DOL, R^N
RH55	LOCR	LFA; associated with c+ Rh:−26
RH56	CENR	LFA; associated with (C)C^w(e) NR
RH57	CEST	HFA; antithetical to JAL
RH58	CELO	HFA; antithetical to Crawford
RH59	CEAG	HFA; associated with e variant
RH60	PARG	LFA
RH61	ceMO	HFA; absent from homozygous *RHCE*ceMO*

LFA and HFA, low and high frequency antigens.
Obsolete: RH13 (Rh^A), RH14 (Rh^B), RH15 (Rh^C), RH16 (Rh^D), RH24 (E^T), RH25 (LW, now system 16), RH38 (Duclos, now RHAG1).

Numerous variants exist that involve aberrant expression of one or more Rh antigens. These rare haplotypes often produce one or more characteristic low frequency antigens (Section 5.17) and, in the homozygous state, may result in absence of high frequency antigens.

Abnormal expression of D or CcEe antigens may be caused by missense mutations in *RHD* or *RHCE*, but often involve exchange of genetic material between the two Rh genes.

The Rh-associated glycoprotein (RhAG) is a member of the Rh protein family and is produced by *RHAG* on chromosome 6 and expresses the three antigens of the RHAG blood group system (Section 5.20). Complexes, probably heterotrimers, of RhAG, RhD, and RhCcEe are part of the band 3 red cell glycoprotein macrocomplex (Section 5.5.7). No Rh antigens are expressed in the absence of RhAG.

Red cells of the Rh deficiency phenotype Rh_{null} express none of the Rh system antigens (Section 5.16). Rh_{null} has two genetic backgrounds:

1 homozygosity for an *RHD* deletion together with an inactive *RHCE* gene; and

2 homozygosity for inactivating mutations in *RHAG*.

Functions of the Rh proteins are unknown, although there is evidence that RhAG is involved in transport of neutral gases, ammonium and carbon dioxide (Section 5.22).

5.2 History

In 1939, Levine and Stetson [1] investigated a haemolytic reaction, which resulted from the transfusion of a woman with blood from her husband. She had recently given birth to a stillborn baby. An antibody in the mother's serum agglutinated her husband's red cells and those of 80% of ABO compatible blood donors. Levine and Stetson [1] showed that this new antigen, which they did not name, was independent of the known blood groups, ABO, MN, and P.

In 1940, Landsteiner and Wiener [2,3] made antibodies by injecting rhesus monkey red cells into rabbits and guinea pigs. The antibodies, called anti-Rh, agglutinated rhesus monkey red cells, but also agglutinated the red cells from 85% of white New Yorkers. Studies of 60 families showed that Rh-positive was inherited as a dominant character [3]. In the same year, Wiener and Peters [4] identified antibodies of apparently identical specificity in the sera of patients who had transfusion reactions after receiving ABO compatible blood. Levine and Stetson's antibody also appeared to be the same as anti-Rh [5].

As early as 1942, Fisk and Foord [6] demonstrated a difference between animal and human anti-Rh: red cells from all newborn babies, whether Rh+ or Rh– as defined by human anti-Rh, were positive with animal anti-Rh. It took another 20 years to prove that human and animal Rh antibodies did not react with the same antigen. The name Rh for antigens recognised by human antibodies could not be changed since it appeared in thousands of publications and so Levine [7] proposed that the antigen defined by animal anti-rhesus be called LW in honour of Landsteiner and Wiener. The accumulated information illustrating the differences between LW and Rh (D), and the genetic independence of Rh and LW, is described in Chapter 16.

Meanwhile, the complexity of the Rh groups had increased. By 1943 Race *et al.* [8] had four antisera of different Rh specificities, which defined seven alleles; in New York, Wiener [9] with three different antisera could define six alleles.

When Levine and his colleagues [5,10,11] confirmed that incompatibility between mother and fetus was the cause of HDFN, one of the success stories of prophylactic medicine began. The story culminated in the 1960s with the discovery that primary D immunisation caused by an incompatible pregnancy can be prevented by the passive administration of anti-D immunoglobulin shortly after delivery. Only a quarter of a century had elapsed between the identification of the cause and the introduction of an effective preventive measure for the disease.

5.3 Notation and genetic models

Two symbolic notations were developed to explain the increasing complexity of the Rh groups. These notations were based on different genetic theories: the Fisher–Race theory postulated three closely linked loci, *C*, *D*, and *E*, whereas Wiener's Rh-Hr theory predicted multiple alleles of a single gene.

5.3.1 Fisher's synthesis

In 1943, when Fisher [12] noticed that the reactions of two of the four antibodies being used by Race were antithetical, he suggested that the antigens they detected were encoded by alleles, *C* and *c*. The reactions of the other two antibodies did not suggest an allelic relationship and he called these anti-D and -E. Three closely linked loci

Table 5.2 Eight Rh haplotypes and their frequencies in English, Nigerian, and Hong Kong Chinese populations.

Haplotype			Frequencies		
CDE	Rh-Hr	Numerical	English	Nigerian	Chinese
DCe	R^1	*RH*1,2,–3,–4,5*	0.4205	0.0602	0.7298
dce	*r*	*RH*–1,–2,–3,4,5*	0.3886	0.2028	0.0232
DcE	R^2	*RH*1,–2,3,4,–5*	0.1411	0.1151	0.1870
Dce	R^o	*RH*1,–2,–3,4,5*	0.0257	0.5908	0.0334
dcE	*r″*	*RH*–1,–2,3,4,–5*	0.0119	0	0
dCe	*r′*	*RH*–1,2,–3,–4,5*	0.0098	0.0311	0.0189
DCE	R^z	*RH*1,2,3,–4,–5*	0.0024	0	0.0041
dCE	r^y	*RH*–1,2,3,–4,–5*	0	0	0.0036

Results of testing with anti-D, -C, -c, -E, and -e, red cells from 2000 English donors [13], 274 Yoruba of Nigeria [14], and 4648 Cantonese from Hong Kong [15].

producing *D* or *d*, *C* or *c*, and *E* or *e* were postulated and these could be assembled into eight different gene complexes or haplotypes [12] (Table 5.2). Subsequent identification of anti-e [16] and of the rare haplotype *dCE* [17] supported Fisher's hypothesis, but anti-d has never been found. Although some of the rare haplotypes found later could not be accommodated easily, the Fisher–Race CDE language is the clearest for interpretation of the majority of serological reactions and for the communication of results. Where applicable, it is this notation that will be used in this book.

5.3.2 Wiener's theory

In an alternative theory, Wiener suggested multiple alleles (R^1, R^2, R^o, etc., Table 5.2) at a single locus, each encoding an agglutinogen (antigen) composed of several blood factors (serological determinants). For example, the agglutinogen produced by R^1 expresses at least three blood factors, Rh_o, rh′, and hr″ (D, C, and e in Fisher–Race terminology).

5.3.3 Numerical notation

Rosenfield *et al.* [18] considered that the descriptive notations based on different genetical theories had obstructed critical immunological interpretation. They introduced a numerical terminology that recorded serological data 'free of bias and divorced from speculative implication', and which was ideal for computer storage and manipulation. This system avoided the assumptions often made in the older notations: for example, in CDE language the presumed genotype DCe/DCe is often used to describe D+ C+ c– E– red cells, even though they may not have been tested with anti-e.

D in CDE language, Rh_o in Rh-Hr notation, became Rh1. D+ phenotype was Rh:1 and D– was Rh:–1. The alleles producing these phenotypes were designated R^1 and R^{-1}, respectively. This numerical notation, slightly modified, is now the basis for the ISBT terminology for all blood groups (Chapter 1).

5.3.4 Tippett's two-locus model

In 1986, Tippett [19] proposed a new model, based on a wealth of serological data, proposing only two structural Rh genes: one encoding D, the other encoding the CcEe antigens. Mutation within each gene and recombination between the two genes to produce fusion genes comprising part of the D gene and part of the CcEe gene, were considered as possible explanations for some of the rare Rh phenotypes that involve aberrant expression of Rh antigens.

Within a few years, molecular analysis of the Rh genes disclosed the accuracy of Tippett's two-locus and fusion gene theories (Section 5.5.2). As with the MNS system (Chapter 3), the multifarious Rh variants appear to arise from processes involving mutation, unequal

crossing-over, gene conversion, post-translational modification of proteins, and interaction with unlinked genes.

5.4 Haplotypes, genotypes, and phenotypes

5.4.1 Frequencies

From Fisher's analysis [12], eight different Rh haplotypes were predicted and these have all been identified. The frequencies of these haplotypes vary in different populations (for summaries of data from many populations see [14,20]). Haplotype frequencies tend to differ little among Europeans, with *dce* slightly lower and *DcE* slightly higher in southern Europe than in northern Europe. In sub-Saharan Africa *Dce* dominates; in East Asia, the Pacific area, and among the indigenous people of the Americas, haplotypes lacking *D* are either rare or absent. Estimates for three selected populations are given in Table 5.2.

5.4.2 Genotypes and phenotypes

The eight haplotypes shown in Table 5.2 can be paired into (8/2)(8+1) or 36 genotypes, but, by using anti-D, -C, -c, -E, and -e, only 18 phenotypes can be distinguished (Table 5.3). Only eight of these phenotypes represent a single genotype, the other 10 represent two, three, or six possible genotypes. In the CDE notation, phenotypes are often expressed in the form of genotypes (e.g. DcE/dce). Unless demonstrated by family or molecular analysis this is not a true genotype (and not italicised), but the genotype deemed most likely on the basis of gene frequencies for the appropriate population. For example, a white English donor whose red cells give the reactions D+ C– c+ E+ e+ is 16 times more likely to be *DcE/dce* than *DcE/Dce*, and 180 times more likely than *Dce/dcE*. Consequently, the probable genotype would be DcE/dce. In Africans, however, *Dce* is more frequent than *dce* and the probable genotype for D+ C– c+ E+ e+ would be DcE/Dce.

The phenotype D+ C+ c+ E+ e+ covers six genotypes, but can be subdivided by the use of anti-ce, -Ce, -CE, or -cE (Table 5.3), though these antibodies are rare and in short supply (Section 5.10).

Molecular techniques have made it possible to distinguish *D/D* from *D/d* (see Section 5.7.2). In *D/d* individuals who are also heterozygous for *RHCE*, it is not possible to determine which *RHCE* allele is *in cis* with the active *RHD*.

5.5 Biochemistry and molecular genetics of the Rh polypeptides

5.5.1 Identification and isolation of the Rh polypeptides

In early investigations, Green and his colleagues [21–25] found that D is associated with protein and dependent on phospholipid and intact sulphydryl groups. Although isolation of membrane proteins in deoxycholate led to loss of Rh antigen activity, Lorusso *et al.* [25] noted that immune complexes of D with anti-D remained intact in the presence of the detergent. In 1982, Moore *et al.* [26] in Edinburgh and Gahmberg [27] in Helsinki exploited this protective property of anti-D on the integrity of D in the presence of detergent to isolate D antigen. They sensitised ^{125}I surface-labelled D+ red cells or membranes with IgG polyclonal anti-D, solubilised the membranes in non-ionic detergent, and precipitated immune complexes with protein A-Sepharose. SDS PAGE and autoradiography revealed a major component of apparent MW 30 kDa [26–30]; a very hydrophobic protein which, unlike most mammalian cell surface proteins, is not glycosylated and is not phosphorylated [26,27,31]. The D polypeptide is fatty acylated: palmitic acid chains are attached through thioester linkages to cysteine residues located near to the cytoplasmic leaflet of the lipid bilayer [32,33].

Immunoprecipitation of radioiodinated membrane proteins by polyclonal or monoclonal anti-c or -E, or by a monoclonal antibody to a non-polymorphic epitope associated with CcEe, demonstrated that the CcEe antigens are also associated with a membrane protein of apparent MW about 30 kDa [26,28,30,34]. This component resembles the D polypeptide: it is hydrophobic [26], palmitoylated [32,33], and not glycosylated [30]. Its electrophoretic mobility differs slightly from that of the D polypeptide, with an apparent MW about 2 kDa higher [26,28,30].

Confirmation that D and CcEe antigens are expressed on similar, but distinctly different polypeptides, came from one- and two-dimensional peptide mapping. This involved the use of either iodolabelled peptides produced by protease degradation of MW 30 kDa polypeptides from D+ and D– red cells [35,36] or of Rh polypeptides immunopurified with monoclonal anti-D, -c, or -E [37,38].

RhD and RhCE polypeptides were purified by large-scale immunoprecipitation with monoclonal antibodies [37,39] and amino acid sequences obtained for the N-terminal 41 residues of the two polypeptides were identical [40].

Table 5.3 Rh phenotypes with possible genotypes and their frequencies in an English population. Reactions with some antibodies to compound antigens and with anti-G are also provided.

Antigens									Frequency	Other antigens				
D	C	c	E	e	Phenotype		Genotypes		%	ce	Ce	CE	cE	G
+	+	−	−	+	DCe/DCe	R_1R_1	*DCe/DCe*	R^1/R^1	17.68	−	+	−	−	+
							DCe/dCe	R^1r'	0.82					
+	−	+	+	−	DcE/DcE	R_2R_2	*DcE/DcE*	R^2R^2	1.99	−	−	−	+	+
							DcE/dcE	R^2r''	0.34					
+	−	+	−	+	Dce/dce	R_or	*Dce/dce*	R^or	2.00	+	−	−	−	+
							Dce/Dce	R^oR^o	0.07					
+	+	−	+	−	DCE/DCE	R_zR_z	*DCE/DCE*	R^zR^z	<0.01	−	−	+	−	+
							DCE/dCE	R^zr^y	<0.01*					
+	+	+	−	+	DCe/dce	R_1r	*DCe/dce*	R^1r	32.68	+	+	−	−	+
							DCe/Dce	R^1R^o	2.16					
							Dce/dCe	R^or'	0.05					
+	−	+	+	+	DcE/dce	R_2r	*DcE/dce*	R^2r	10.97	+	−	−	+	+
							DcE/Dce	R^2R^o	0.73					
							Dce/dcE	R^or''	0.06					
+	+	−	+	+	DCe/DCE	R_1R_z	*DCe/DCE*	R^1R^z	0.20	−	+	+	−	+
							DCE/dCe	R^zr'	<0.01					
							DCe/dCE	R^1r^y	<0.01*					
+	+	+	+	−	DcE/DCE	R_2R_z	*DcE/DCE*	R^2R^z	0.07	−	−	+	+	+
							DCE/dcE	R^zr''	<0.01					
							DcE/dCE	R^2r^y	<0.01*					
+	+	+	+	+	DCe/DcE	R_1R_2	*DCe/DcE*	R^1R^2	11.87	−	+	−	+	+
							DCe/dcE	R^1r''	1.00	−	+	−	+	+
							DcE/dCe	R^2r'	0.28	−	+	−	+	+
							DCE/dce	R^zr	0.19	+	−	+	−	+
							Dce/DCE	R^oR^z	0.01	+	−	+	−	+
							Dce/dCE	R^or^y	<0.01*	+	−	+	−	+
−	+	−	−	+	dCe/dCe	r′r′	*dCe/dCe*	$r'r'$	0.01	−	+	−	−	+
−	−	+	+	−	dcE/dcE	r″r″	*dcE/dcE*	$r''r''$	0.01	−	−	−	+	−
−	−	+	−	+	dce/dce	rr	*dce/dce*	rr	15.10	+	−	−	−	−
−	+	−	+	−	dCE/dCE	r_yr_y	*dCE/dCE*	r^yr^y	<0.01*	−	−	+	−	+
−	+	+	−	+	dCe/dce	r′r	*dCe/dce*	$r'r$	0.76	+	+	−	−	+
−	−	+	+	+	dcE/dce	r″r	*dcE/dce*	$r''r$	0.92	+	−	−	+	−
−	+	−	+	+	dCe/dCE	$r'r_y$	*dCe/dCE*	$r'r^y$	<0.01*	−	+	+	−	+
−	+	+	+	−	dcE/dCE	$r''r_y$	*dcE/dCE*	$r''r^y$	<0.01*	−	−	+	+	+
−	+	+	+	+	dcE/dCe	r″r′	*dcE/dCe*	$r''r'$	0.02	−	+	−	+	+
							dCE/dce	r^yr	<0.01*	+	−	+	−	+

*Extremely rare.

5.5.2 Cloning of the Rh genes

In 1990, Avent *et al.* [40] in Bristol and Chérif-Zahar *et al.* [41] in Paris utilised the N-terminal amino acid sequence of D polypeptide to isolate and clone an Rh gene from human bone marrow cDNA libraries. Both teams isolated an identical gene with an open reading frame representing 417 amino acids, but differing from the RhD polypeptide sequence. The encoded amino acid sequence of this cDNA, which represents *RHCE* [42,43], is shown in Figure 5.1.



Figure 5.1 Amino acid sequences of the RhCE and RhD polypeptides as deduced from cDNA nucleotide sequences [40,41,44–47]. Where the amino acid of both polypeptides is the same, that of the D polypeptide is shown by a dash (-). The N-terminal methionine (m at position 1) is cleaved from the mature proteins. Cc and Ee polymorphisms are shown.

In 1992, cDNA representing *RHD* was cloned by Le Van Kim *et al.* [44] after further screening of a human bone marrow cDNA library with *RHCE* cDNA. The nucleotide sequence also predicted a 417 amino acid polypeptide, with 92% sequence identity with the RhCE polypeptide (Figure 5.1).

5.5.3 The Rh polypeptides

Although the *RHD* and *RHCE* cDNA open reading frames encode 417 amino acid polypeptides, the N-terminal methionine, which represents the mRNA translation-initiation signal, is cleaved from the mature proteins [40,41]. The RhD and RhCcEe polypeptides differ by between 31 and 35 amino acids, depending on the *RHCE* allele. The N- and C-terminal regions are well conserved (Figure 5.1).

The calculated MW of the Rh polypeptides is 45.5 kDa [40,41,44]. The much lower MW of 30 kDa estimated from SDS PAGE probably results from the abnormally high level of SDS binding to these very hydrophobic proteins. Results of hydropathy analyses on the amino acid sequences suggested that the Rh polypeptides traverse the membrane lipid bilayer 12 times [40,45]. The sequence of the N-terminal domain, together with the lack of a cleaved N-terminal signal sequence, indicated that the N-terminus is located within the cytoplasm [40,41] and immunochemical analyses on intact cells and on leaky ghosts with rabbit antibodies directed at the C-terminal domain confirmed the cytoplasmic location of the C-terminus [42,48,49]. Subsequently, homology modelling based on crystal structures of bacterial homologues of the Rh proteins and on the human non-erythroid Rh family glycoprotein RhCG, have supported the model of 12 membrane-spanning domains and six extracellular domains [50–54] (Figures 5.2 and 5.3). These models predict the presence of an extracellular vestibule in the regions of the third and fourth external loops and sixth, seventh, and eighth membrane-spanning domains that penetrates the cell membrane permitting access to IgG antibodies [50,55,56].

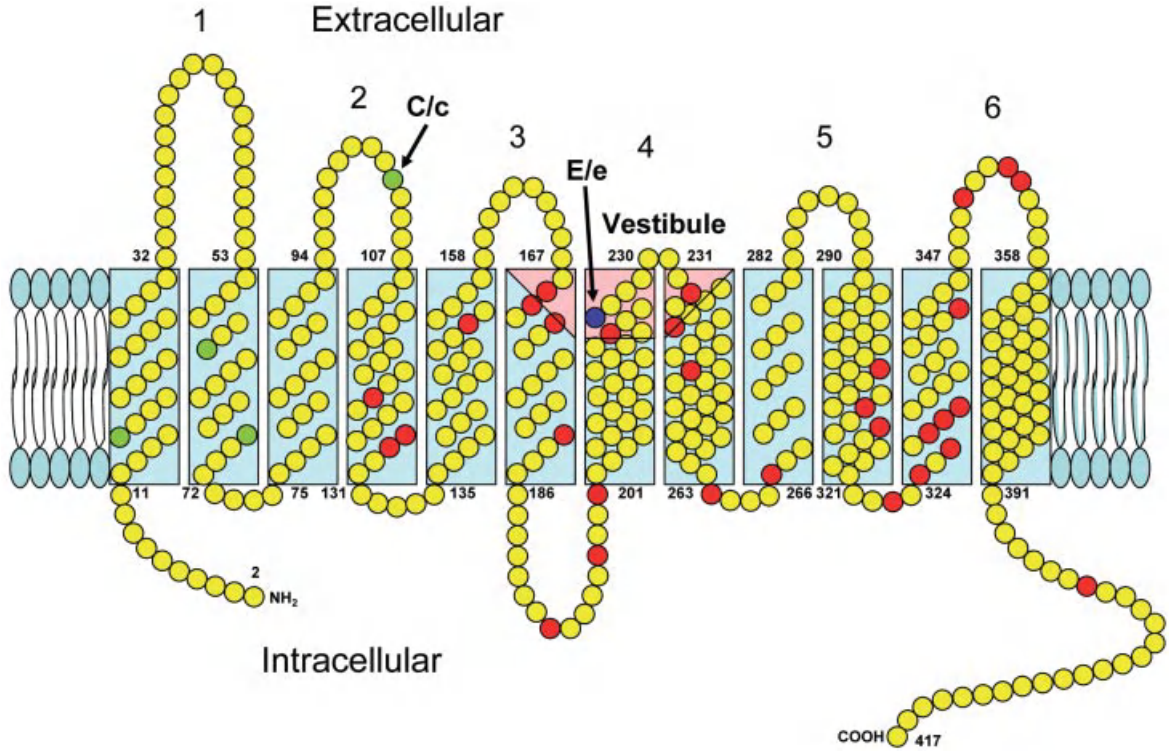


Figure 5.2 Diagrammatic representations of the possible topology of the RhD and RhCE polypeptides within the red cell membrane showing 12 membrane-spanning domains, cytoplasmic N- and C-termini, and the proposed extracellular vestibule region (pink) (after [55]). The yellow circles represent sites where the amino acid residues of the RhD and RhCE polypeptides are the same and the red circles where they may differ; those in green and blue represent the positions of the Cc and Ee polymorphisms, respectively, with the most important C/c residue arrowed.

The RhD and RhCE polypeptides have five and six cysteine residues, respectively. Three of the cysteine residues in the RhCE polypeptide and two in the RhD polypeptide form Cys-Leu-Pro motifs at the point of entry of the polypeptide into the cytoplasmic leaflet of the lipid bilayer and probably represent the major sites for attachment of palmitic acid [32,33]. Expression in erythroleukaemic (K562) cells of RhD polypeptide with the cysteine residues at positions 12, 186, 315, and 316 converted to alanine by site-directed mutagenesis demonstrated that intracellular cysteine residues are not essential for translocation and membrane assembly of RhD [57]. Palmitoylation may play a role, however, in maintaining the tertiary structure of the protein as 11 of 20 monoclonal anti-D showed reduced reactivity with the mutated protein.

The D– phenotype results from absence of the RhD polypeptide from the membrane (Section 5.6.1). Most other Rh antigens are dependent on the conformation of the Rh protein in the membrane and may involve interactions between more than one extracellular domain. Their expression can be affected by relatively minor changes to the protein, such as a single amino acid substitution in a membrane-spanning domain of the protein, which could be remote from the sequence that is primarily responsible for expression of the antigen.

5.5.4 Chromosomal assignment and organisation of the Rh genes

The second autosomal linkage reported in humans was that between the Rh blood group polymorphism and a gene for elliptocytosis, which results from mutations in *EPB41*, the gene encoding the cytoskeletal protein band 4.1 [58,59]. When the gene for peptidase C (*PEPC*) was assigned to chromosome 1 by somatic cell hybridisation [60], it was already known to be syntenic with the linkage

group containing the Rh genes, the gene for the Scianna blood group (*ERMAP*), *PGM1*, *UCK2*, and *FUCA1*. *In situ* hybridisation and somatic cell hybrid analysis located *RHCE* at 1p36.1 [61,62].

The two Rh genes, *RHD* and *RHCE*, have almost identical genomic organisation, each consisting of 10 exons, with exons 1–7 encoding 50–60 amino acids each and exons 8–10 encoding the last 58 residues [63,64] (Table 5.4). The regions of the Rh polypeptides encoded by the exons, according to the 12 membrane-spanning domain model, are shown in Figure 5.4. *RHD* and *RHCE* share 93.8% homology over all introns and coding exons [65]. The most notable difference is in intron 4, where *RHD* has a deletion of about 600 bp, relative to *RHCE* [46,65–67]. Four other intronic insertions or deletions over 100 bp were detected [65].

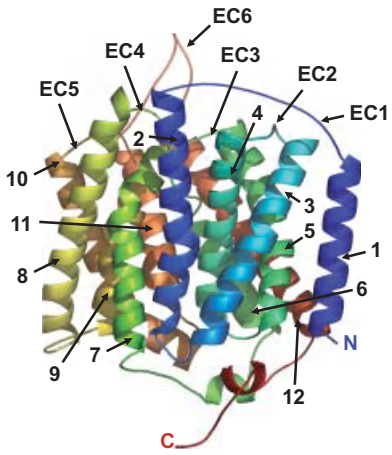


Figure 5.3 Homology model of an Rh protein (RhD or RhCE), showing the intracellular N and C termini, the 12 membrane-spanning domains, and the six extracellular (EC) domains. (Figure provided by Nicholas Burton.)

The transcription initiation site of *RHCE* is located 83 bp upstream of the translation initiation codon [63]. The 5′ flanking regions of *RHD* and *RHCE* contain at least two GATA-1 and Sp1, Ets, E2F, and putative Aly/REF transcription-factor binding sites, and GATA-1, which could play a key part in expression of the Rh genes [63,68,69].

RHD and *RHCE* consist of 57 295 and 57 831 bp, respectively [65], and are separated by about 30 kb, which contains the gene *TMEM50A* (previously *SMP1*) [70] (Figure 5.5). They are very unusual for genes in 'tandem' as they are in opposite orientation: *pter–5′RHCE3′–5′TMEM50A3′–3′RHD5′–cen* (Figure 5.5) [62,70]. *RHD* is flanked by two 9 kb regions of 98.6% homology, the *Rh boxes*. Deletion of *RHD*, the usual cause of the D– phenotype in white people, appears to have occurred between a 1463 bp region of identity in each of the *Rh boxes* [70] (Figure 5.5).

5.5.5 Genomic rearrangement of *RHD* and *RHCE*

Unequal crossing-over and gene conversion, as mechanisms for generating genomic recombination between

Table 5.4 Organisation of *RHD*, *RHCE*, and *RHAG*.

	Codons		3′ intron size (bp)		
Exons	*RHD & RHCE*	*RHAG*	*RHD*	*RHCE*	*RHAG*
1	1–49	1–52	11 857	11 758	17 700
2	50–112	53–114	5269	5575 *C* 5 318 *c*	1000
3	113–162	115–164	10 131	10 437	2300
4	163–211	165–213	426	1075	800
5	212–267	214–269	1627	1627	2100
6	268–313	270–315	3134	3133	1200
7	314–358	316–356	10 276	10 268	3800
8	359–384	357–379	4843	4826	200
9	385–409	380–404	6942	7918	900
10	410–417	405–409			

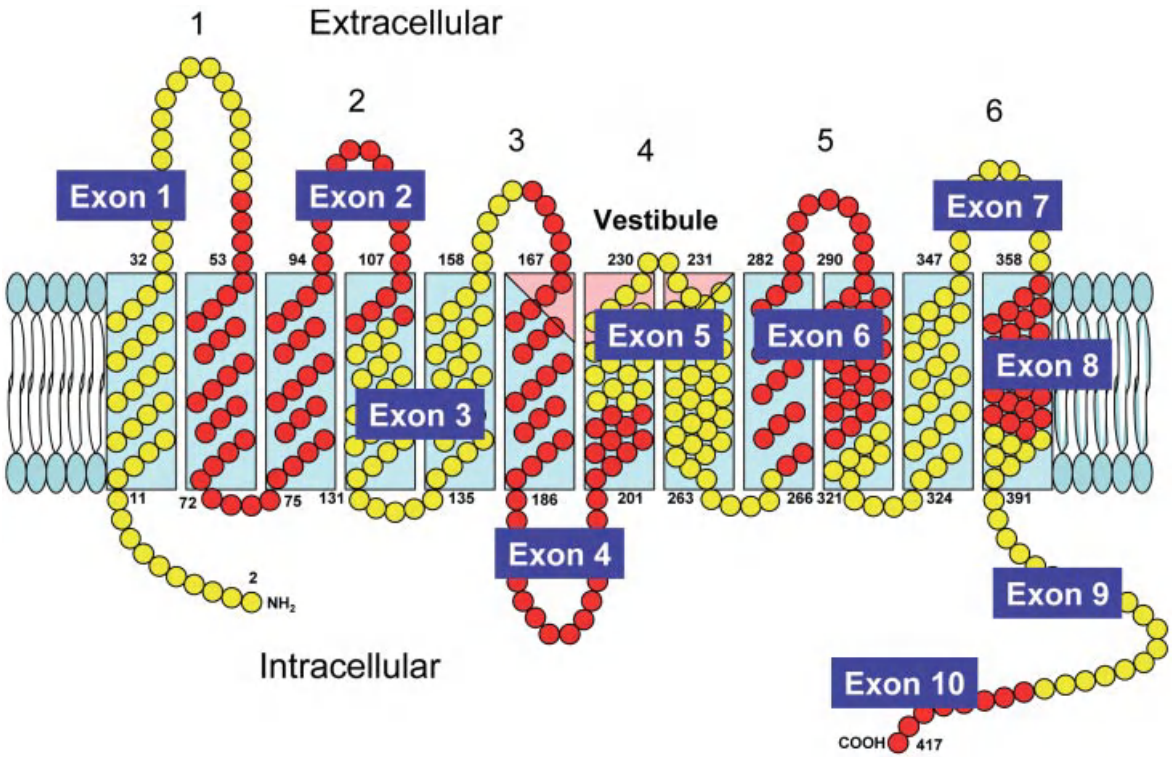


Figure 5.4 Model of the RhD and RhCE polypeptides in the membrane showing the regions encoded by the 10 exons.

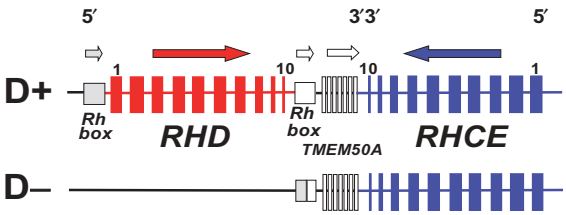


Figure 5.5 Genomic organization of the Rh genes in typical D+ (above) and D– (below) haplotypes, showing *RHD*, the two homologous *Rh boxes*, and *TMEM50A* in 5′ to 3′ orientation, and *RHCE* in 3′ to 5′ orientation. In the D– haplotype, there is a deletion of part of each *Rh box* and of *RHD*.

closely linked homologous genes, are described in Section 3.9 for *GYPA* and *GYPB*. Genomic rearrangements are associated with many examples of variant Rh phenotypes, with gene conversion appearing to be the predominant mechanism and resulting in the creation of *RHD–CE–D* and *RHCE–D–CE* hybrids. Macroconversion events give rise to an Rh gene in which a substantial segment is replaced by the equivalent segment from its homologue, whereas microconversion events can result in exchange of one or more small regions, often leading to single amino acid changes. In addition, gene conversion events are often associated with untemplated mutations, which change nucleotides to those not derived from either gene. *D–CE* or *CE–D* breakpoints for *RHD*DVI*, *RHD*DFR*, R^N, and *Dc–* all occur within a recombination hotspot located in an *Alu*-S sequence and the 100 basepairs immediately downstream of it, within intron 3 (see Figures 5.8 and 5.10) [71].

RHD and *RHCE* are in opposite orientation, so it is likely that the gene conversion events responsible for the generation of Rh hybrid genes occurred following the pairing of *RHD* and *RHCE*, *in cis* (Figure 5.6). Consistent with this supposition, mutations in exons 5 and 7 of the *d(C)ce*^{s} haplotype are present both in the *RHCE* gene and the *RHD–CE(3–8)–D* gene (Section 5.13.2). Likewise, in the DVI-1 haplotype, exons 5 of both *RHCE* and *RHD–CE(4–5)–D* encode Pro226, characteristic of an *RHCE*E* allele (Section 5.6.4.5).

5.5.6 The Rh-associated glycoprotein (RhAG, CD241)

During the original isolation of the 30 kDa Rh polypeptides, Moore *et al.* [26] noticed co-precipitating minor components of lower electrophoretic mobility. These components, with an apparent MW varying between 35 and 100 kDa were shown to be heterogeneously *N*-glycosylated glycoproteins with endo-β-galactosidase-degradable carbohydrate moieties that carry ABH determinants [30,39,72]. Treatment with endoglycosidase-F, which cleaves *N*-glycans, reduced their apparent MW to about 30 kDa [72,73]. This glycoprotein is now named the Rh-associated glycoprotein (RhAG).

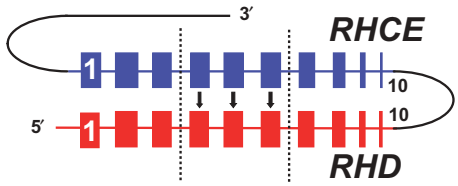


Figure 5.6 Probable mechanism for Rh gene conversion, with pairing between *RHD* and *RHCE*, *in cis*. In this example exons 4–6 of *RHD* are being replaced by the equivalent exons from *RHCE* to produce the *RHD–CE(4–6)–D* gene characteristic of DVI-2 (see Figure 5.8).

Nucleotide sequences of cloned PCR products, amplified from genomic DNA with degenerate primers derived from the N-terminal amino acid sequence of RhAG, predicted a very hydrophobic, 409-amino acid integral membrane protein with two potential extracellular *N*-glycosylation sites, no sites for palmitoylation, and a high degree of sequence similarity with the Rh polypeptides. Hydropathy analysis and *in silico* homology modelling suggested that RhAG closely resembles the Rh polypeptides with 12 membrane-spanning domains [74,75] (Figure 5.7). Only Asn37, on the first extracellular loop, is *N*-glycosylated [49].

Based predominantly on sedimentation velocity through sucrose gradients of membrane components isolated by solubilisation in non-ionic detergent, the Rh core complex was considered to be a tetramer comprising two molecules of Rh polypeptides and two molecules of RhAG [33]. More recent homology modelling based on crystal structures of bacterial Rh homologues and the human Rh family glycoprotein RhCG, the Rh and RhAG proteins appear to form trimers in the membrane (Figure 5.7). These most likely comprise homotrimers of RhAG and heterotrimers of RhAG with RhD or RhCcEe, but least likely to be heterotrimers of RhAG with RhD and RhCE [50–54].

Organisation of *RHAG*, the gene encoding RhAG, is very similar to that of *RHD* and *RHCE* (Table 5.4)

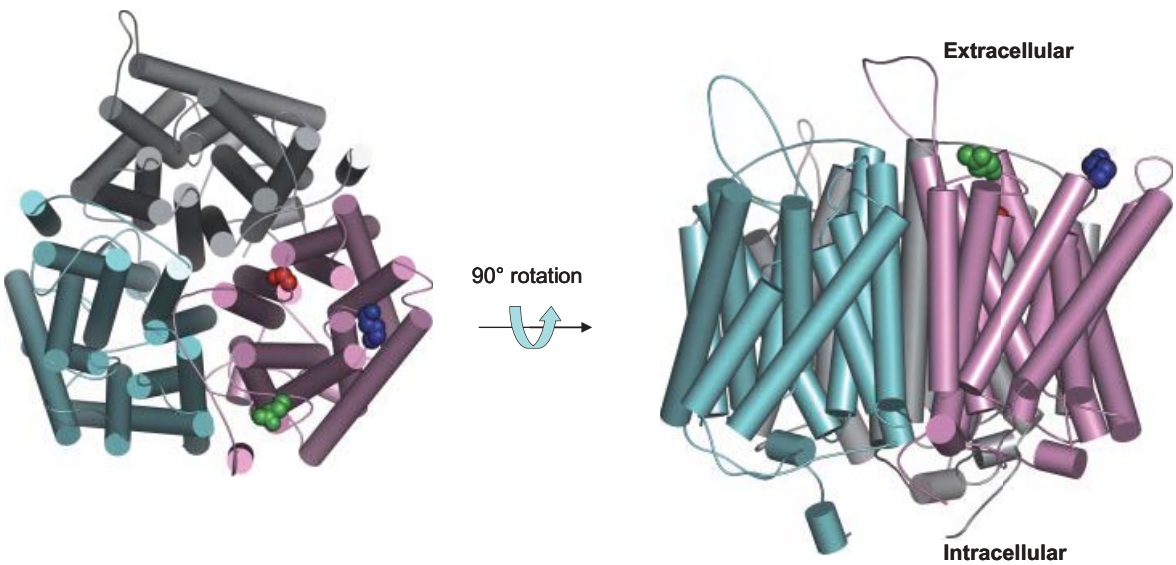


Figure 5.7 Homology model of a heterotrimer of RhAG (pink), RhCE (cyan), and RhD (grey) (reproduced from Tilley *et al.* [76], with permission from John Wiley & Sons). Also shown are the residues associated with Ol^a (red), Duclos (blue), and DSLK (green). In fact, this heterotrimer is unlikely to exist, with heterotrimers containing two molecules of either RhD or RhCE, with RhAG, more probable.

[78–80]. Most of the sequence homology is confined to exons 2–9. The 5′ promoter region contains several putative *cis*-acting elements including an inverted GATA sequence at position −58 to −53, which acts as an erythroid-specific promoter [78,81]. In addition, there is an erythroid-specific hypersensitive site 10 kb upstream from the translation-initiating codon, with powerful *RHAG*-transcription enhancing activity in K562 cells [82]. This site could be responsible for the erythroid-dominant expression of *RHAG*. In contrast to the Rh genes, which are on chromosome 1, *RHAG* is located at 6p12.3 [83].

Presence of RhAG in the red cell membrane is a requirement for expression of Rh antigens. Homozygosity for inactivating mutations in *RHAG* is the most common cause of the rare Rh_{null} phenotype, in which none of the antigens of the Rh system are expressed (Section 5.16.2). Knock-down of RhAG during early erythropoiesis was accompanied by reduced expression Rh proteins at the cell surface [84]. Red cells of *Rhag* knockout mice lack the Rhd protein [85]. Dependency on RhAG for expression of Rh antigens was also demonstrated by transfection experiments in K562 erythroleukaemic cells and human embryonic kidney cells [86–88]. Rh_{null} cells resulting from inactive Rh genes have detectable levels of RhAG [81].

During erythropoiesis *ex vivo*, RhAG appears on early erythroblasts. Initial reports suggest that RhAG appears before the Rh antigens [89–91], whereas a later study shows that they appear concomitantly [84]. The complex of RhAG and the Rh proteins is assembled within the membrane at this early stage of erythropoiesis [84]. The *N*-glycan of RhAG is smaller in Rh_{null} cells, however, inferring that RhAG moves to the surface faster in the absence of the Rh polypeptides [73].

RhAG carries the antigens of the RHAG blood group system described in Section 5.20.

Human homologues of RhAG, named RhBG and RhCG, have been recognised in non-erythroid cells, and many other members of this family are present in other species [80,92–94]. Thus, the Rh family in humans comprises five proteins encoded by the genes *RHD*, *RHCE*, *RHBG* (chromosome 1), *RHAG* (chromosome 6), and *RHCG* (chromosome 15).

5.5.7 The band 3 macrocomplex

RhD, RhCE, and RhAG are part of a macrocomplex of red cell membrane proteins with tetramers of band 3 at its core [95] (see Section 10.7 and Figure 10.2). Other constituents of this complex are the LW glycoprotein, glycophorins A and B, and CD47, and the complex is linked to the red cell cytoskeleton through attachment of band 3 to ankyrin R and protein 4.2.

RhAG and Rh proteins are co-precipitated with band 3 from solubilised membranes [95]. Red cells with band 3 Coimbra, which contain only trace levels of band 3 (~2% of normal), have about 7% and 21% of normal levels of RhAG and Rh proteins, respectively. Band 3 knockout mice have much reduced RhAG and little or no Rh [95]. K562 cells transfected with *RHD* or *RHCE* cDNA express Rh antigens, and subsequent transfection of these cells with cDNA encoding band 3 substantially increased levels of Rh antigens and of endogenously produced RhAG [96,97]. This enhancing effect of band 3 expression appears to be greater on RhCE–RhAG complexes than on RhD–RhAG complexes. The effect is reduced when K562 cells are transfected with band 3 cDNA containing the South-East Asian ovalocytosis (SAO) mutation, providing a possible explanation for reduced expression of Rh antigens on SAO red cells (see Section 10.9). The interaction between RhAG and band 3 in erythroblasts appears to occur inside the cell, followed by transfer to the membrane and subsequent interaction between RhAG and the Rh proteins at the cell surface [84]. Protein 4.2-deficient red cells have normal quantities of the proteins, although hyperglycosylation of RhAG in these cells suggests delayed trafficking from the Golgi [98,99]. There is evidence that direct interaction between ankyrin R and the C-terminal cytoplasmic domains of Rh and RhAG is important for the stability of the Rh complex in the red cell membrane [100]. Amino acid substitutions in those regions of RhD and RhAG could be responsible for weak D and Rh_{null} phenotype, respectively.

Another protein complex first recognised in mice, known as the junctional or 4.1R complex, contains band 3 dimers, Rh proteins, but not RhAG, plus Kell, Xk, and Duffy proteins, attached to the cytoskeleton through glycophorin C, protein 4.1R, and p55 [101] (see Section 10.7 and Figure 10.2).

Monoclonal anti-LW co-precipitates LW glycoprotein and Rh polypeptide [102]. LW glycoprotein is totally absent from Rh_{null} cells (Section 5.16.5) and LW antigens have a higher level of expression on D+ than D− cells (Chapter 16).

Rh_{null} cells have a 60–70% reduction in glycophorin B (GPB) [103], reflected as reduced levels of S, s, and U antigens (Section 3.20). GPB might facilitate transport of RhAG to the cell surface membrane. The RhAG of GPB-deficient (S− s− U−) red cells is more heavily glycosylated

than normal, probably as a result of RhAG remaining in the intracellular membrane system longer, permitting more glycosylation [73], and red cells of individuals homozygous for *GYP*Mur*, in which GPB is replaced by a GP(B–A–B) molecule, have significant reductions in cell surface expression of RhAG and Rh antigens [104].

CD47 is a widely distributed glycoprotein of the immunoglobulin superfamily (IgSF), with an apparent MW of 47–52 kDa in red cell membranes, five membrane-spanning domains, and an extracellular N-terminal domain containing a single IgSF V domain [105]. *CD47* is located on chromosome 3q13.1-q13.2 [106,107]. CD47 is present in reduced quantity (75% of normal) on Rh_{null} red cells [72,106,107] and also on cells expressing RhD, but no RhCE (D–– and related phenotypes, Section 5.15) [98]. CD47 is almost absent from band 3-deficient and protein 4.2-deficient red cells [95,98,99], suggesting that it is closely associated with RhCE in the band 3 macrocomplex. In contrast to mice, alteration or deficiency of CD47 in 4.1R-deficient red cells suggests that CD47 may also be located in a putative human junctional protein complex [108]. CD47 functions as a marker of self by binding signal regulatory protein α (SIRPα) on macrophages, generating a negative signal that prevents phagocytosis of the red cells, prolonging red cell survival in the circulation. This interaction could be important in limiting the destruction of host cells in AIHA [109]. There is evidence that CD47 expression decreases on circulating red cells throughout their lifespan and during storage as blood products [110]. In contrast, however, CD47 on stored red cells may undergo a conformational change, bind thrombospondin 1, and act as an "eat me" signal [111].

Fy5 antigen of the Duffy system (Section 8.4.4) is absent from Rh_{null} cells, though there is no evidence of reduced expression of the Duffy glycoprotein or other Duffy antigens. This supports the presence of a human junctional complex containing Rh proteins and the Duffy glycoprotein (see Section 10.7 and Figure 10.2).

5.6 D and variants of D

D is the most immunogenic of the Rh antigens and is the most important clinically. About 80% of D– healthy volunteers and 20–30% of D– patients who received large volumes of D+ blood made anti-D [112–115]. Until the introduction of immunoglobulin prophylaxis, anti-D was a common cause of severe HDFN.

D+ and D– phenotypes are often referred to as Rh+ and Rh–. Between 82 and 88% of Europeans and North American Caucasians are D+; around 95% of black Africans are D+ [14,20]. D is a high frequency antigen in East Asia, reaching 100% in some populations. By normal blood grouping techniques, 99.7% of Hong Kong Chinese [16] and a similar proportion of Japanese [14] appear D+, but a substantial proportion of those classified as D– have a very weak D antigen called DEL (Section 5.6.4.13).

D antigen expression varies quantitatively, with a continuum of antigen strength from the greatly enhanced expression associated with D–– to weak D, the most extreme of which is DEL. Even among the common phenotypes there is readily detectable quantitative variation of D. Less D is expressed in the presence of C [116–122]: in titrations with anti-D, DcE/DcE cells give higher scores than DCe/DCe cells. Fluorescence flow cytometry with monoclonal and polyclonal anti-D demonstrated the following decreasing order of strength of D antigen: DcE/DcE > DCe/DcE > DCe/DCe > DcE/dce > DCe/dce [123–125]. Further discussion on quantitative aspects of D is found in Section 5.6.8.

There is a plethora of *RHD* genes encoding variants of the D antigen and some producing no D antigen at all. It is not possible to list them all here, but they can be found in the web sites of Wagner and Flegel [126].

5.6.1 Molecular genetics of the D– phenotype

D– phenotype represents a total absence of D polypeptide from the red cell membrane and, consequently, absence of all epitopes of the D antigen. This explains why d, an antigen allelic to D, has never been found.

The most common cause of the D– phenotype in people of European origin is homozygosity for a complete deletion of *RHD* (Figure 5.5); D+ Caucasians are either homozygous or hemizygous for the presence of *RHD* [44,46,127]. The deletion occurs between two 1463 bp regions of identity within the *Rh boxes* that flank *RHD* [70]. Other causes of D– in Caucasians are rare.

In black Africans a common molecular background for D– is homozygosity or hemizygosity (with an *RHD* deletion) for a complete, but inactive *RHD* called the *RHD* pseudogene or *RHD*Ψ*, which contains a 37 bp sequence duplication consisting of the last 19 nucleotides of intron 3 and the first 18 nucleotides of exon 4 [128]. This duplication could generate a reading frameshift and introduce a premature translation stop codon. Alternatively, if a potential splice site at the 3′ end of the inserted intronic sequence in exon 4 were utilised, the sequence of exon 4 would remain unchanged. *RHD*Ψ* also has a nonsense mutation in exon 6 (Tyr269stop), which ensures that no

RhD protein is present in the red cell membrane. No transcript derived from *RHD*Ψ* was detected. *RHD*Ψ* is usually *in cis* with *RHCE*ce*.

Other abnormal genes that are relatively common in D– Africans are called *RHD-CE-D*^{s}. These hybrid genes usually comprise exons 1, 2, and the 3′ end of exon 3 of *RHD*, the 5′ end of exon 3 and exons 4–7 of *RHCE*, and exons 8–10 of *RHD* (type 1), but occasionally the whole of exon 3 is derived from *RHD* (Type 2) [129–132]. These genes produce no D, but probably produce abnormal C. They are associated with the VS+ V– phenotype and the *d(C)ce*^{s} (*r*′^{s}) haplotype (Section 5.13).

In black South Africans and Malians, respectively, the following frequencies of the D– alleles were estimated: *RHD* deletion, 0.10 and 0.14; *RHD*Ψ*, 0.07 and 0.065; *RHD-CE-D*^{s}, 0.04 in both [128,131,133]. Of 82 D– black Africans, 67% had *RHD*Ψ*, 15% had *RHD-CE-D*^{s}, and 18% had no *RHD*. For 54 D– African Americans, the corresponding figures were 24%, 22%, and 54% [128].

In East Asia D– phenotype is rare. Between 62 and 77% of East Asian (Japanese, Chinese, Korean) individuals whose red cells are negative by routine D typing methods are homozygous for the *RHD* deletion [67,134–138]. Most of the remainder, almost all of whom are C+, have a gene for DEL, a variant of D so weak that it can only be detected by very sensitive techniques (Section 5.6.4.13). Between 3 and 8% have an *RHD–CE–D* hybrid gene, *RHD–CE(2–9)–D*$_2$, containing exons 2–9 of *RHD* and producing no D antigen [136–138].

In addition to the *RHD* deletion, *RHD*Ψ*, *RHD-CE-D*^{s}, and *RHD–CE(2–9)–D*$_2$ there are numerous rare *RHD* genes and hybrid genes, which contain exons of *RHD* but produce no RhD protein and, therefore, no D antigen (catalogued in [126]). As a result of screening DNA from 8442 apparent D– European blood donors, 50 (0.59%) contained *RHD*, including three *RHD*DEL* alleles, two missense mutations (encoding Trp16stop or Tyr330stop), a splice site mutation (IVS8+1g>a), *RHD*Ψ*, *RHD-CE-D*^{s}, and various *RHD–CE–D* hybrids, the most common of which was *RHD–CE(2–9)–D*$_2$ [139]. All were linked to either *RHCE*Ce* or *RHCE*cE*, with the exceptions of *RHD*Ψ* and *RHD-CE-D*^{s}, which were linked to *RHCE*ce*.

5.6.2 Weak D (D^{u}) and partial D: a false dichotomy?

Stratton [140] first coined the term D^{u} for a D antigen detected by only some anti-D. The definition of D^{u} evolved to become the D of those red cells that are not agglutinated by IgM anti-D, but which react with IgG anti-D in an antiglobulin test. With the introduction of more potent anti-D reagents, most red cells that would previously have been classified as D^{u}, would not now be considered as having an abnormal D by routine testing. D^{u} has been considered a purely quantitative variant of D, differing from normal D purely on the number of antigen sites per red cell [141–145]. Consequently, there can be no D^{u} antigen and no anti-D^{u}, so 'D^{u}' was replaced with the term 'weak D' in the 1990s [146]. The definition of weak D has often depended on the anti-D reagents and techniques used and so it is difficult to provide frequencies for the weak D phenotype. One estimate gave frequencies for weak D as 0.3% and 1.7% in white and black North London donors, respectively [147].

If weak D is considered a purely quantitative variant of D, then another type of D variant, now usually referred to as partial D, is a qualitative variant. Since the publication by Argall *et al.* [148] in 1953, it has been clear that rare D+ individuals can make a form of alloanti-D. Many different types of partial D antigen have been identified. The D antigen can be regarded as a mosaic of epitopes; individuals whose red cells lack part of the D mosaic can, when exposed to a complete D antigen, make antibody to the missing epitopes. This antibody behaves as anti-D when tested against normal (complete) D.

This distinction between weak D and partial D has come to assume an inappropriate level of importance in transfusion medicine as it is often used as the basis for making the decision on whether to transfuse a patient with D+ or D– red cells and whether or not to give a woman anti-D immunoglobulin after delivery of a D+ baby. The definitions of these two terms are, however, imprecise and the terminology used to identify individual variants is confusing, often representing a mixture of phenotype and genotype.

Two types of differentiating definitions are often used, but neither is satisfactory.

1 'Individuals with partial D antigens can make anti-D; those with weak D antigens cannot.' The problem with this is that a D variant might be called weak D because no person identified with that variant has made anti-D. That does not mean, however, that any patient with that D phenotype will not make anti-D following transfusion with D positive red cells or a D positive pregnancy.

2 'In partial D, the RhD proteins have amino acid changes (from normal RhD) within one or more of the predicted external loops of RhD polypeptide, whereas weak RhD proteins have one or more amino acid substitutions within either the membrane-spanning domains

or the cytoplasmic loops of the protein, but not exposed to the outside of the membrane.' One major problem with this definition is that the precise locations of the amino acid residues of the RhD protein within the membrane are not known. Also, this definition is not functional from the point of view of transfusion practice as *RHD* genotyping is still not performed routinely in many hospitals or even reference laboratories.

The so-called partial D phenotypes have been given symbols such as DIIIa, DVI, DBT, and DFR. They can usually be distinguished from each other by serological means as well as by molecular methods and at least one individual with each phenotype has made alloanti-D. So-called weak D types have been numbered weak D type 1 to weak D type 81 (weak D-1 to weak D-81 here), plus a few intermediate numbers (e.g. weak D-4.2), with the numbers continuing to increase [126,149]. These weak D types are assumed to be associated with weakness of D expression, but it is only possible to distinguish them from each other by molecular genetic testing. Furthermore, in some cases, especially weak D-4.2, -15, and -21, they are found in patients who have made alloanti-D [150,151].

In some cases symbols suggestive of variant D antigen activity may represent *RHD* mutations, without any evidence of abnormal D expression. For example, DMA (Leu207Phe) is encoded by an *RHD* gene that has only been found *in trans* with normal *RHD*, so there is no information on the effect on D expression [133], and weak D-19 (Ile204Thr) and -20 (Phe417Ser) have normal D antigens as determined by serological tests, despite reduced numbers of D antigen sites [152].

As an example of the failure of the current terminology, the D variants DAR and weak D-4.2 have both been found in individuals with alloanti-D and both have the same three nucleotide changes encoding three amino acid substitutions [150,153]. They only differ by a single silent mutation, which does not affect the amino acid sequence of the protein. Consequently, the terms DAR and weak D-4.2 represent D variants with identical phenotypes and almost identical genotypes, yet the former is considered partial D and the latter weak D.

The weak D/partial D dichotomy is artificial and obsolescent, and the terminology used to describe these variants is misleading [154]. The terms weak D and partial D should be abolished and replaced with a single collective name, such as D variant. In the current absence of any internationally established terminology, however, the commonly used terminology will continue to be used in this chapter.

5.6.3 Partial D and the epitopes of D

The pioneering work on the subdivision of D, from which our current understanding of the immunologic profile of the D antigen is derived, was by Tippett and Sanger in the 1960s and 1970s [155,156]. They divided partial D into six categories (I–VI) from the patterns of reactions between the red cells and antibodies of D+ people who had made anti-D. Family studies showed all categories to be inherited. A seventh category was added later and category I is obsolete [157,158]. Associated low frequency antigens have subdivided categories IV, V, and VI, and assist in the definition of DIII, DVII, DFR, DBT, DAU-5, and DHAR (Table 5.5).

In the 1980s, serological analysis of D antigens was revolutionised by the introduction of monoclonal antibody technology. A plethora of monoclonal anti-D was produced. Testing these antibodies against red cells with partial D antigens led to different patterns of reactions, considered to represent different epitopes of the D antigen (epD). Lomas *et al.* [206] defined seven D reaction patterns (epD1 to epD7) by testing 29 monoclonal anti-D against red cells representing most of the category D antigens, and two more epitopes were added later [207–209]. Although epD6 and epD7 had been shown to differ by inhibition studies with radiolabelled antibodies [210], they could not be distinguished by agglutination techniques and were referred to as epD6/7. Eighteen of the 29 antibodies were anti-epD6/7 [206].

In 1994, Lomas *et al.* [187] gave the name DFR to a new partial D antigen, identified with the assistance of monoclonal antibodies. They refrained from naming DFR category VIII because it was no longer possible to carry out all the necessary cross-testing as some of the defining anti-D made by D category members were no longer available. Since then, numerous other partial D antigens have been identified (Table 5.5 [126]), creating many subsplits of the original reaction patterns. The pattern of reactions between partial D antigens and monoclonal anti-D, shown in Table 5.6, was produced in 2001 for the fourth international workshop on monoclonal antibodies to red cell surface antigens [211]. It includes 30 epitopes, with a terminology consisting of the original epD1 to epD9 (excluding epD7) [206,207,209], followed by numbers representing subdivisions of the epitopes (e.g. epD6.4). It is important to remember that the D epitopes really only represent reaction patterns and are not absolute. Reaction patterns may be dependent on antibody concentrations, particularly with epitopes of low affinity, so different batches of the same antibody could produce different results. Other factors, such as

Table 5.5 Selected variant D antigens.

Name	Molecular basis	Exons	EC domain*	LFA‡	*RHCE**	anti-D	No. D sites/ cell × 10^3	Ethnic group	References
DII	A354D	7	6		*Ce*	Yes	3	White	[158,159]
DIIIa (DIII-5)	L62F, A137V, N152T[§], T201R[§], F223V[§]	2,3,4,5	ECV	DAK	*ce*	Yes	12	Black	[158,160,161]
DIIIb (DIII-7)	*RHD–CE(2)–D*, A137V, N152T[§], T201R[§], F223V[§]	2	2, ECV		*ce*	Yes		Black	[158,162–164]
DIIIc	*RHD–CE(3)–D*	3	0		*Ce*	Yes	22	White	[158,165]
DIII-4	L62F, A137V, N152T[§]	2,3	0			Yes	33	White	[150]
DIII-6	A137V, N152T[§], T201R[§], F223V[§]	3,4,5	ECV		*ce*			Black	[163]
DIII-8	A137V, N152T[§]	3	0			Yes	11	White	[166]
DIII-9	N152T[§], P323H	3,7	0			Yes			[166]
DIVa-1	L62F, N152T[§], D350H[§]	2,3,7	6	Go^a	*ce*	Yes	9	Black	[158,167]
DIVa-2	L62F, A137V, N152T[§], D350H[§]	2,3,7	6	Go^a	*ce*			Black	[183]
DIVb	D350H[§], G353W[§], A354N[§], E398V	7–9	6	Evans	*Ce*, *cE*	Yes	4	White	[158,167]
DIV-3	*RHD–CE(6–9)–D*	6–9	6		*Ce*		0.6	White	[149,150]
DIV-4	D350H[§], G353W[§], A354N[§]	7	6		*Ce*		4		[168]
DIV-5	*RHD–CE(7–9)–D*	7–9	6					Japanese	[169]
DV-1 (Kou)	F223V[§], E233Q[§]	5	ECV	D^w	*Ce*			White, Japanese	[167,170]
DVa-2	*RHD–CE(5)–D*	5	ECV	D^w	*ce*, *Ce*, *CE*	Yes	9	White, Black, Japanese	[158,167,171, 172]
DVa-4	E233Q[§]	5	ECV	D^w	*Ce*			Japanese	[170,171]
DVa-6	F223V[§], E233Q[§], V238M[§]	5	ECV	D^w	*Ce*			Japanese	[170,171]
DVI-1	*RHD–CE(4–5)–D*	4,5	ECV		*cE*	Yes	0.3	White	[158,173]
DVI-2	*RHD–CE(4–6)–D*	4–6	ECV	BARC	*Ce*	Yes	2	White, Japanese	[158,174]
DVI-3	*RHD–CE(3–6)–D*	3–6	ECV	BARC	*Ce*		14	White, Chinese	[175,176]
DVI-4	*RHD–CE(3–5)–D*	3–5	ECV	BARC	*Ce*		8	Spanish	[177]
DVII	L110P	2	0	TAR	*Ce*	Yes	3	White	[158,178]
DAR (weak D-4.2)	T201R[§], F223V[§], I342T	4,5,7	ECV		*ce*	Yes	1	Black	[150,153]
DARE	T201R[§], F223V[§], E223Q[§], I342T	4,5,7	ECV		*ce*			Black	[163]
DAU-1	S230I, T379M	5,8	ECV		*ce*		2	Black	[179]
DAU-2	R70Q, S333N, T379M	2,7,8	0		*ce*		0.4	Black	[179]
DAU-3	V279M, T379M	6,8	0		*ce*	Yes	11	Black	[179]
DAU-4	E233K, T379M	5,8	ECV		*ce*		2	Black	[179]
DAU-5	F223V, E233Q, T379M	5,8	ECV	D^w	*ce*		10	Black	[180–182]
DAU-6	S333N, T379M	5,8	0					Black	[181]
DAU-7	V279M, S333N, T379M	6,7,8	0					Black	[183]
DBA	L227P	5	ECV				6	White	[126]
DBT-1	*RHD–CE(5–7)–D*	5–7	ECV	Rh32	*Ce*, *ce*	Yes	4	White, Black, Japanese	[184,185]
DBT-2	*RHD–CE(5–9)–D*	5–9	ECV, 6	Rh32	*Ce*			Japanese	[184,186]
DCS-1	F223V[§], A226P[§]	5	ECV		*cE*		3	White	[182]

(*Continued*)

Table 5.5 (*Continued*)

Name	Molecular basis	Exons	EC domain*	LFA‡	*RHCE**	anti-D	No. D sites/ cell × 10^3	Ethnic group	References
DCS-2	A226P§	5	ECV		*cE*		0.8	White	[182]
DCS-3	F223V§, A226P§, E233Q§	5	ECV					Chinese	[176]
DFR-1	M169L§, M170R§, I172F§	4	ECV	FPTT	*Ce*, *cE*	Yes	5	White	[167,187,188]
DFR-2	*RHD–CE(4)–D*	4	ECV						[189]
DFR-3	M169L§, M170R§, I172F§, G180A	4	ECV					White	[189]
DFR-4	M169L§, M170R§	4	ECV					Chinese	[176]
DFV	F223V	5	ECV		*Ce*	Yes		White, Indian	[163,182]
DFW	H166P	4	ECV		*Ce*			White, Sri Lankan	[190]
DHAR	*RHCE–D(5)–CE*	5	ECV	Rh33, FPTT	*ce*	Yes		White	[191–194]
DHK (DYO)	E233K	5	ECV		*ce*			Japanese	[170–172]
DHMI	T283I	6	5		*cE*	Yes	2	White	[195,196]
DHO	K235T	5	ECV		*Ce*		1	White	[197]
DHR	R229K	5	ECV		*cE*		4	White	[198]
DIM	C285Y	6	ECV		*cE*		0.2	White	[150]
DMH	L54P	1	1		*ce*	Yes		White	[199]
DMI	M170I	4	ECV		*Ce*	Yes		White	[190]
DNB	G355S	7	6		*Ce*	Yes	6	White	[168]
DNU	G353R	7	6		*Ce*		10	White	[159]
DOL-1	M170T, F223V§	4,5	ECV		*ce*	Yes	5	Black	[190]
DOL-2	M170T, F223V§, L378V	4,5,8	ECV		*ce*	Yes		Black	[190,340,699]
DVL-1	R229del	5	ECV		*cE*		5	White	[200]
DVL-2	K235del	5	ECV		*Ce*		0.15	White	[200]
DWI	M358T	7	6		*Ce*	Yes	8	White	[201]
DYU	R234W	5	ECV		*ce*		3	White	[180]
Weak D-1	V270G	6	0		*Ce*	Yes	1	White	[149,150]
Weak D-1.1	L18V, V270G	1,6	0		*Ce*		0.6	White	[202]
Weak D-2	G385A	8	0		*cE*	Yes	0.5	White	[149,150]
Weak D-3	S3C	1	0		*Ce*		2	White	[149,150]
Weak D-4	T201R§, F223V§	4,5	ECV		*ce*		2	White	[149,150]
Weak D-4.1	W16C§, T201R§, F223V§	1,4,5	ECV				4	White	[150]
Weak D-15	G282D	6	5		*cE*	Yes	0.3	White	[149,150]
DEL-1	K409K, splice site	9	0		*Ce*		<0.02	E. Asian	[136,203]
DEL-5	*RHD*(IVS3+1g>a)	Intron 3			*Ce*	Yes	<0.02	White	[139,203,204]
DEL-ex 8 del	Exon 8 del	8	0		*Ce*	Yes		Black	[205]

*Extracellular loops involved or proposed extracellular vestibule (ECV).
‡Associated low frequency antigens.
§Encoded by *RHCE* sequence.

Table 5.6 Reactions of monoclonal antibodies defining 30 epitopes of D with partial D antigens (Fourth International Workshop on Monoclonal Antibodies against Human Red Cell Surface Antigens [211]).

Anti-EpD	Partial D antigen																			
	DII	DIII	DIVa	DIVb	DVa1	DVa2	DVa3	DVa4	DVa5	DVI	DVII	DFR	DBT	DHAR	DHMI	DNB	DAR	DNU	DOL	DHK
1.1	+	+	−	−	−	−	−	−	−	−	+	+	−	−	+	+	V	V	V	−
1.2	+	+	−	−	−					−	+	−	−	−	+					
2.1	+	+	−	−	+	+	+	+	−	−	+	+	−	−	+	+	+	+	+	V
2.2	+	+	−	−	+	+	+	+	−	−	+	−	−	−	−	+	−	+	+	V
3.1	+	+	−	−	+	+	+	+	+	+	+	+	−	−	+	V	+	+	+	+
4.1	−	+	+	−	+	+	+	+	+	+	+	+	−	−	+	+	+	+	+	+
5.1	+	+	+	+	−	−	−	−	−	−	+	+	−	+	+	+	+	+	+	
5.2	+	+	+	+	+	+	−	−	−	−	+	+	−	−	+	+	+	−	+	−
5.3	+	+	+	+	−	−	−	−	−	−	+	−	−	+	+	+	−	+	−	
5.4	+	+	+	+	+	−	−	−	−	−	+	−	−	−	+	+	+	+	V	−
5.5	+	+	+	+	−					−	+	−	−	−	−					
6.1	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+
6.2	+	+	+	+	+	+	+	+	+	−	+	+	+	−	+	+	+	+	+	V
6.3	+	+	+	+	+	+	+	+	+	−	+	+	−	−	+	+	+	+	+	V
6.4	+	+	+	+	+	+	+	+	+	−	+	−	+	+	+	+	+	+	+	V
6.5	+	+	+	+	+	+	+	+	+	−	+	−	+	−	+	+	+	+	+	+
6.6	+	+	+	+	+	+	+	+	+	−	+	−	−	+	+	+	+	+	V	V
6.7	+	+	+	+	+	+	−	−	−	−	+	−	−	−	V	+	+	+	+	V
6.8	+	+	+	+	+	+	+	+	+	−	+	−	−	−		+	+	+	+	V
8.1	+	+	+	+	+	+	+	+	+	−	−	−	−	−	V	+	−	−	+	V
8.2	+	+	+	+	+	+	+	+	+	−	−	−	+	−	V	+	+	+	+	+
8.3	+	+	+	+	+	+	−	−	−	−	−	−	+	−		−				
9.1	−	+	−	−	+	+	+	+	+	+	+	+	−	−	+	+	+	−	+	+
10.1	+	+		−	−					−	−	−	−	−	−					
11.1	+	+	+	+	−					−	−	−	−	−	−					
12.1	+	+	+	+	+					−	+	−	+	−	−					
13.1	+	+	+	−	+	+	+	+	+	−	+	+	−	−	−	+	+	+	+	
14.1	+	+	+	−	+					−	+	+	−	−	+					
15.1	+	+	+	−	+	+	+	+	+	+	+	+	−	−	+	+	+	+	+	−
16.1	+	+	+	+	+					+	+	+	+	−	+					

V, variable reactions with different antibodies.
For five types of DVa, see Figure 5.8.

reaction temperature or pH might appear to alter specificity.

Many of the partial D phenotypes have been subdivided on the basis of genotype by molecular sequencing (Table 5.5).

5.6.4 Characteristics of D variants

Listed below and in Table 5.5 are serological and molecular characteristics of selected D variants. Much of the serological information is taken from a variety of publications [163,166–168,172,173,175,177] and from unpublished observations. The molecular bases for some D variants involving gene rearrangements are shown in Figure 5.8.

5.6.4.1 DII, DNU, DNB

DII originally contained three unrelated propositi with *RHD*DII* travelling with *RHCE*Ce* in the two families tested. The rediscovery of the original Category II propositus led to the subdivision of epD3 [209]. DII is associated with Ala354Asp in the sixth extracellular loop of RhD [159]. DNU (Gly353Arg) [159] and DNB (Gly355Ser) [168] are D variants with similar epitope profiles to DII. DNB is common in Central Europe, with frequencies up to 1 in 292 in Switzerland [168].

5.6.4.2 DIII and the DAK (RH54) antigen

DIII red cells react with all monoclonal anti-D (Table 5.6), yet must lack at least one D epitope as some individuals with DIII phenotype make anti-D. There are at least seven sub-divisions of DIII, all having Asn152Thr in the fifth membrane-spanning domain.

DIIIa is quite common in people of African origin: tests on 93 African Americans and 63 African Brazilians revealed frequencies for *RHD*DIIIa* of 0.11 and 0.19, respectively [212], although DIIIa phenotype is not apparent when *RHD* is present on the opposite chromosome. DIIIa was initially associated with three amino acid substitutions [161], but reanalysis plus sequencing of 58 other DIIIa samples revealed six nucleotide changes encoding five amino acid substitutions: Leu62Phe, Ala137Val, Asn152Thr, Thr201Arg, and Phe223Val [213] (also known as DIII-5). None of these amino acids is predicted to be in an extracellular domain, though position 223 could be in the proposed extracellular vestibule (Figures 5.2 and 5.3). *RHD*DIIIa* is usually inherited with variant *RHCE*ce*, usually *RHCE*ce*^{S} (Section 5.13) [213]. Of 39 DIIIa patients in one study, 16 had anti-D and 27 anti-e or anti-hr^{B} [213]. DIIIa cells express the low frequency antigen DAK, which is also expressed by cells

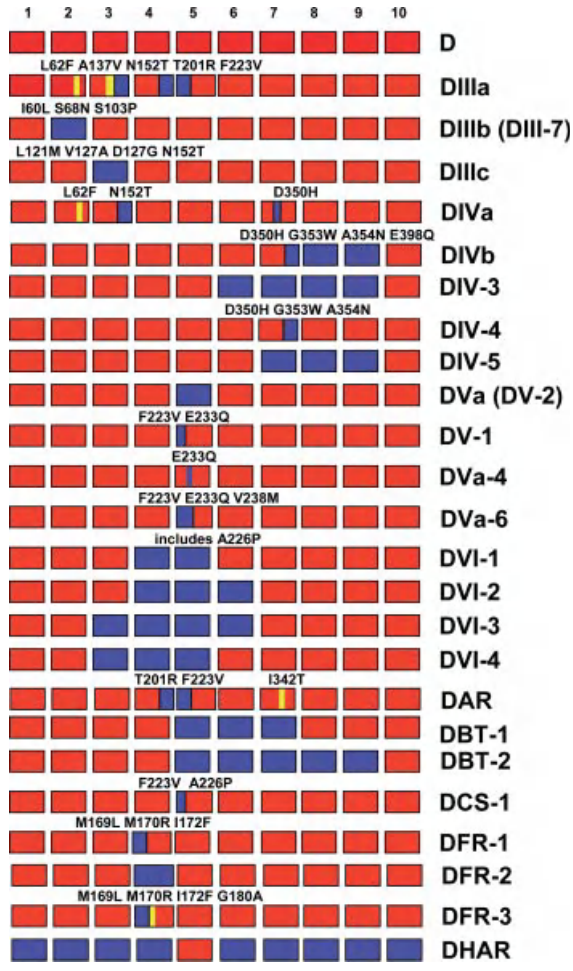


Figure 5.8 Representation of some genes responsible for variant D antigens. Red boxes, exons derived from *RHD*; blue boxes, exons derived from *RHCE*; yellow lines, untemplated amino acid substitutions. All these genes are usually paired with *RHCE*, except *RHD*HAR*.

with the DIIIb (DIII-7) and DOL variants and with the R^{N} phenotype (Section 5.14.1.1) [160,164]. Although Val223 is common to these DAK+ phenotypes, it also occurs in other unusual Rh complexes that do not express DAK. Anti-DAK reacted with red cells of 4% of African American blood donors.

Unlike most D+ red cells, DIIIb cells are G–. Two DIIIb individuals have *RHD* in which exon 2 is replaced by exon 2 from *RHCE*c* [162], but it is likely that DIIIb is identical to the variant named DIII-7, in which, in addition to having exon 2 derived from *RHCE*c*, has Ala137Val,

Asn152Thr, Thr201Arg, and Phe223Val [163,164]. DIIIb has Pro103, as a result of the conversion of *RHD* exon 2 to that of *RHCE*c*, and expresses no G, which is dependent on Ser103 encoded by either normal *RHD* or *RHCE*C* (see Section 5.11).

DIIIc propositi are white and DIIIc is inherited with *RHCE*Ce* in two families [165,214]. In one family, DIIIc is associated with *RHD* in which exon 3 is replaced by exon 3 from *RHCE* [165]. DIII types 4, 6, 8, and 9 are associated with various other amino acid substitutions, but all include Asn152Thr (Table 5.5). DIII type 5 is the same as DIIIa [213].

5.6.4.3 DIV and the Go^a (RH30) antigen

DIV, originally called D^{Cor} [215], appears to be elevated as judged by a few selected incomplete anti-D, which agglutinate saline suspensions of DIV red cells. DIV was initially subdivided by reactions with anti-Go^a, an antibody to a low frequency antigen [216–218]. DIVa and DIVb cells, which are Go(a+) and Go(a–), respectively, can be distinguished by monoclonal anti-D: DIVb, but not DIVa cells, lack epD4 (Table 5.6).

DIVa individuals are mostly black. DIVa travelled with *RHCE*ce* in most families tested, but is also present in the very rare complex DIV(C)– (Section 5.15.5). Three amino acid changes distinguish DIVa-1 from D [167] (Table 5.5): two, encoded by exons 3 and 7, probably represent microconversion events; Leu62Phe (exon 2) represents an untemplated mutation. DIVa-2 has an additional Ala137Val [183]. Asp350His (exon 7) in the sixth extracellular loop is a characteristic of all DIV subcategories.

Approximately 2% of African Americans are Go(a+) [219,220].

DIVb is heterogeneous; there is great variation in the strength of the variant D antigen. At least some DIVb cells have the low frequency antigen Evans (RH37) [221]. All known DIVb individuals have been white. DIVb travelled with *RHCE*Ce* in two families and with *RHCE*cE* in three families [158]. DIVb is associated with an *RHD–CE–D* gene in which the 3′ end of exon 7, and exons 8 and 9 are derived from *RHCE* [167].

DIV-3, -4, and -5 were defined primarily by molecular testing. All have an *RHD–CE–D* gene: in DIV-3 exons 6–9 have the *RHCE* sequence [149]; in DIV-4 only part of exon 7 is exchanged [168]; and in DIV-5, initially considered DIVb in four of 5 million Japanese blood donors [222], exons 7–9 are *RHCE*-derived [169].

DIVb and DIV-3, -4, and -5 have Asp350His, Gly353Trp, and Ala354Asn in the sixth extracellular loop.

5.6.4.4 DV and the D^w (RH23) antigen

DVa red cells are D^w+ [223]. *RHD*DVa* is usually linked to *RHCE*ce* in black families and *RHCE*Ce* in white and Japanese families; *RHD*DVa* linked to *RHCE*cE* is rare [158,224]. The strength of DVa antigen is very variable. The molecular background to DVa is heterogeneous, but always involves replacement of all or part of exon 5 of *RHD* by the equivalent region of *RHCE* (Figure 5.8) [167,170–172,197,224]. All forms involve Glu233Gln, predicted to be in the fourth extracellular loop, and one (DVa-4) has only Glu233Gln. All give rise to a typical DVa phenotype, so the Glu233Gln substitution must be the key to D^w expression. Red cells with the DHK phenotype, in which there is a Glu233Lys substitution, lack epD1 and some epD5 epitopes like DVa (Table 5.6), but are D^w– [170]. DAU-5 red cells, which have Glu233Gln, express D^w, whereas DAU-4 cells, which have Glu233Lys, do not [182]. One of the DVa variants, DVa (E) (Figure 5.8), has Pro226, suggesting that the *RHCE*-derived segment of exon 5 originated from *RHCE*E* [225]. The red cells expressed an abnormal E antigen, despite no *RHCE*E* allele being present, suggesting that the *RHD–CE–D* hybrid produced some E.

Subcategory DVb contained one propositus, whose red cells did not react with any anti-D from DVI individuals and were D^w– [226].

5.6.4.5 DVI and the BARC (RH52) antigen

DVI has very few D epitopes and most monoclonal anti-D do not react with DVI cells (Table 5.6). A minority of anti-D from D– people react with DVI cells, which may reflect a quantitative rather than a qualitative effect [158].

Most DVI propositi are white. DVI travels with *RHCE*Ce* in most families and less commonly with *RHCE*cE*. Anti-BARC is a marker for the *D^{VI}Ce* haplotype: 76 of 78 *D^{VI}Ce* samples were BARC+; all of 21 *D^{VI}cE* samples were BARC– [158,227].

Four types of *RHD–CE–D* encoding DVI have been recognised (Figure 5.8). The gene for DVI-1, which is always linked to *RHCE*cE*, has exons 4 and 5 derived from *RHCE*E* (encoding Pro226) [173,228]. Genes for DVI-2, -3, and -4, which are linked to *RHCE*Ce*, have exons 4–6, 3–6, and 3–5, respectively, derived from *RHCE*e* (Ala226) [173–175,177]. Genes for DVI-2, -3, and -4, but not -1, produce BARC, suggesting that the presence of Ala226 in the hybrid Rh protein is important in BARC expression. Apart from the polymorphism at residue 226, all three types have the same amino acid changes from normal D in predicted extracellular loops

or in the extracellular vestibule: Met169Leu, Met170Arg, and Ile172Phe in or adjacent to loop 3; Glu233Gln in or adjacent to loop 4. Quantitative differences, in terms of numbers of D sites per cell, exist between these DVI types: DVI-1 is low with about 300–500 sites; DVI-2 has about 2000 sites; DVI-4 about 8000 sites; and DVI-3 is about normal for a DCe complex with 12 000–14 000 sites [150,175,177].

By screening with monoclonal antibodies, the incidence of DVI in Europe, the USA, and Australia has been estimated between 0.015 and 0.04% and represents between 5 and 16% of weak D samples [229–233]. Only one DVI was found in over 5 million Japanese donors [222]. DVI-3 is the most common cause of DVI in Chinese [176] and DVI-4 the most frequent in Spain [177].

5.6.4.6 DVII and the Tar (RH40) antigen

Characteristic of DVII is a positive reaction with anti-Tar [234–236]. Of eight DVII individuals with anti-D, two were untransfused males and in four a weak anti-D was accompanied by a strong anti-E [158,237]. Red cells of one of 1585 D+ British donors failed to react with anti-D from a DVII individual; this sample also had DVII phenotype [237]. DVII results from a mutation in *RHD* exon 2 encoding Leu110Pro in the second extracellular loop of RhD [178]. Of over 60 000 German blood donors, 68 had DVII phenotype; of 33 DVII donors analysed, all had the Leu110Pro mutation [238]. An unusual *RHD* (DVII-2) in two unrelated individuals encoded Leu110Pro and Pro103Ser (characteristic of c in *RHCE*), and produced Tar and partial c [239].

5.6.4.7 DFR and the FPTT (RH50) antigen

Red cells with the DFR phenotype react with an antibody to the low frequency Rh antigen FPTT [187,188]. In 23 propositi DFR was associated with *RHCE*Ce* and in two propositi with *RHCE*cE*. Two DFR propositi had produced anti-D. Two of 3967 Australians, but only one of 60 000 Germans, had DFR [187,233].

DFR (DFR-1) is associated with an *RHD–CE–D* gene in which the 5′ end of exon 4 is *RHCE*-derived [167]. The three amino acid substitutions, Met169Leu, Met170Arg, Ile172Phe, are located in the third extracellular loop or extracellular vestibule. The molecular basis of FPTT expression is described in Section 5.17.2. DFR-2 has the whole of exon 4 derived from *RHCE* [189], DFR-3 is the same as DFR-1, but with an additional Gly180Ala substitution [189], DFR-4 only has Met169Leu and Met170Arg, and DFR-5 has segments of exons 3 and 4 from *RHCE*. All have Met169Leu and Met170Arg.

5.6.4.8 DBT and the Rh32 antigen

Rh32 is a low frequency antigen associated with the partial D antigen DBT [184] and with the R^N complex, which has normal D, but weak C and e (Section 5.14.1.1). Of eight DBT propositi tested, five had normal C and c, two had weak C (one of whom had a weak e), and one was C– c+. Three had made anti-D. The propositi were mostly of white European origin, but one was Moroccan, one Thai, and one Japanese.

In the Moroccan family, DBT-1 is associated with *RHD–CE–D* in which the whole of exons 5–7 are *RHCE*-derived [185]. This gives rise to Glu233Gln in loop 4 or extracellular vestibule and Asp350His, Gly353Trp, and Ala354Asn in loop 6. In the Japanese DBT-2 family, exons 5–9 of *RHCE* are present [186]. The molecular basis of Rh32 expression is described in Section 5.17.2.

5.6.4.9 DAR, DARE, and weak D type 4.2

DAR is a partial D associated with three amino acid substitutions in RhD: two, Thr201Arg and Phe223Val, are *RHCE*-derived; Ile342Thr is untemplated [153]. Val223 is predicted to be in the extracellular vestibule, close to loop 4. DAR is relatively common in Africans: of 326 black South African donors, 16 (4.9%) had *RHD*DAR* and five (1.5%) of these had the DAR phenotype. Of the 16 individuals with *RHD*DAR*, all but two had an *RHCE* variant, *RHCE*ceAR*, in which most of exon 5 and the 5′ end of exon 6 is *RHD*-derived and expresses some D epitopes (Sections 5.6.4.12 and 5.13.2).

The genes encoding weak D-4.2.1, -4.2.2, and -4.2.3 are identical apart from different synonymous mutations, which have no obvious effects on the protein product or phenotype [150]. Both DAR and weak D-4.2 phenotypes are associated with anti-D production, emphasising the difficulties of the partial D/weak D terminology. DARE, a variant found in Ethiopians, has an additional Glu233Gln substitution in the extracellular vestibule [163]. Another variation is DAR with an additional Thr203Ala [77].

5.6.4.10 Variants designated as weak D

As mentioned in Section 5.6.2, weak D is generally considered a complete D antigen, with all epitopes present, but expressed weakly. This, however, is often difficult to determine because monoclonal anti-D could fail to react with weak D cells because of low avidity of the antibody, rather than complete loss of the epitope.

In 1999, Wagner *et al.* [149,150] sequenced the 10 *RHD* exons from 161 weak D samples from Germany, all with between 70 and 4000 antigen sites per cell, and found nucleotide changes encoding amino acid substitutions in all of them. Based on amino acid sequence differences a numerical classification was established, numbering antigens from weak D type 1 to weak D type 81 (abbreviated to weak D-1 to weak D-81 here), and the list is maintained on the Rhesus web site [126]. All the amino acid substitutions associated with these weak D types are considered to be in the predicted membrane-spanning or cytoplasmic domains of the RhD protein; none was extracellular, but in some cases this could be dependent on the conformational model of RhD protein applied and whether the putative RhD vestibule is considered extracellular or intramembranous. Some of the weak D types have been subdivided; for example, weak D-1 has Val 270Gly, whereas weak D-1.1, which has weaker D expression than weak D-1, has Val 270Gly plus Leu18Val [202]. Further subdivisions (e.g. weak D-4.2.1) accommodate synonymous mutations.

Weak D of types 1–3 were the most frequent, representing, respectively, 70%, 18%, and 5% of the weak D samples tested [149]. Of 50 DCe/dce Australian blood donors with weak D, 76% had the type 1 mutation and 6% the type 3 mutation, whereas of 48 DcE/dce donors with weak D, 96% had the type 2 mutation [240]. Frequencies differ in other populations: the very rare weak D-38 (Gly278Asp) is relatively common in Portuguese [241]; weak D-42 is the most common in Quebec, Canada [242] and weak D-3 the most common in the Zagreb region of Croatia [243]. Weak D-4.3 associated with C^x and VS was found in 1 in 854 apparently D– Upper Austrian blood donors [244] (Section 5.12.2). Some of these phenotypes, such as weak D-2 and -38, have very weak expression of D giving very weakly positive results with routinely used anti-D typing reagents, whereas in some others the weakness could be barely detectable with routine reagents.

The definition of these weak D types is molecular rather than practical. They do not fit the early definitions of D^u and weak D, which assume that all epitopes are expressed and consequently cannot be associated with anti-D production. Alloanti-D has been found in individuals with weak D type 15 [150] and weak D type 4.2 is functionally identical to DAR [153] (Section 5.6.4.9), which is often associated with anti-D production. In addition, there are rare cases of patients with the two most common weak D types, types 1 and 2, with anti-D [154,245–248]. Although these antibodies are often dismissed as autoantibodies [249], if the red cells give a negative DAT, no anti-D can be eluted from them, and they do not adsorb the anti-D from the patient's own plasma, they should be considered to be alloanti-D. There are single reports of weak D types 21, 33, and 41 with anti-D [151,250,251].

5.6.4.11 Clusters of D variants

Phylogenetic studies have led to the concept of four clusters of variant *RHD* alleles, each of which is defined by a single allele that differs from the consensus *RHD* allele, and each of which may comprise many alleles that encode additional amino acid substitutions [179,190].

Eurasian Cluster. Contains the consensus or 'normal' *RHD* as its primordial allele. Includes *RHD* alleles encoding DFR-1, -2, and -3, DBA, DFV, DHQ, DMI, DFW, DFL, and weak D-17, -33, and -62 together with *RHCE*Ce* and *RHD* alleles encoding DCS-1 and -2, DHR, DFV, and weak D-16 together with *RHCE*cE* [190].

*African DAU Cluster. RHD*DAU-0* encodes Thr379Met, which has no obvious affect on D phenotype. Seven D variants, numbered DAU-1 to DAU-7, have Thr379Met plus one or two addition substitutions resulting in variant D antigen expression and associated with, at least in the case of DAU-3, anti-D production [133,179–181,183]. DAU cluster alleles are linked to *RHCE*ce*.

African weak D type 4 Cluster. Contains alleles for weak D-4 and its subdivisions, DAR and DARE, DFV, DOL-1 and -2, DTO, and the inactive allele *RHD*Ψ*, all of which encode Phe223Val and are linked to *RHCE*ce* [190].

African DIVa Cluster. Contains alleles for DIVa, DIIIa, and DIII-4, and *RHD–CE–D^S* (type 1), all of which encode Asn152Thr and are linked to *RHCE*ce* [190].

5.6.4.12 DHAR, R_o^{Har}, the Rh33 antigen and other D variants expressed in the absence of *RHD*

R_o^{Har} is a rare complex consisting of a weak partial D (DHAR), c, a very weak e, no G, and two low frequency antigens, Rh33 and FPTT [191–193] (Section 5.14.2.4). Generally, weak D antigens are most efficiently detected by an antiglobulin technique, but DHAR is different: only 7% of anti-D reacted with DHAR cells by the antiglobulin technique, although 27% reacted with enzyme-treated DHAR cells [191]. All four IgM, but only five of 24 IgG human monoclonal anti-D reacted with DHAR cells [207]. Individuals with DHAR have produced anti-D, one of which caused mild HDFN [193,252].

The 'haplotype' responsible for R_o^{Har} consists of only one gene: there is no *RHD* or *RHCE*, but an *RHCE–D–CE* hybrid with exon 5 representing *RHD* [194] (see Figure

5.10). Exon 5 of *RHD* encodes four amino acids, Phe223, Glu233,Val238, and Val245, characteristic of RhD that are in the extracellular vestibule region, and also encodes Ala226, the amino acid characteristic of e, explaining the weak e expression.

Exon 5 of *RHCE*ceCF* encodes two amino acid substitutions characteristic of RhD, Glu233 and Val245. The ceCF complex expresses partial c and e, the low frequency antigen Crawford (RH43), but not its antithetical high frequency antigen CELO (RH58), and some epitopes of D [253,254]. The ceCF phenotype was present in 0.056% of African Americans [253].

Exons 3 of *RHCE*ceRT* and *RHCE*ceSL* each encode a single amino acid substitution, Arg154Thr and Ser-122Leu, respectively, that result in expression of epD6 in the absence of *RHD* [255,256]. Neither Thr154 nor Leu12 is present in RhD and neither is expressed externally. Consequently, it is likely that the substitutions affect the conformation of the RhCE protein so that it mimics epD6. *RHCE*ceAR*, which is an *RHCE–D–CE* gene containing part of *RHD* exons 5 and 6, is usually linked to *RHD*DAR* in people of African origin (Section 5.6.4.9), but red cells of a Caucasian blood donor with *RHCE*ceAR*, but no *RHD*, expressed some D epitopes [257].

5.6.4.13 DEL

DEL is a very weak form of D that can only be detected serologically by adsorption and elution tests. Between 10 and 33% of Japanese, Chinese, and Korean red cell samples shown to be D– by conventional serological techniques were found to be DEL [116,134,135,258]. In eastern Asia the most common *RHD* gene responsible for DEL has 1227G>A in the most 3′ nucleotide of exon 9 [136–138,259–261]. This synonymous mutation in the codon for Lys409 disrupts splicing, resulting in transcripts lacking exon 9 and no normal *RHD* transcript [259,260]. Exon 9 skipping predicts an RhD protein containing 79 new amino acids at its C-terminus. *RHD*DEL* is almost exclusively linked *RHCE*Ce*.

Of 104 Chinese pregnant women with anti-D, none had the DEL phenotype and of 199 apparent D– Chinese women with a history of pregnancy, 44 had the DEL variant and no anti-D and 155 were truly D–, 38 of whom had anti-D [262].

Numerous rare *RHD* alleles are responsible for DEL phenotypes, many of them in Europeans, and almost all linked to *RHCE*Ce* or *RHCE*cE* [126,139,203–205, 261,263]. The most common DEL allele in Caucasians encodes Met295Ile and the second most common has the splice site mutation *RHD*(IVS3+1g>a) [139,204]. Four individuals with *RHD*(IVS3+1g>a) had anti-D and one of the antibodies caused mild HDFN [203,264]. A Lebanese woman with DEL red cells and *RHD* with a deletion of exon 8 also had anti-D [205]. The maximum antigen site density per red cell in DEL was 36, though in most cases there was less than 22 [203]. A deletion of four nucleotides in intron 5 of *RHD* (IVS5-38del4) was initially considered responsible for DEL phenotype [203,265], but was later shown to be a polymorphism with no effect on phenotype [189].

5.6.5 A molecular approach to the structure of D epitopes

The problem of locating D epitopes on a model of the RhD protein was approached by site-directed mutagenesis and the expression of cDNA constructs in K562 cells. Liu *et al.* [266,267] expressed cDNA representing *RHCE*cE* after having changed nucleotides encoding amino acids characteristic of the third, fourth, or sixth extracellular loops of RhCE to those characteristic of RhD. D epitope expression was then evaluated by flow cytometry. The overall conclusions were that there are six epitope clusters, some of which are overlapping, but are located predominantly on the third, fourth, and sixth RhD loops. None of these epitope clusters is more than 25Å in diameter. According to this model, some anti-D recognise a 'footprint' consisting of a single extracellular loop, with others the 'footprint' could comprise two, three, or four loops. More recent modelling of RhD based on crystal structures of homologous proteins has led to the concept of the extracellular vestibule in the region of loops 3 and 4 (Figures 5.2 and 5.3), which may permit access of antibodies to internal regions of the protein, but this does not significantly affect the model of Liu and her colleagues.

Application of phage-display technology, with Fab/phage anti-D libraries from the B cells of an anti-D producer [268], provided an alternative view of the way that monoclonal antibodies define D epitopes. Information from a genetic and serological analysis of 53 unique anti-D chosen from 83 random clones demonstrated extensive genetic homology between antibodies directed against different D epitopes [269]. Chang and Siegel [269] suggested that these antibodies would not have such similar sequences if they recognised spatially discrete and structurally unrelated regions of the D protein. They proposed that antibodies to the various D epitopes bind to an 'identical footprint', which represents most or all of the extracellular expression of the protein, rather

than spatially distinct epitopes. The specificity differences with partial D antigens would result, therefore, from conformational changes within the 'footprint'.

Any further attempts to analyse the nature of the D epitopes must take into account the architecture of the RhD-RhAG heterotrimers [77].

5.6.6 Weak D caused by the *trans* effect of *RHCE*C*

A weak D phenotype that is not inherited in a regular fashion often occurs when the haplotype encoding the D antigen is *in trans* (on the opposite chromosome) with *dCe* or, rarely, *dCE* or *d(C)ce*^{s} [270,271]. That is, there is a *trans* effect from a haplotype encoding C, but not D. When the haplotype producing the weakly expressed D is partnered by a haplotype that encodes neither C nor D (*dce* or *dcE*) in another family member, the D is expressed normally. Some examples of DEL could result from the effect of *dCe* on a haplotype containing a weak D gene [139,272].

5.6.7 Elevated D antigens

Extra strong D antigens are detected by direct agglutination of red cells by incomplete anti-D (IgG antibodies that do not directly agglutinate red cells with normal D expression). Elevation of D associated with *D––* and related haplotypes (*Dc–*, *DC*^{w}*–*, *D••*) results from an increased number of D sites (Section 5.15). Elevation of D is also associated with some *D(C)(e)* haplotypes (Section 5.14.1) and with DIVa (Section 5.6.4.3). Apparent elevation of D antigen, and of other Rh antigens, is observed in red cells with reduced sialic acid levels resulting from glycophorin A deficiency (Chapter 3).

5.6.8 Quantity of D antigen sites

The number of D sites on red cells, estimated by the use of radiolabelled anti-D and by fluorescence flow cytometry, has demonstrated that the site density differs within different phenotypes [122,141,150,273–276] (Table 5.7). Estimates for D site density on red cells with variant D phenotypes are shown in Table 5.5. Some partial D phenotypes, such as DIII and DIVa, have normal numbers of D sites per cell, whereas others, such as DVI-1 and -2, have very low D site density [145,150,273]. DEL red cells usually have less than 22 D sites per cell, with a maximum of 36, whereas weak D-26 cells, with 70 sites, were agglutinated by anti-D in an antiglobulin test [203,204,265]. In any individual, there is a wide range in the number of D sites in each red cell [123,124].

Table 5.7 Estimated number of D antigen sites per red cell for various Rh phenotypes [150,273,274,276].

Phenotype	D sites per cell (range)
DCe/dce	9900–14 600
DcE/cde	12 000–19 700
Dce/dce	12 000–23 200
DCe/DCe	14 500–22 800
DCe/DcE	23 000–31 000
DcE/DcE	15 800–33 300
D––/D––	110 000–202 000

For numbers of C, c, and e sites, see Table 5.8.

5.6.9 D variants and transfusion practice

The recommended practice in the United Kingdom is that patients should be tested in duplicate by direct agglutination with potent IgM monoclonal anti-D [277]. These antibodies are selected to give a negative reaction with DVI red cells. Consequently, patients who have the DVI phenotype and are, therefore, prone to make anti-D, are typed as D–, are transfused with D– red cells, and are given anti-D immunoglobulin following delivery of a D+ baby. Patients with very weak expression of D, including those with DEL phenotype, will also be typed as D– and will receive D– red cells or receive anti-D immunoglobulin, which will not be detrimental and could prevent them from making anti-D. An antiglobulin test for detection of some weak D phenotypes is not recommended for patient testing in the UK or USA [278], but is mandatory in some countries. Some D variants, for example DIII, in which D is expressed strongly, will be typed as D+ and so patients with these variants will be at risk of making anti-D.

If significantly weak or equivocal results are obtained on routine D typing of a patient's red cells, then the patient should be considered D– until the cause of the aberrant result is resolved. Further testing with a panel of D monoclonal antibodies will identify most of the more common variants, especially weak D-1, -2, and -3 and DAR. More definitive identification can be achieved by molecular testing, either with kits or arrays designed to identify many or most of the known variants, or by sequencing *RHD* (which will also identify new variants). Once the variant is identified, then a policy should be in place for transfusion and giving anti-D prophylaxis. It is well established that individuals with weak D-1, -2, and -3 make alloanti-D only extremely rarely, if at all, so it is

a reasonable policy to treat patients with those variants as D+, in order to conserve stocks of D– red cells, and to treat those with any other variant as D–. This will not adversely affect D– stocks as all of the other variants are relatively rare.

For typing blood donors it is essential that red cells capable of immunising a D– patient to make anti-D are labelled D+. Consequently, reagents are selected that give a positive reaction with DVI red cells and those of most other D variants. DVI individuals, therefore, are D+ donors, but D– patients. This leads to the thorny question of those variants, such as DEL, that are not detected by agglutination tests, even in the presence of antiglobulin. There is limited evidence that DEL [265,279] can cause primary immunisation of D– patients, as can other very weak forms of D, including weak D-2 [280], weak D-26 [204], weak D-1 with *dCe in trans* [281], and a D–/D+ chimera with a proportion of D+ cells too low to be detected by routine serological testing [139]. This has led some transfusion services in a few parts of Europe to introduce screening of D– donors for *RHD*, so that transfusion of D– patients with DEL red cells, especially girls and women of childbearing age, can be avoided [263]. In an international forum, however, most contributors were of the opinion that routine testing for *RHD* should not be recommended [282]. Since *RHD*DEL* is usually linked to *RHCE*Ce* or *RHCE*cE*, several contributors to the forum were in favour of transfusing D– C– E– red cells to D– patients, especially girls and women of childbearing age. In a survey from Denmark, testing of 5058 D– donors for *RHD* revealed 13 with *RHD*, all with DEL phenotype [283]. In a subsequent look-back of the 136 transfusions with these red cells to D– patients, only one recipient had made anti-D and that patient had also received D+ platelets. Eleven of the recipients had made antibodies to other blood groups, showing that they are responders. The conclusion from this study is that it would be very difficult to justify the expense of the introduction of screening all D– donors for *RHD*.

DVa stimulated production of anti-D in a D– woman during her first pregnancy and resulted in mild HDFN of her second baby, who also appeared to have the partial D antigen [284]. The mother had received anti-D immunoglobulin after the first pregnancy. A weak D-3 fetus appears to have stimulated anti-D production in a *dCe/dce* mother, causing the neonatal red cells to give a positive DAT [285].

Anti-D in women with variant D red cells has been responsible for severe HDFN [165,184,222,286–290]. Anti-D immunoglobulin should be given to women known to have partial D during and after pregnancy, because the anti-D constituent that does not bind to the mother's own partial D cells should suppress immunisation by binding to the normal D of the fetus [291]. This is particularly important in DVI mothers, whose red cells lack most D epitopes, although in most cases the mother will be typed as D– and receive anti-D automatically.

5.7 Predicting D phenotype from DNA

It is possible to predict, with a high level of accuracy, a person's D phenotype by testing their DNA. Since in most cases the D– phenotype arises from homozygosity for a deletion of *RHD*, at the simplest level this can be achieved by amplifying a region of *RHD* to determine whether the gene is present. Owing to the high level of sequence identity between *RHD* and *RHCE*, primers must be selected so that they only initiate amplification from *RHD*. The regions often used to discriminate between the two genes are exon 7, where there are multiple sequence differences, the 3′ untranslated region of exon 10 where there is a sequence in *RHD* that is not present in *RHCE*, and intron 4, which is 600 bp in *RHD* and 1200 bp in *RHCE*. To avoid false results arising from hybrid *RHD* genes that contain regions with the *RHCE* sequence (see Figure 5.9), at least two different regions must be tested. In addition, any test must also be designed to ensure that *RHD*Ψ*, the D– gene common in people of African origin, is detected [128].

Many of the early publications on PCR-based D phenotype prediction are listed by Flegel *et al.* [292]. There are now several commercial products that will identify all of the most important D variants. These platforms involve multiplex PCR followed by gel electrophoresis or the application of microarray technology, with oligonucleotides probes immobilised on chips or coloured beads.

5.7.1 Fetal D typing

It is beneficial to be able to predict D phenotype from DNA of the fetus of a D– pregnant woman with anti-D: if the fetus is D+ it is at risk from HDFN (Section 5.18.1.4) and the pregnancy can be managed appropriately; if D–, it is not at risk and no further action is required. When the molecular basis for the D– phenotype was first elucidated, fetal DNA was obtained by amniocentesis or chorionic villus sampling, invasive procedures that present a significant risk to the fetus. Since the discovery that cell-free fetal DNA is present in the blood of pregnant women [293], representing between 3 and 6% of cell-free DNA

in the maternal plasma [294], this is the preferred source of fetal DNA in D– pregnant women. Fetal D typing in alloimmunised women is now routine practice in many countries, particularly in Europe. The technology employed is usually real-time quantitative PCR targeting *RHD* exon 4, designed to give a negative result with *RHD*Ψ*, exon 5 or 7, and exon 10, so that *RHD–CE–D*^{S} will be revealed. Lists of published methods can be found in references [295,296].

One potential source of error is a previous solid organ transplant in the mother. Tests on DNA isolated from the plasma of a D– pregnant woman predicted a D+ fetus, whereas DNA isolated from amniocytes gave a D– result. The woman, who had received a kidney transplant from a D+ donor, delivered a D– baby [297].

It is policy in many countries to offer one or two doses of anti-D immunoglobulin to pregnant women at around 28–34 weeks gestation, to protect against antenatal D immunisation. In a predominantly Caucasian population, however, about 38% of these women would be carrying a D– fetus and so receive the treatment unnecessarily. Trials have shown that high-throughput methods for fetal D typing, including robotic isolation of plasma DNA, are accurate from 11 weeks gestation and that fetal D typing of all D– pregnant women is feasible [298–301]. Screening of fetal D type has been introduced as routine practice in Denmark [302] and the Netherlands [700].

The main advantage of carrying out fetal RhD screening is that it eliminates unnecessary treatment of pregnant women with blood products and the associated inconvenience, discomfort, and perceived risks of infection from pooled donor blood products that such injections entail. Fetal *RHD* screening provides a way of significantly reducing the quantity of blood products given routinely to pregnant women. In addition anti-D immunoglobulin is in short supply and produced by immunising volunteers with blood products. There may also be a small financial saving, with the cost of the test being less than that of the anti-D immunoglobulin, especially if testing takes place early in pregnancy and so avoids unnecessary anti-D injections following sensitising events such as trauma or threatened miscarriage.

A method for testing single blastomeres obtained from cleavage-stage embryos has been developed for preimplantation diagnosis to prevent severe HDFN [303].

5.7.2 *RHD* zygosity testing

When a pregnant woman has anti-D, knowledge of the *RHD* zygosity of the father will assist in predicting fetal D phenotype. If the father is homozygous for *RHD* then the fetus will be D+ and no fetal testing is required; if the father is hemizygous for *RHD*, there is an even chance that the fetus will be D+ or D–. Prediction of *RHD* zygosity from serologically determined probable genotypes is notoriously inaccurate and of little value, but there are molecular technologies available. Identification of the DNA sequence across the breakpoint within the upstream and downstream *Rh boxes* that occurs with the deletion of *RHD* in the D– haplotype (Figure 5.5) has led to the design of PCR-based tests that detect the *RHD*-deletion haplotype directly, either by the use of sequence-specific primers or *Psf*I restriction endonuclease [70,304,305]. Although this may be a suitable test for Caucasians, it is far from accurate in individuals of African origin because of considerable sequence variation in the upstream and downstream boxes [306–308]. Consequently, a better method for determining *RHD* zygosity is quantitative PCR, comparing *RHD* with a gene of known zygosity, such as *RHCE* [304,309]. It is important that a test for *RHD*Ψ* is also carried out.

5.8 C and c

5.8.1 C and c antigens

C and c are the products of alleles. C has a frequency of 68% and c a frequency of 81% in English blood donors, giving gene frequencies of 0.4327 for *RHCE*C* and 0.5673 for *RHCE*c* [13]. In black Africans the frequency of c is much higher and the frequency of C much lower, whereas in eastern Asia the opposite is the case, C approaching 100% and c of low incidence [14,21].

Table 5.8 shows the estimated numbers of C, c, and e antigen sites on red cells of various Rh phenotypes [276,310]. The number of C sites is partially dependent on the nature of the anti-C used. The figures in Table 5.8 were obtained with anti-C serum that had been adsorbed with DcE/DcE cells to remove all traces of anti-G [310].

The *DCE* haplotype produces weak C. DCE/dce cells react weakly with most anti-C sera and DCE/DcE cells have a low number of C antigen sites [310] (Table 5.8). K562 cells transfected with cDNA representing *RHCE*Ce* reacted strongly with three monoclonal anti-C, but when cDNA representing *RHCE*CE* was used, two of the anti-C did not react and the other reacted weakly [311]. It appears, therefore, that the Pro226Ala (E/e) polymorphism causes conformational changes to the protein that affect C expression.

Table 5.8 Estimated numbers of C, c, and e antigen sites per red cell [276,310].

	Antigen sites per cell (range)		
Phenotype	C	c	e
DCe/DCe	45 700–56 400		
dCe/dCe	42 200		
DCe/DcE	25 500–39 700	40 000–53 000	14 500
DCe/DCE			13 400
DCe/dce		37 000–42 000	24 400
DCe/dce C^w+	21 500–40 000		
dCe/dce	31 100		
DcE/DCE	8500–9800		
DcE/DcE		78 000–80 000	
dce/dce		70 000–85 000	18 200
d(C)ce^s/dce	7200		

For numbers of D antigen sites, see Table 5.7.

Table 5.9 The C/c and E/e polymorphisms: amino acid substitutions in the RhCcEe polypeptide deduced from DNA sequences. The sequence of the D polypeptide is shown for comparison.

Polypeptide	Amino acid residue				
	16	60	68	103	226
ce	Trp	Leu	Asn	Pro	Ala
Ce	Cys	Ile	Ser	Ser	Ala
cE	Trp	Leu	Asn	Pro	Pro
CE	Cys	Ile	Ser	Ser	Pro
D	Trp	Ile	Ser	Ser	Ala

Amino acid residue 16 is encoded by exon 1 of *RHCE*; residues 60, 68, 103 by exon 2; residue 226 by exon 5.

5.8.2 The C/c polymorphism

The C/c polymorphism is usually associated with six nucleotide substitutions in *RHCE* resulting in four amino acid changes (C/c): Cys16Trp (48C/G) encoded by exon 1; Ile60Leu (178A/C), Ser68Asn (203G/A), and Ser103Pro (307T/C) encoded by exon 2 [43,47] (Table 5.9). Only residue 103 is predicted to be outside the membrane, in the second extracellular loop (Figure 5.2).

Ser103 is essential, but not sufficient, for C specificity. Exons 2 of *RHD* and *RHCE*C* are identical and both encode Ser103. Any antibody specific for Ser103 would react with the products of *RHD* and *RHCE*C*, so would be called anti-G, not anti-C (Section 5.11). C, therefore, is a very conformational antigen and anti-C are heterogeneous. For full expression of C, the protein must have Ser103, Cys16, and some other downstream amino acids characteristic of the RhCE protein. Cys16 is not a requirement for all epitopes of C. *RHD–CE–D^s*, part of the *d(C)ce^s* haplotype that is relatively common in Africans, has exons 1, 2, and the 5′ end of exon 3 derived from *RHD*, and so encodes Trp16 and Ser103 from *RHD*, but Thr152 and the amino acids of the third extracellular loop from *RHCE* [130]. *RHD-CE-D^s* produces weak partial C [129–131,312]. Likewise, the aberrant *RHCE* gene associated with r^G, which also produces weak partial C, encodes Ser103, but Trp16 [313]. Conversion of the codon for Cys16 to Trp by site-directed mutagenesis of cDNA representing *RHCE*Ce*, and expression of the construct in K562 cells, resulted in about 50% reduction in binding of two anti-C, compared with wild type *RHCE*Ce* cDNA [311]. The abnormal C associated with Ser103 and Trp16, could represent the antigen called C^G (RH21) [314,315].

The c antigen is determined, almost entirely, by the presence of Pro103, which is encoded by *RHCE*c*, but not *RHD* (Table 5.9). The Cys16Trp polymorphism does not affect c expression – 74% of C– c+ black Africans, with normal c, have Cys16 [316] – though Cys16 encoded by an *RHCE*ce* allele does affect expression of some epitopes of e [317]. Cys16 was also present in 68% of white people with the Dce phenotype, but in none with dce/dce or DcE/DcE phenotypes [318]. Non-human primates with *RHCE* encoding Pro103, but no other amino acids characteristic of c, have c+ red cells [319,320]. Site-directed mutagenesis experiments have revealed that different anti-c have different amino acid requirements [267].

5.8.3 Predicting C/c phenotype from DNA

The defining difference between the *RHCE*C* and *RHCE*c* is a 307T/C polymorphism in exon 2. Exons 2 of *RHD* and *RHCE*C* have an identical sequence, so it is not possible to design allele-specific primers for identification of *RHCE*C* (except in D– samples), though it is relatively straightforward to design them for *RHCE*c* [128,318,321]. C expression does not correlate closely enough with 48C in intron 1, encoding Cys16, for use in predicting C phenotype (Section 5.8.2). A size polymorphism in intron 2 of *RHCE* correlates closely with

C/c expression [322]. *RHCE*C/c* genotyping has been achieved by performing two PCRs: one incorporating a primer complementary to a sequence within an *RHCE*C*-specific 109 bp insert in intron 2; the other utilising a primer specific for 307C (*RHCE*c*) in exon 2 [128,321,323]. The *d(C)ce*^{s} haplotype gives a negative result in the intron 2 test despite producing C, because intron 2 from the *RHD–CE–D*^{s} gene that produces the abnormal C is *RHD*-derived (Section 5.13.2). Quantitative real-time PCR has been applied to predicting C/c phenotypes from fetal DNA in maternal plasma [296].

5.8.4 Cc antibodies

The well-known heterogeneity of anti-C sera (lacking anti-D and -G) has been demonstrated by tests against a variety of C+ phenotypes. This heterogeneity can only be interpreted partly in terms of varying quantities of anti-C, -Ce, and -CE [324]. Most immune 'anti-C' made by D+ people are predominantly anti-Ce (Section 5.10.2). Grouping reagents made from IgM anti-C found in sera of D– people, together with 'incomplete' anti-D, often contain anti-G [324].

5.9 E and e

5.9.1 E and e antigens

E and e, another pair of antithetical antigens within the Rh system, are encoded by *RHCE*. In all populations e has a significantly higher frequency than E [14,21]. The following are antigen and gene frequencies in an English population: E 29%; e 98%; *RHCE*E* 0.1554; *RHCE*e* 0.8446 [13]. The figures for most other populations do not differ substantially from these.

E antigen site densities vary considerably, depending both on red cell phenotype and source of anti-E [276]. Masouredis *et al.* [325] recorded 27 500 E sites on DcE/DcE cells and 17 900 on DcE/dce cells. Estimates of e antigen site densities are shown in Table 5.8.

5.9.2 The E/e polymorphism

The E/e polymorphism is associated with 676C/G in exon 5 of *RHCE*, predicting Pro226Ala in the extracellular vestibule of the RhCE protein [43,47] (Table 5.9). The e antigen is not solely dependent on Ala226 because Ala226 is also encoded by *RHD*, yet the *RHD* product does not express e. Many different changes to *RHCE* will affect e expression, as will become apparent in subsequent sections of this chapter. For example, the presence of VS antigen, which results from Leu245Val encoded by an *RHCE*e* allele, is associated with e weakness (Section 5.13) [130,131,326].

When the same *RHCE* gene encodes C and E, as in the relatively rare *DCE* haplotype, the expression of C is often very weak (Section 5.8.1). Pro226, therefore, appears to suppress C expression [311]. The C/c polymorphism, however, has no obvious effect on the expression of E [311].

5.9.3 Predicting E/e phenotype from DNA

The presence of *RHCE*E* can be detected by PCR with an allele-specific primer, but detecting *RHCE*e* is slightly more complex because 676G, the nucleotide characteristic of the *RHCE*e*, is also present in *RHD*. This difficulty is easily resolved by using a primer specific for 676G paired with a primer to an *RHCE*-specific nucleotide in exon 5 (e.g. 787A) [318,327]. Alternatively, *RHCE*E/e* genotyping could be achieved by *Mnl*I digestion of an *RHCE*-specific PCR product. Occasionally anti-E causes HDFN (Section 5.18.2), so methods have been developed for predicting fetal E phenotype from fetal DNA in maternal plasma [296].

5.9.4 E variants

Red cells with the very rare antigen E^{w} (RH11) are reactive with some, but not all, anti-E sera. One E^{w}+ patient has made alloanti-E [328]. All known examples of anti-E^{w} are red cell immune and anti-E^{w} has been responsible for HDFN [329,330]. E^{w} is always associated with *DcE* and, in two E^{w}+ patients, with 500T>A in exon 4 of *RHCE* encoding Met167Lys in the third extracellular loop [328].

Four categories of E variants (EI to EIV) were revealed by testing monoclonal anti-E against red cells with unusual E antigens [331,332]. The variant E proteins all had Pro226. EI had a Met167Lys, and so is identical to the partial E antigen associated with E^{w} [328]. EII had exons 1–3 derived from *RHD*, EIII had Gln233Glu and Met238Val in the extracellular vestibule, encoded by a segment of exon 5 derived from *RHD*, and EIV had an Arg201Thr exchange in a cytoplasmic domain. Screening of over 140 000 Japanese blood donors with monoclonal anti-E revealed 15 (0.011%) with E variants: nine (EKM) with the EIII mutation; one (EKK) with the EII mutation; and five (EKH) with a new mutation, encoding Arg-154Thr close to the third extracellular loop [333]. Both EKK (EII) and EKH were associated with very weak expression of c and are described further in Section 5.14.3 and Table 5.10.

Weak E, in which the red cells failed to react with four of 14 monoclonal anti-E, had an *RHCE*cE* allele

containing 939G>A in the last (3′) nucleotide of exon 6 [334]. Analysis of cDNA derived from cultured erythroblasts revealed a transcript lacking exon 6 and encoding a protein with a loss of 46 amino acids. Whether this abnormal protein is responsible for the aberrant E, or whether a very small quantity of normal protein was produced by an undetected normally spliced transcript, was not determined.

5.9.5 e Variants – hr^S, hr^B, and related antigens

Some e+ people, most of them of African origin, have made antibodies that resemble anti-e in tests with red cells of common Rh phenotypes. The categorisation of e variants has been prevented by the scarcity of avid e reagents, the complexity of the antibody response by e+ people who make 'anti-e', and the large variety of these aberrant e antigens [335]. Two broad categories can be defined: (i) those with partial e who make an e-like antibody; and (ii) those with partial e and partial Hr_o (RH17) who make antibodies that react with all red cells save those of similar unusual phenotypes, Rh_{null} cells, and cells with D−− and related phenotypes. A separable e-like antibody may also be present in the sera of the second type. Cells of the first category come from individuals heterozygous for a rare *Dce* haplotype, encoding a partial e antigen, and for normal *DcE* (or *dcE*), which produces no e, but does produce the related high frequency antigen. Cells of the second category come from individuals homozygous for a rare haplotype, which produces partial e and no related high frequency antigen (or heterozygous for two similar haplotypes of this type). Two of the possible categories, hr^S and hr^B, are well investigated and are described in more detail below. As will become clear from the text below, to consider these antigens strictly as e variants is a gross oversimplification.

5.9.5.1 hr^S (RH19) and Hr (RH18)

The antibody that defined the blood factor hr^S was the first alloanti-e-like found in the serum of a e+ person (Shabalala) [336]. The serum reacted with all cells of common Rh phenotype, but more strongly with E− e+ cells than with E+ e− cells. After adsorption with E+ e− cells, the serum no longer reacted with e− cells, but still reacted with e+ cells, with the exception of the red cells of about 1% of e+ black South Africans, which were considered hr^S−. Shapiro [336] estimated that about 6% of Bantu Rh haplotypes were *Dce* or *dce* encoding no hr^S. The antibody removed by adsorption of Shabalala serum with E+ e− cells defines a high frequency antigen called Hr (also Hr^S and RH18) [335,336]. Hr is present on all red cells except those rare e+ cells that lack hr^S, cells with D−− and related phenotypes, and Rh_{null} cells. Anti-Hr may also be a component of anti-Hr_o, the immune response of immunised D−− individuals (Section 5.15.6).

Other examples of anti-hr^S have been described [337], though many other antibodies labelled anti-hr^S will have been inaccurately identified. The immune response of hr^S− people is highly variable and antibodies behaving like anti-e in the serum of e+ hr^S− individuals are often assumed to be anti-hr^S even when the critical cells for correct identification are not available. Anti-hr^S appears to be a component of many anti-e sera [336].

Anti-Hr has caused fatal HTRs [338] and HDFN requiring exchange transfusion [336,337,339].

As might be expected from the serological description, the molecular basis for the e+ hr^S− phenotype is heterogeneous. Four *RHCE* genes (see Table 5.10), *RHCE*ceAR*, *RHCE*ceEK*, *RHCE*ceBI*, and *RHCE*ceSM*, encode Met238Val and weak e, but no hr^S or Hr [338, 340], whereas *RHCE*ceMO* also produces weak e and no hr^S or Hr, but encodes Val223Phe and not Met238Val [341].

5.9.5.2 hr^B (RH31) and Hr^B (RH34)

Description of the blood factor hr^B is very similar to the hr^S story. Anti-hr^B was found in the serum of a black South African (Bastiaan), together with an antibody to a high frequency antigen, anti-Hr^B [342]. Although anti-hr^B and -Hr^B in Bastiaan serum may not have been completely separable by adsorption [343], separable anti-hr^B and -Hr^B are found in other similar sera and sera containing either anti-hr^B or -Hr^B also exist [335,344].

Neither hr^B nor Hr^B is produced by the *d(C)ce*^{s} type 1 or 2, or $D^{IIIa}ce^s$ haplotypes, which produce VS, but not V (Section 5.13.2) [344]. Consequently, individuals homozygous for these haplotypes, or with compound heterozygosity, are hr^B− Hr^B− and can make anti-hr^B and -Hr^B; individuals heterozygous for any of these haplotypes and for *DcE* are hr^B− Hr^B+ and can make anti-hr^B. Almost 40 years after the original study on the hr^B− and Hr^B− index case, another blood sample from Bastiaan became available, which revealed homozygosity for *RHCE*ce* typical of *d(C)ce*^{s} (*RHCE*ceVS.03*, see Table 5.10) [345].

Anti-hr^B was detected in the sera of two patients who had transient loss of hr^B [346]. The apparent alloantibodies were later found to be autoantibodies when hr^B reappeared.

5.9.5.3 Transfusion of patients with e variant red cells

Red cells with variant e antigens, especially hr^S– and hr^B–, are commonly found in patients of African origin and, therefore, in transfusion-dependent patients with sickle cell disease (SCD). Protecting these patients from producing Rh antigens, or providing suitable blood if they have already done so, can prove difficult. Often DcE/DcE red cells are selected for transfusion for prevention of production of anti-hr^S or -hr^B, but this can lead to production of anti-E and, because hr^S– and hr^B– phenotypes are often associated with a D variant (DIII) phenotype that remains undetected by routine serology, with production of anti-D [344,347]. Molecular testing of patients with SCD assists in the selection of the most suitable available red cells for transfusion [348,349].

5.9.5.4 STEM (RH49), a low frequency antigen associated with some hr^S– and hr^B– phenotypes

Further heterogeneity of hr^S– and hr^B– phenotypes was revealed by anti-STEM, an antibody to a determinant present on the red cells of 3–6% of South Africans, but only very rarely present in white people [350]. Family studies suggested that STEM is associated with some *Dce* haplotypes that produce neither hr^S nor hr^B. Red cells of approximately 65% of hr^S– Hr– and 30% of hr^B– Hr^B– individuals are STEM+. People with hr^S+ STEM+ or hr^B+ STEM+ red cells may be heterozygous for an hr^S-deficient or hr^B-deficient *Dce* haplotype. STEM, therefore, may be a marker for some partial e or 'partial Hr_o' antigens.

5.9.6 Ee antibodies

Anti-E occurs more commonly than anti-C in the sera of D+ people. Unlike other Rh antibodies, anti-E often appears to be 'naturally occurring'. Many anti-E are only reactive with protease treated E+ red cells [351–354] (Section 5.18.3).

Anti-e is not a common antibody since only 3% of people are e–. Sera containing anti-e often contain anti-Ce or anti-ce (Section 5.10).

5.10 Compound CE antigens

Some Rh antigens, known as compound antigens, are only expressed when c and e, or C and e, or C and E, or c and E are produced by the same *RHCE* gene. Reactions of antibodies to these antigens are shown in Table 5.3. Antibodies to these compound antigens probably recognise conformational differences in the RhCE protein that result from the amino acid substitutions associated with both the C/c and E/e polymorphisms.

5.10.1 ce or f (RH6)

Anti-ce (also known as anti-f) reacts with the products of *RHCE*ce*. It also reacts with d(C)ce^s cells (Section 5.13). Reactions of the products of some aberrant haplotypes with anti-ce are contrary to predictions based on their reactions with anti-c and -e. For example, some *Dc*– haplotypes express ce antigen, but no e (Section 5.15.3).

The first two examples of anti-ce were detected in multi-transfused DCe/DcE patients [355,356]. Anti-ce is a common component of anti-c and -e sera [355,357], but is only rarely found as a single specificity. Three cases of HDFN caused by anti-ce have been reported [358–360] (although one of the sera also contained anti-c). Anti-ce has also been implicated in a delayed HTR [361]. One autoanti-ce in a DCe/dce patient could only be detected in an acidified antiglobulin test; its role in the patient's transfusion reaction was uncertain [362].

5.10.2 Ce (RH7) and Rh41

Recognition of the compound antigen Ce (also known as rh_i) [363], provided an explanation for the observation that certain anti-C sera distinguish C produced by *DCe* and *dCe* from that produced by *DCE* and *dCE* [364]. The original anti-Ce, together with a weak anti-e, was found in the serum of an immunised DcE/DcE woman; the anti-e was removed by adsorption with Dce/dce or dce/dce cells [363]. Most immune anti-C made by D+ people are predominantly anti-Ce and most anti-C and -C+D sera contain at least some anti-Ce [324].

Anti-Ce has caused HDFN requiring treatment by fetal or neonatal transfusion [365–367] and may have contributed to an HTR [368]. IgA anti-Ce in a DCe/DCe woman was responsible for AIHA [369].

Anti-Rh41, an antibody made by a DCe/DcE C^w+ woman, was similar to anti-Ce in reacting with cells from people carrying C and e produced by the same gene, but differed from anti-Ce by reacting with d(C)ce^s/dce cells, but not with C^w+ red cells of DCe/dce, DCe/DcE, or dCe/dce phenotypes [370].

5.10.3 CE (RH22)

Anti-CE is a very rare antibody that reacts with the products of the rare haplotypes *DCE* and *dCE*. The first example was found together with anti-C in the serum of a dce/dce woman who had never been transfused and had a dce/dce husband and child [371]. A second example of

anti-CE plus a weak anti-C also appeared to be 'naturally occurring'.

5.10.4 cE (RH27)

Anti-cE (together with anti-E and -S) was first identified in the serum of a DCe/DCe woman [372]. Another example of anti-cE, detected in the serum of a DCe/DCE C^w+ man, appeared to be a 'naturally occurring' antibody and was unusual for an Rh antibody because it bound complement [373].

5.11 G (RH12)

Anti-G reacts with red cells that express D or C, or both D and C. Ser103, encoded by *RHD* and by *RHCE*C*, is the key to G reactivity (see Table 5.9). The importance of Ser103 in G expression has been demonstrated by rare phenotypes that break the rule that all cells that have D and/or C are G+.

DcE producing normal D, but no G, was found in three generations of a white family, studied because of HDFN caused by anti-C+G [374]. In two unrelated individuals with DcE/dce G– red cells, also with apparently normal D, *RHD* had 307T>C encoding Ser103Pro [375].

Red cells with the variant D antigen DIIIb express most epitopes of D, but are G– (Section 5.6.4.2) and their immune response sometimes includes a separable anti-G [156,157]. DIIIb is produced by *RHD* in which exon 2 is replaced by exon 2 from *RHCE*c* and encodes Leu60, Asn68, and Pro103 [162–164].

A phenotype (designated r^G, probably erroneously) in which the red cells are D– C– E–, but G+, and probably have weak c and e, was associated with *RHCE* encoding Trp16 (characteristic of *c* and *RHD*) in exon 1 and Ile60, Ser68, and Ser103 (characteristic of *RHCE*C* and *RHD*) in exon 2 [313]. This gene could represent *RHCE*ce* in which exon 2 is substituted by exon 2 from *RHD* or from *RHCE*C*.

The 'haplotype' inappropriately labelled r''^G produces G, E and possibly very weak C and D, but no c or JAHK [376–378] (see Table 5.10). The r''^G gene represents *RHD–CE–D* in which exons 4–8 derive from *RHCE*E* [375]. This gene encodes Trp16 and Ser103.

The gene named r^G produces G, very weak C detected by about one in three anti-C from D+ individuals (initially called anti-C^G [315]), and weak e [314,315,379]. It also produces the low frequency antigen JAHK (RH53) [377]. Homozygosity for r^G was found in a man whose parents were first cousins [314]. The r^G gene is *RHCE*Ce* with 365C>T in exon 3 encoding Ser122Leu in the fourth membrane-spanning domain, adjacent to the second extracellular loop, which contains Ser103 [380]. It is likely that conformational changes resulting from the substitution of a neutral serine by a hydrophobic leucine is responsible for weakness of C and e and for JAHK expression, but G is expressed strongly because it is much less dependent on protein conformation than C.

Estimates for the number of G antigen sites per red cell [381]: DCe/DCe 9900–12 200; dCe/dCe 8200–9700; DCE/DCE 5400; DcE/DcE 3600–5800; Dce/Dce 4500–5300; DcE/dce 4200; and four unrelated dcE/dce G+ (r''^G/r) 600–3600. Anti-G blocks C and D sites [379], and, on occasion, e sites [382].

Anti-G was originally found in the sera of dce/dce people, together with anti-D and/or -C [379,383,384] and was detected in 30% of single donor 'anti-CD' sera and in all commercial anti-CD and -CDE reagents [324]. Anti-G can be isolated by adsorption/elution techniques with r^G/dce or r''^G/dce cells [379,381]. Since these phenotypes are rare, a double elution method has proved useful: 'anti-CD' is adsorbed onto and eluted from dCe/dce (D– C+ G+) cells to isolate anti-C and -G, then this eluate, which contains no anti-D, is adsorbed onto and eluted from Dce/dce (D+ C– G+) to isolate anti-G [385]. A thorough serological analysis of 27 sera from immunised women with apparent anti-D+C revealed three anti-D+C, 13 anti-D+C+G, seven anti-D+G, and four anti-C+G [386]. The clinical significance of anti-G is discussed in Section 5.18.2.

The concept of G has helped to sort out some previous serological puzzles. It explained why some dce/dce women immunised only by pregnancy appeared to have made anti-C+D, even though their husbands were C–; their antibody was anti-D+G. It provided an explanation for the apparent anti-C+D in the serum of two dce/dce mothers who had delivered dCe/dce children [383,384]; their antibodies were anti-C+G. Recognition of anti-G also explained the apparent ability of Dce/dce cells to adsorb anti-C activity from anti-C+D sera; such sera were anti-D+G. An apparent anti-C+D made by a D– woman transfused with eight units of D– blood was shown to be anti-C+G and one of the donors was identified as dCe/dce [387].

5.12 C^w, C^x, and MAR

C^w and C^x are antigens of relatively low frequency that have an allelic relationship with the high incidence

antigen MAR [388]. C^w and C^x result from single nucleotide changes in exon 1 of *RHCE* (usually *RHCE*Ce*) encoding amino acid substitutions in the first extracellular loop: 122A>G, Gln41Arg in C^w; 106G>A, Ala36Thr in C^x [389]. These amino acid changes probably cause conformational alterations in the protein that are responsible for quantitative and qualitative abnormalities of C.

5.12.1 C^w (RH8)

Callender and Race [390] found the first example of anti-C^w in the serum of a DCe/DCe patient who had been transfused with DCe/DCe C^w+ red cells. In an English population C^w has an occurrence of 2.6% [13]; similar frequencies are found in most other northern European and white American populations, but it is very much lower in most other populations [14,20]. The highest frequency of C^w (7–9%) has been found in Latvians, Lapps, and Finns.

C^w+ red cells are almost always C+, but the C antigen associated with C^w is weaker than normal C, though recognition of this weakness depends on the anti-C used. Although C^w is usually produced by a *DCe* haplotype, C^w associated with *dCe*, *dCE*, and *DCE* have also been found. A person with apparently normal DCC^we/dce red cells made anti-C that did not react with DCC^we/dce or dCC^we/dce cells [391]. Similar antibodies have been detected in the sera of DCC^we/DCC^we and DCC^we/DcE individuals [392,393].

Studies with ^{125}I-labelled human monoclonal anti-C^w provided the following estimates of C^w sites per red cell: DCC^we/DCC^we 32 000; DCC^we/DCe 15 200; DCC^we/DcE 19 800; DCC^we/dce 15 300; dCC^we/dce 26 200 [394]. Similar studies with monoclonal anti-C did not reveal any obvious reduction in C antigen density in C^w+ cells compared with C^w– cells [394].

Very rarely, C^w is produced by *RHCE* that produces c and e [389,395,396]. In one individual with C^w+ C– c+ red cells, the cells were also D– and G– (dcC^we/dce). C^w is usually produced by *RHCE*Ce* encoding Arg41; C^w associated with c is produced by *RHCE*ce* encoding Arg41 and Cys16 [389]. *RHCE*CC^{w}e* and *RHCE*cC^{w}e* alleles, therefore, have exons 1 of identical sequence (see Table 5.9) and *RHCE*cC^{w}e* could have arisen by recombination between *RHCE*CC^{w}e* (exon 1) and *RHCE*ce* (exons 2–10). The *DC^w*– haplotype, which produces C^w but no C, c, E, or e, is described in Section 5.15.4.

Anti-C^w is not an uncommon antibody and often results from no known red cell immunising stimulus. One in 1100 pregnant Manitoban women had anti-C^w [397]. Anti-C^w has been responsible for several of cases of HDFN [397], a very few have been severe [398–402]. Anti-C^w is not usually detected in pregnant women as red cells for antibody screening are often C^w–.

5.12.2 C^x (RH9)

Like C^w, C^x is usually produced by a *DCe* haplotype that produces abnormal C. C^x+ red cells react with some, but not all, anti-C. Very rare haplotypes that encode C^x include *dCC^{x}e*. A haplotype containing *RHD* encoding weak D-4.3 and *RHCE*ce* encoding c, no C, C^x, and VS was found in about 0.12% of Upper Austrian donors [244]. A similar phenotype, but D–, was present in four of 513 unrelated Somalis [403].

Seven C^x-positives were found among 5919 (0.12%) British donors [404,405] and 202 were found among 70 503 (0.29%) Americans [406]. C^x has a much higher incidence in Finland: 37 of 2060 (1.8%) Finns were C^x+ [388].

The first anti-C^x caused mild HDFN, as have other examples since [404,405]. Some anti-C^x appear to be 'naturally occurring' [407]. Anti-C in the serum of a transfused DCC^xe/dce patient reacted with most C+ cells, including DCC^we/dce cells, but not with DCC^xe/dce, DCC^xe/DcE, or DCC^xe/DCC^xe cells [408].

5.12.3 MAR (RH51)

Anti-MAR was found in a Finnish woman whose red cells were C^w+ C^x+ D+ C+ c– E– e+ and who was probably heterozygous *DCC^{w}e/DCC^{x}e* [388]. Testing of 10 045 Finnish donors revealed 21 MAR-negatives: nine were C^w+ C^x– (probably *RHCE*C^w/C^w*), three were C^w– C^x+ (*RHCE*C^x/C^x*), and nine were C^w+ C^x+ (*RHCE*C^w/C^x*). In eight families, all 20 children of MAR– parents were either C^w+ or C^x+. Anti-MAR reacted weakly with many examples of C^w+ C^x– and C^w– C^x+ cells. It did not react with Rh_{null}, D––, or DC^w– cells. As C^w and C^x usually result from Gln41Arg and Ala36Thr in the RhCE protein, respectively [389], it is probable that both Gln41 and Ala36 are required for MAR expression.

Two other antibodies to high frequency antigens, produced in probable *RHCE*C^x/C^x* [406] and *RHCE*C^w/C^w* [409] women, resembled anti-MAR in their serological reactions. However, the antibody from the *RHCE*C^w* homozygote reacted weakly with DCC^xe/DCC^xe cells and the antibody from the *RHCE*C^x* homozygote reacted strongly with DCC^we/DCC^we cells. The antibody of the probable *RHCE*C^x* homozygote caused HDFN [406].

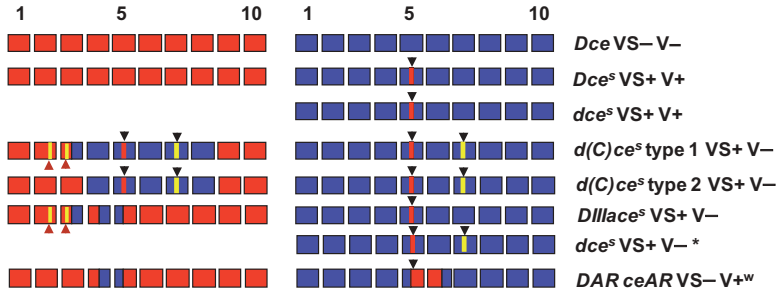


Figure 5.9 Representation of the 10 exons of *RHD* (red boxes) and *RHCE* (blue boxes) in VS+ and/or V+ phenotypes. ▼ Codons for Val245 in exon 5 and Cys336 in exon 7. ▲ Codons for Phe62 (exon 2) and Val137 (exon 3) characteristic of *RHD*DIIIa*. Yellow lines, untemplated amino acid substitutions. * Genotype not confirmed by transcript analysis.

5.13 VS (RH20) and V (RH10)

5.13.1 Serology

Anti-V, first reported in 1955 [410], and anti-VS, described five years later [411], define antigens that are common on the red cells of Africans, but are rare in other populations. V and VS are associated with a partial e called e^s, though e and e^s are often difficult to distinguish. As a general rule, VS is produced by the haplotypes *Dce*^{s}, *dce*^{s}, and *d(C)ce*^{s}, whereas V is produced by *Dce*^{s} and *dce*^{s}, but not *d(C)ce*^{s}.

The haplotype *d(C)ce*^{s} (previously known as *r′*^{s}) produces c, e^s, ce (f), a weak partial C (sometimes called C^G), G, and VS; it does not produce D, Ce, or V. In addition, anti-Rh42, an antibody found in the serum of a DCe/Dce mother of Dce/d(C)ce^s and DCe/d(C)ce^s children, only reacts with the product of *d(C)ce*^{s} [412]. Many individuals with d(C)ce^s phenotype have produced alloanti-C [312,517]. In addition, despite having apparently normal expression, the c and ce might be partial antigens as one *d(C)ce*^{s}*/DCe* patient produced anti-c, which reacted more strongly with ce than c [413]. VS is also associated with the hr^B– phenotype (Section 5.9.5.2): neither hr^B nor Hr^B is encoded by *d(C)ce*^{s} [132,344].

Many examples of anti-VS and -V have been found, often in sera containing other antibodies. The heterogeneity of anti-VS has been thoroughly investigated [414]: some antibodies react preferentially with VS+ V– cells, some prefer VS+ V+ cells, and others react equally well with both types. Heterogeneity of anti-VS probably provides an explanation for the specificity called anti-hr^H (RH28) [415]. Heterogeneity of V antibodies is also observed when they are tested against red cells of unusual phenotypes [414]. No clinically significant anti-VS or -V has been reported. Anti-VS and -V are usually reactive by an antiglobulin technique, but a saline active anti-V is recorded [416].

Some phenotypes do not fit the serological rules described above. A D– C– V– VS+ donor [131] and the VS– V+ (ceAR) phenotype [131,153] are described below (Section 5.13.2). The *DCe*^{s} haplotype, producing an apparently normal C, no c, and VS and V is extremely rare [417].

Of 100 black South African blood donors, 34 were VS+ V+, 9 were VS+ V–, and 4 were VS– V+, with weak V (ceAR) [131]. The incidence of V in two surveys of African Americans was 27% [410] and 39% [418], and was 40% in West Africans [410]. V is not an exclusively African characteristic: there was no trace of African ancestry in two English families with V antigen [410]. In white Argentineans, of 33 *Dce* haplotypes, 12 (36%) were *Dce*^{s}, reflecting the contribution of African alleles in that genetic pool [419].

Weakness of D in red cells of DcE/d(C)ce^s individuals demonstrated that *d(C)ce*^{s} has a depressing effect on *D in trans*, similar to that of *dCe* (see Section 5.6.6).

5.13.2 Molecular genetics

VS is associated with 733C>G in exon 5 of *RHCE*ce* encoding Leu245Val [130,131,326]. In *dce*^{s} and *Dce*^{s} haplotypes, no other mutation has been detected (Figure 5.9). Val245 is present in the RhD protein, so a microconversion event may have been involved in the formation of the VS gene. Amino acid 245 is predicted to be buried in the membrane in the eighth membrane-spanning domain, adjacent to the vestibule region, which contains Ala226, the residue primarily responsible for e expression (see Figure 5.2). It is likely that VS expression and the

associated weakness of e (e^s) result from conformational changes to the protein resulting from Leu245Val. Avent [56] suggested that residue 245 may be involved in stabilisation of the RhCE-RhAG-RhAG trimer.

The typical *d(C)ce*^{s} haplotype (type 1) contains a hybrid gene, *RHD–CE–D*^{s}, consisting of exons 1, 2, part of 3 (including codons 121, 127, and 128), 9, and 10 from *RHD* and the remainder of exon 3 (including codon 152) and exons 4–8 from *RHCE*e* [129,130] (Figure 5.9). As *RHD–CE–D*^{s} (type 1) also encodes Leu62Phe (exon 2) and Ala137Val (exon 3), exons 1–3 are probably derived from *RHD*DIIIa*, so the hybrid could be written *RHDIIIa–CE(4-8)–D*^{s}. The hybrid gene also encodes Leu245Val (exon 5), and Gly336Cys (exon 7, 1006G>T). It is paired with *RHCE*ce* that also encodes Leu245Val (VS) and Gly336Cys. The presence of Ser103 encoded by exon 2 of *RHD–CE–D*^{s} together with downstream sequences characteristic of *RHCE*, is probably responsible for C expression by d(C)ce^s, its weakness being due to tryptophan, and not cysteine, at position 16 (Section 5.8.2). Both *RHD–CE–D*^{s} and its paired *RHCE* gene encode Val245 and Ala226 (e) and are likely to produce VS and e^s. Both genes also encode Gly336Cys, a substitution not present in VS+ V+ phenotypes [131]. It is likely that anti-VS and -V both recognise Val245 in an Rhce protein, but that anti-V is more conformationally dependent than anti-VS and does not react with the protein when Cys336 is also present.

Another *RHD–CE–D*^{s} gene, part of the *d(C)ce*^{s} type 2 haplotype, resembles the type 1 hybrid apart from having the whole of exon 3 derived from *RHCE* (Figure 5.9) [132]. In contrast to *d(C)ce*^{s} type 1, *d(C)ce*^{s} type 2 encodes a much weaker C and does not produce Rh42.

VS, but not V, is also encoded by a haplotype comprising *RHD*DIIIa* paired with *RHCE*ce*^{s} encoding Val245 and Cys336 (typical of *d(C)ce*^{s}) [132].

Pham *et al.* [132] suggest that the origin of the *d(C)ce*^{s} type 1 haplotype is the result of a gene conversion event involving *RHCE*ce*^{s} and *RHD*DIIIa*, whereas the origin of the *d(C)ce*^{s} type 2 involved *RHCE*ce*^{s} and *RHD* (see Section 5.5.5 and Figure 5.6).

*RHCE*ce* encoding Trp16Cys, Leu245Val (typical of VS), Val314Ala, and Gly336Cys (typical of *d(C)ce*^{s}) encoded c, VS (though the V type was not clear), and variant e and hr^B [420]. This allele was found in 5.5% of African Americans. It may be the same as that found in a D– C– c+ E– e+ G– V– VS+ black English blood donor, with an *RHCE* allele encoding Val245 and Cys336 [131] (Figure 5.9). This strongly supports the suggestion that *RHD–CE–D*^{s} produces weak C and that the presence of Cys336, and not the hybrid Rh protein, is responsible for the absence of V in the d(C)ce^s phenotype.

*RHCE*ceAR* encodes partial c and e, weak V, but no VS. Individuals with *RHCE*ceAR* have made anti-c [421,422]. *RHCE*ceAR* is an *RHCE–D–CE* gene with codons for Val238, Val245 (VS), Gly263, and Lys267 derived from exon 5 of *RHD* and Val306 from exon 6 of *RHD* [131,153] (Figure 5.9). *RHCE*ceAR* is usually linked to *RHD*DAR* (Section 5.6.4.9). In a survey of 326 black South Africans, 20 (6.1%) had *RHCE*ceAR* and 14 of these also had the *RHD*DAR* [153].

An Austrian haplotype encoding VS and C^x [244] is described in Section 5.12.2.

5.14 Variants with abnormal Cc and Ee antigens

Gathered together in this section are some haplotypes that result in abnormal expression of C or c and E or e antigens. There are many of these variants known and they are continuing to be found [423], and only selected examples are listed in Table 5.10 and described below. It should be remembered that weak antigens (denoted by parentheses) can only be observed on red cells of people homozygous for the rare haplotype or on those of people with a suitable antithetical Rh haplotype: for example, weak C will not be detected if a normal C is produced by the opposite gene, but will be detected if the opposite gene produces c. Some of these haplotypes also produce low frequency marker antigens. Some details of antibodies to low frequency Rh antigens are provided in Section 5.17 and Table 5.14. Some complexes involving partial e antigens, described in Section 5.9.5, are omitted from this section. Homozygosity for some of the haplotypes described in this section has revealed that they do not encode certain high incidence Rh antigens, such as Hr_o, Rh46, CEST, and CELO, which are produced by all normal Rh haplotypes. Haplotypes producing neither E nor e are described in Section 5.15.

5.14.1 Variant *RHCE*Ce* alleles

5.14.1.1 R^N ($\overline{\overline{R}}^N$) incorporating *RHCE*CeRN;* Rh32 and Rh46 antigens

The symbol $\overline{\overline{R}}^N$ given to a haplotype encoding weak C and weak e in an African American family [424] has been replaced by R^N to avoid word processing complications. In addition to weak C and weak e, R^N encodes D and G, which have been shown to be elevated in some studies

Table 5.10 Some *RHCE* variants.

Name	Antigens	Exons	Amino acid changes or hybrid gene	Paired *RHD*	Ethnic group	References
*RHCE*CeRN.01*, R^N	(C) (e) Rh32, DAK Rh:–46	4	*RHCE–D(4)–CE*	*RHD*	Black	[424,425]
*RHCE*CeRN.02*, R^N	(C) (e) Rh32, DAK Rh:–46	3, 4	*RHCE–D(4)–CE* + T152N	*RHD*	Black	[424,425]
*RHCE*CeJAL* (*RHCE*CeMA*)	(C) (e) (hr^S) (hr^B) JAL CEST–	3	R114W	*RHD*	White	[426–428]
*RHCE*CeVA*	(C) (e) Rh33 FPTT	5	*RHCE–D(5)–CE*	*RHD*	White	[429]
*RHCE*CeNR*	(C) (e) C^w CENR	1, 6–10	*RHCE(1–5)–RHD(6–10)* + Q41R	*RHD*	White	[430,431]
*RHCE*CeJAHK*	(C) (e) G JAHK	3	S122L	None	White	[379,380]
*RHCE*Ce667*	(C) (e)	5	V223F			[423]
*RHCE*ceJAL*	(C) (e) (hr^S) (hr^B) (VS) (V) JAL CEST–	3, 5	R114W, L245V	*RHD*DAU0*	Black	[426–428]
*RHCE*ceBE*	(c) (e) (ce) Be^a	5	P221R	None	White	[432,433]
*RHCE*ceLOCR*	(c) (e) LOCR Rh:–26	2	G96S	None	White	[434–436]
*RHCE*ceHAR*, R_o^{Har}	c (e) (ce) Rh33 FPTT (D)	5	*RHCE–D(5)–CE*	None	White	[191,194]
*RHCE*ceCF*	(c) (e) Crawford VS (D) CELO–	1, 5	W16C, Q233E, L245V	None	Black	[253,254]
*RHCE*ceRT*	(c) (D)	3	R154T	None	White	[255]
*RHCE*ceSL*	(D)	1, 3	W16C S122L	None	White	[256]
*RHCE*ceAR*	(c) (e) (V) (D) VS– hr^S– Hr–	1, 5, 6	W16C, M238V, L245V, R263G, M267K, I306V	Often with *RHD*DAR*	Black	[153]
*RHCE*ceVS.01*, ce^s	(c) (e) VS V hr^B– Hr^B–	5	L245V	*RHD* or none	Black	[130,131,326]
*RHCE*ceVS.02*, ce^s	(c) (e) VS V hr^B– Hr^B–	1, 5	W16C, L245V	*RHD*DIIIa* or none	Black	[130,131,326]
*RHCE*ceVS.03*, ce^s	(c) (e) VS V– hr^B– Hr^B–	1, 5, 7	W16C, L245V, G336C	*RHD–CE–D*^{s} or *RHD*DIIIa*	Black	[130,131,326]
*RHCE*ceC*^{x}*VS*	c e C^x VS	1, 5	W16C, A36T, L245V	*RHD*weak 4.3*	White	[244]
*RHCE*ceMO*	(c) (e) hr^s– hr^B– Hr– Hr^B–	1, 5	W16C, V223F	Often with *RHD*DAU0*	Black	[338,341]
*RHCE*ceEK*	(c) (e) hr^s– Hr–	1, 5	W16C, M238V, R263G, M267K	Often with *RHD*DAR*	Black	[338]
*RHCE*ceBI*	c (e) STEM hr^s– Hr–	1, 5, 6, 8	W16C, M238V, A273V, L378V	Often with *RHD*DOL*	Black	[338,699]
*RHCE*ceSM*	(e) (STEM) hr^s– Hr–	1, 5, 6	W16C, M238V, A273V	Often with *RHD*DOL*		[340]
*RHCE*ceRA*	(c) (e) hr^S–	1, 4	W16C, G180R	None	Indian	[437]
*RHCE*ceAG*	(e) (hr^B)	2	A85G		Black	[438]
*RHCE*ceBP*	c (e) ce–	5	R229del	*RHD* or none	White	[697]
*RHCE*ceTI*	(e) (C)	1, 7	W16C, T342I	Usually *RHD*DIVa-2*		[698]
*RHCE*cEKH*	(c) (E)	3	R154T		Japanese	[333]
*RHCE*EKK*	(E)	1–3	*RHD(1–3)–CE*	With *RHCE(1–3)–D*	Japanese	[333]
*RHCE*cE734C*	(c)(E)	5	L245P	*RHD*	White	[459]

[425,441,442], and the low frequency antigens Rh32 and DAK (also associated with DIIIa) [160]; R^N does not produce any c, E, ce, or, usually, Ce. One individual with R^N produced anti-C [443]. The Hr_o-like high frequency antigen Rh46, produced by most Rh haplotypes, is not produced by R^N [442].

R^N is most commonly found in people of African origin. It was estimated that Rh32 is present on red cells of about 1% of African Americans, but it has only been found as a rarity in white people [443]. Numerous R^N homozygotes are recorded; some found because of immune antibody in their serum (anti-Rh46), others because their weak C and e antigens were detected during routine Rh typing [29,442].

In two R^N homozygotes, R^N represented *RHD* paired with an *RHCE–D–CE* gene (*RHCE*CeRN.01*) with exon 4 derived from *RHD*, the remainder of the gene having the *RHCE*Ce* sequence [425]. One other individual, apparently heterozygous for R^N, had a similar hybrid gene (*RHCE*CeRN.02*), but with the 3′ end of exon 3, encoding Asn152, also derived from *RHD* [425] (Figure 5.10). These hybrid genes encode Leu169Met, Arg170Met, and Phe172Ile changes close to the third predicted extracellular loop of RhCE. The molecular basis for Rh32 expression is described in Section 5.17.2.

Homozygous R^N individuals can make, if immunised by pregnancy or transfusion, anti-Rh46, which reacts with red cells of almost all Rh phenotypes except R^N, Rh_{null}, D−−, and related phenotypes [157,442]. Anti-Rh46 has been responsible for serious HDFN [442]. Mouse and human monoclonal antibodies with Rh46 specificity have been produced [444,445].

5.14.1.2 *RHCE*CeJAL* and JAL (RH48)

A multilaboratory investigation of the serum of a mother whose baby had DAT-positive red cells culminated in the recognition of a new low frequency antigen, JAL (RH48) [426]. In Caucasians JAL is associated with weak expression of C, e, hr^S, and hr^B [427,446,447]. *RHCE*CeJAL* contains 340C>T in exon 3 encoding Arg114Trp in the fourth membrane-spanning domain and is paired with *RHD* [427,428]. It is apparently identical to the previously described *RHCE*CeMA*, though red cells were not tested with anti-JAL [429]. *RHCE*CeJAL* (identified serologically) has a frequency of 0.0003 in French-speaking Swiss; no JAL+ individual was found among more than 50 000 German-speaking Swiss [446].

JAL associated with a haplotype producing weak c and weak e in JAL+ individuals of African origin is described in Section 5.14.2.1.

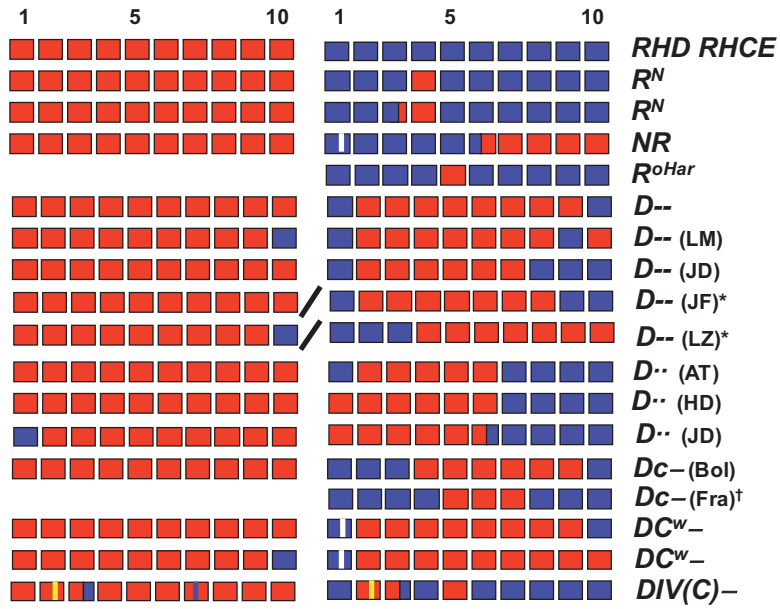


Figure 5.10 Representation of the 10 exons of *RHD* (red boxes) and *RHCE* (blue boxes) in some haplotypes containing rearranged genes encoding either partial Hr_o or no Hr_o. White line, exon 1 encoding Arg41 and C^w. Yellow line, untemplated amino acid substitutions. *Proposed separate haplotypes in two D−− propositi (JF and LZ). †Presence or absence of *RHD* not stated (Fra).

5.14.1.3 FPTT-associated haplotypes

Several new unusual Rh phenotypes were recognised during the investigation of a serum (Mol), which contained antibodies to several low incidence antigens [188]. Results of adsorption and elution tests suggested that a single antibody, anti-FPTT (-RH50), was reacting with cells of three propositi, each with a 'new' phenotype. None of the families was large enough to define the new complexes completely. FPTT is also associated with R_o^{Har} (Sections 5.6.4.12 and 5.14.2.4) and DFR (Section 5.6.4.7).

One haplotype encoded weak C, detected by one of six anti-C, very weak e, and FPTT, but no ce. The haplotype was not paired with *dce* or *DCe*, so whether it produces D and/or G is not known and lack of c and E is not proven. The reactions were similar to those of r^G cells (Section 5.14.1.5), but r^G cells are not FPTT+. The family of the second propositus showed that their FPTT-associated haplotype produced D, G, weak C, weak e, and atypical VS; it did not produce any E, ce, or V. Red cells of the third propositus expressed weak e, but normal C [188].

FPTT antigen is very variable in expression and is inherited in a normal way. FPTT has a frequency of about 0.01% in the South of France [188].

5.14.1.4 Other depressed DCe haplotypes

*RHCE*CeVA* represents an *RHCE-D-CE* hybrid allele in which exon 5 from *RHCE*C* is replaced by exon 5 from *RHD*. It produces weak C and weak e, Rh33, and probably FPTT [429]. Consequently it resembles R_o^{Har} (Sections 5.6.4.12 and 5.14.2.4) apart from producing weak C instead of weak c and by being linked to *RHD*, so it cannot be determined whether *RHCE*CeVA* produces any D.

Red cells of a white woman (NR) with anti-Hr_o, which caused severe HDFN in her second child, were D+ C+ c– E– e+ C^W+, with weak expression of C and e, and were Rh:–32, but reacted with an antibody to a new low frequency antigen CENR (RH56) [430,431]. She was considered to have the genotype *D(C)C^w(e)/D––*, confirmed by molecular analysis. The *RHCE*(C)C^w(e)* allele (*RHCE*CeNR*) is an *RHCE–D* hybrid, with exons 1–5 derived from *RHC*CC^{w}e* and exons 6–10 (or 9) derived from *RHD* [431]. Also present were transcripts representing *RHCE–D*, with only exon 1 derived from *RHCE* (reported previously in *D‥* and *DC^w–* [448,449]) (Figure 5.10) and *RHD*. A family study showed that *RHCE*CeNR* is paired with *RHD*.

A depressed haplotype similar to R^N, but not producing Rh32, was found in six unrelated Swedes [450]. Selected anti-C and -e sera distinguished the Swedish D(C)(e) from R^N, even before anti-Rh32 had been identified [451]. Four more propositi with the Swedish *D(C)(e)* haplotype were reported [452], but in one propositus and his mother, unlike the others, the depressed haplotype was associated with enhanced D expression. Existence of two Swedish *D(C)(e)* haplotypes was confirmed by the identification of an antibody, anti-Rh35, specific for Swedish D(C)(e) complex with normal D [441]. The original anti-Rh35 is still the only example and very little of it remains.

5.14.1.5 r^G or *RHCE*CeJAHK* and JAHK (RH53)

The inappropriately named r^G is an *RHCE*Ce* allele that produces G, very weak C, weak e, and the low frequency antigen JAHK [314,315,377,379] (see Section 5.11). It contains 365C>T encoding Ser122Leu [380]. It is not linked to *RHD*.

Anti-JAHK was found in several sera containing multiple antibodies to low frequency antigens.

5.14.2 Variant *RHCE*ce* alleles

5.14.2.1 *RHCE*ceJAL* and CEST (RH57)

In contrast to *RHCE*CeJAL* found in Caucasians (Section 5.14.1.2), in people of African origin JAL is produced by a variant *RHCE*ce* allele that produces weak c, e, ce, VS, V, hr^S, and hr^B [426,427,447]. Alloanti-c and -e have been produced by c+ e+ JAL+ individuals [447,453] and an anti-Hr-like antibody, named anti-CEST (-RH57) was made by an *RHCE*ceJAL* homozygote [447]. Like *RHCE*CeJAL*, *RHCE*ceJAL* encodes Arg114Trp, but also encodes Leu245Val, which is responsible for VS expression (Section 5.13.2) [427,428]. Westhoff *et al.* [428] propose that the loss of a hydrogen bond between Arg114 and Ala 226 and the conformational modification caused by the tryptophan side chain are responsible for the weak expression of C, c, and e associated with JAL. *RHCE*ceJAL* is identical to the previously reported ce^s(340) [338], although red cells were not tested with anti-JAL. *RHCE*ceJAL* is usually paired with *RHD*DAU0* [427,428].

A different mutation was present in a JAL+ Caucasian and a JAL+ Asian, who had JAL encoded by *RHCE*ce*: 341G>A, Arg114Gln [427]. This allele was linked to *RHD*.

5.14.2.2 *RHCE*ceLOCR*, Rh26, and LOCR (RH55)

A variant c antigen detected by its failure to react with one strong anti-c reagent was numbered anti-Rh26 [434]. Two further c+ Rh:–26 propositi were found in tests with anti-Rh26 on 1900 C+ red cell samples. A DCe/dce Dutch

woman made anti-Rh26 during her first pregnancy. Screening with the antibody revealed a second Dutch propositus with DCe/dce Rh:–26 red cells [435]. Most anti-c sera appeared to contain mixtures of anti-c and -Rh26. Two of 10 monoclonal anti-c behaved as anti-Rh26 as they did not react with c+ Rh:–26 cells [435]. The c+ Rh:–26 phenotype results from an *RHCE*ce* allele that produces weak c, normal ce, weak e (in one individual), and the low frequency antigen LOCR (RH55) [434–436,454]. *RHCE*ceLOCR* contains 286G>A in exon 2, encoding Gly96Ser in the second extracellular loop [435,436]. It is not linked to *RHD*.

5.14.2.3 *RHCE*ceBE* and Be^a (RH36)

The low frequency antigen Be^a (Berrens) was first described in 1953 [432], but was not shown to be related to the Rh system until 20 years later. Anti-Be^a has been implicated in several cases of severe HDFN [433,455–457]. Be^a is encoded by an *RHCE*ce* allele that produces slightly weakened c, e, and ce and contains 662C>G in exon 5 encoding Pro221Arg in or close to the extracellular vestibule [433].

5.14.2.4 Variant *RHCE*ce* alleles encoding epitopes of D

Several variant *RHCE*ce* alleles – *Ro^{Har}* (*RHCE*ceHAR*), *RHCE*ceCF*, and *RHCE*ceAR* – contain nucleotides in exon 5 characteristic of *RHD* and the encoded protein expresses some D epitopes. Exons 5 of *RHCE*ceRT* and *RHCE*ceSL* encoded amino acid substitutions that are not present in RhD, yet the proteins still express some D epitopes. All of these variants are described in Section 5.6.4.12.

Ro^{Har} (Figure 5.10) produces two low frequency antigens, Rh33 and FPTT. Two Rh:33 individuals, both Germans, appeared to be homozygous for R$_o^{Har}$ [191,458]. Their red cells did not react with about 50% of immune sera from D–– people (anti-Hr$_o$), suggesting they have a partial Hr$_o$ antigen [430]. R$_o^{Har}$ is probably less rare in Germany than elsewhere. Seven of 14 000 apparently D– German donors gave discrepant results with anti-D; all seven turned out to be Rh:33. None of 1060 English blood donors screened with anti-Rh33 was Rh:33 [191]. One of 42 600 donors tested in southern France was Rh:33 and appeared to have the R$_o^{Har}$ haplotype [188].

5.14.2.5 Other variant *RHCE*ce* alleles

A number of variant *RHCE*ce* alleles have been found in people of African origin that do not produce the high frequency antigens Hr or Hr^B (Section 5.9.5). People with these alleles in the homozygous or compound heterozygous state may produce antibodies to high frequency antigens following transfusion, which will make finding blood for future transfusion difficult. This is a particular problem in patients with sickle cell disease. In addition to *RHCE*ceVS* (*ce^s* and *(C)ce^s*, Section 5.13) and *RHCE*ceAR* (Section 5.6.4.9), these alleles include *RHCE*ceMO*, *RHCE*ceEK*, and *RHCE*ceBI* [338,341,423] (Table 5.10).

*RHCE*ceRA*, encoding Gly180Arg, produced profoundly weakened e and no hr^S [437]. Three multiply transfused African American e+ patients made alloanti-e [438]. Their abnormal allele, *RHCE*ceAG*, contained 254G>C in exon 2 encoding Ala85Gly in the third transmembrane domain and absence of the high frequency antigen CEAG (RH59).

5.14.3 Variant *RHCE*cE* alleles

Screening of over 140 000 Japanese blood donors with monoclonal anti-E revealed 15 (0.011%) with E variants [333]. Two of the variants, EKH and EKK (EII) are also associated with weak c (Section 5.9.4 and Table 5.10). The aberrant E of EKK appears to result from a hybrid gene with exons 1–3 derived from *RHD* and exons 4–10 from *RHCE*E* (or *RHCE–D(2,3)–CE*). This gene is linked to the opposite hybrid: exons 1–3 derived from *RHCE*c*, producing the weak c, and exons 4–10 from *RHD* (or *RHD–CE(2,3)–D*) [333].

Two Caucasian men had an *RHCE* allele (*RHCE*cE734C*) encoding Leu245Pro and produced altered c and suppressed E, not detected with standard reagents [459].

5.15 Haplotypes producing neither E nor e; D–– and related phenotypes

This section includes haplotypes that encode neither E nor e; some also produce neither C nor c. All encode D, which is usually exalted in expression. Most of these haplotypes were initially identified in individuals homozygous for the haplotype, who had produced antibodies to high frequency antigens collectively known as anti-Hr$_o$ or -Rh17. When the terms D––, Dc–, DC^w–, etc. are used to denote phenotypes, they usually represent homozygosity for the corresponding haplotype.

These haplotypes often consist of a complete or almost complete *RHD* paired with a hybrid gene containing a

substantial portion of *RHD* (Figure 5.10). This may provide an explanation for the enhanced D expression.

5.15.1 *D––*

The first propositus with the D–– phenotype, in which the red cells expressed D, but no C, c, E, or e, had consanguineous parents and was presumed to be homozygous for a very rare haplotype, *D––* [460,461]. Despite the rarity of this haplotype, many examples of the D–– phenotype have been found in many different ethnic groups. Almost all D–– propositi have been ascertained through the presence of anti-Hr_o in their serum (Section 5.15.6). Many propositi heterozygous for *D––* and a common Rh haplotype have been found, often being disclosed by apparent parentage exclusions [462]. Analysis of a large Canadian family revealed that two sisters with the D–– phenotype, and whose parents were not consanguineous, had the genotype *D––/–––* (heterozygous for *D––* and the Rh amorph gene, Section 5.16.1) [463].

The frequency of the *D––* haplotype was roughly estimated as 0.0005 in Sweden [464], 0.0047 in Iceland [465], and 0.005 in American Hispanics (determined by molecular typing) [439]. Seven of 692 000 (0.001%) Japanese donors had the D–– phenotype, a frequency of 0.0032 for the *D––* haplotype [466].

D–– produces only three Rh antigens: D, G, and Rh29. Red cells of individuals homozygous or heterozygous for *D––* express more D antigen than normal D+ cells and are directly agglutinated by some incomplete (non-agglutinating) anti-D. Most incomplete polyclonal anti-D will directly agglutinate cells of *D– –* homozygotes, but only carefully selected antibodies will distinguish cells of *D––* heterozygotes from DcE/DcE cells. This elevated expression of D is attributed to an increased number of D sites per red cell (Table 5.7).

The molecular genetic background of the D–– phenotype is heterogeneous. Homozygosity for *RHCE–D–CE* hybrid genes appears to account for several examples of D–– (Figure 5.10). In D–– individuals from Iceland, Britain, and Italy, only exons 1 and 10 were *RHCE*-derived [467,468]. Studies on two D–– members of an Italian family (LM) suggested that two hybrid genes were present: *RHCE–D(2–8)–CE–D(10)* and *RHD–CE(10)* [467,469,470]. Transcript analyses on a family (JD) whose propositus was heterozygous for *D––* and *D··* (Section 5.15.2) revealed *RHD* linked to *RHCE–D(*2 or 3–6*)–CE* [448]. Production of D epitopes from both genes of the haplotypes might account for the exalted expression of D.

Homozygosity for a single nucleotide deletion (907delC) in an *RHCE*cE* allele was responsible for D–– in three American Hispanics and heterozygosity for the same haplotype was found in patients and donors from the same ethnic group who were E– yet were predicted to be E+ by molecular testing [439]. One of 100 Hispanic donors had *RHCE*cE(907delC)*.

Two members of a Chinese family appeared to be *D––/Dc–* (Section 5.15.3) [440]. They were heterozygous for *RHD–RHCE(10)* and *RHCE–RHD(4–10)*, with the *RHCE*c* sequence, but neither of these hybrid genes was linked to *RHD* or *RHCE* (Figure 5.10) and the red cells did not have exalted D.

A thorough molecular analysis on a Japanese family (JF) with a D–– propositus, whose parents were not consanguineous, suggested that she was heterozygous for two abnormal Rh haplotypes [471]. In one, inherited from the father, there is *RHCE–D(2 or 3–7)–CE*, but no normal *RHD* (Figure 5.10). The other comprises *RHD*, but no *RHCE*. *D1S80* is a gene marker on chromosome 1, telomeric to the *RH* loci. As the propositus has received no *D1S80* allele from her mother, Okuda *et al.* [471] propose that a region of chromosome 1, containing *RHCE* and *D1S80*, was deleted during maternal gametogenesis.

An *RHCE*cE* allele (*RHCE*cEMI*) in a D+ C– c+ E– e+ black individual contained a 9-nucleotide deletion in exon 3 and appeared to produce no Rh polypeptide at the red cell surface [341]. There is no evidence that it was linked to *RHD*.

An unusual form of *D––* produced Tar (RH40) [235], a low frequency antigen usually associated with DVII (Section 5.6.4.6). The D antigen produced by this *D––* haplotype was stronger than that usually associated with Tar, but weaker than normal D.

Like Rh_{null} cells, D–– cells have a substantial reduction in CD47 content (Section 5.5.7 and see Table 5.13), but only slight reduction in RhAG [83].

5.15.2 *D··* and Evans (RH37)

Similarity between *D––* and the Rh haplotype producing the low frequency antigen Evans, recognised during studies on the first two Evans+ propositi, led to the Evans haplotype being denoted *D··* [472]. Inheritance of the haplotype was straightforward. Testing of apparent D–– red cell samples with anti-Evans led to discovery of the first person homozygous for *D··* (HD) [473] and positive reactions of apparent D–– red cells with immune sera from D–– individuals disclosed a second *D··* homozygote [474]. Families with *D··* have been mostly white British.

The antigens produced by *D··* are D, G, Evans, and the high frequency antigens Rh29 and Dav (RH47) [430], although over three decades after the first report of D··

weak e antigen was detected in one case (JD, see below) [475]. The D antigen is elevated, but less so than that produced by D–– [473]. The number of D antigen sites per red cell were estimated to be 56 000 for *D··/D··*, compared with 110 000–202 000 for *D––/D––* and 21 000 for *DcE/DcE* [473].

Molecular analyses on a Scottish family (AT) with five Evans+ members in three generations demonstrated that *D··* comprised *RHCE–D–CE*, with exons 2 (or 3) to 6 derived from *RHCE*, linked to *RHD* [476] (Figure 5.10). A very similar haplotype, but with exon 1 and the 5′ untranslated region of the hybrid gene also derived from *RHD*, was present in a *D··* homozygote (HD) [470] (though a different result was obtained on the same individual in another study [467]). In another family study, in which the propositus (JD) was heterozygous for *D··* and *D––*, the haplotype producing Evans comprised *RHCE–D*, with only exon 1 derived from *RHCE*, plus *RHD–CE*, with the 3′ end of exon 6 (encoding Cys311) and exons 7–10 derived from *RHCE* [448]. In an Evans+ African American, *RHD(1-6)–CE* appeared to be linked to *RHCE*ceMO* (Section 5.14.2.5) [475].

The amino acid sequence encoded by the junction of the 5′ end of *RHD* exon 6 and the 3′ end of *RHCE* exon 7 creates a unique amino acid sequence in the fifth cytoplasmic domain of the protein (Table 5.11). Conformational changes resulting from this sequence are probably responsible for Evans expression. *RHD*DIVb* also has this exon 6–7 junction and also encodes Evans (Section 5.6.4.3 and Figure 5.8) [221].

5.15.3 *Dc–*

The first homozygous *Dc–* propositus was found in an inbred white American family of French extraction [477]. His parents were double first cousins and two of his four siblings were also *Dc–/Dc–*. Other propositi with the Dc– phenotype have been Japanese [478], French [479], Argentinean [480,481], and Chinese (*D––/Dc–*) [440]. All Dc– propositi were ascertained through the presence anti-Hr_o in their serum.

Dc– produces D, G, c, Rh29, and, sometimes, ce. The strength of the D antigen is usually elevated and that of c is depressed; the c antigen may also differ qualitatively from normal c [477].

Transcript analysis on the French *Dc–* homozygote revealed *RHD* plus *RHCE–D(4–9)–CE* [482] (Figure 5.10). Exon 2 encoded Pro103, explaining the c expression. Other *Dc–* haplotypes contain *RHCE(1–3)–D(4–10)* [440] and *RHCE–D(5–7/8)–CE* [481].

Some haplotypes dubbed *Dc–* should, more accurately, be called Dc(e) or Dc((e)), as e can be detected by adsorption/elution tests or even by direct testing [483,484]. These haplotypes, mostly found in black people, have normal c and either normal or only slightly enhanced D.

5.15.4 *DC^w–*

A propositus identified as homozygous for *DC^w–* was a member of a large Canadian family [485]. Her parents were second cousins and four of her eight siblings were also *DC^w–/DC^w–*. *DC^w–* produces D, G, C^w, and Rh29; the D antigen is elevated in strength [485] and the C^w is depressed [486]. Unlike other haplotypes producing C^w, *DC^w–* makes neither C nor c. One other apparent *DC^w–* homozygote has been reported [449].

The original *DC^w–/DC^w–* propositus had transcripts representing *RHD* and *RHCE–D(2 or 3–9)–CE* [482] (Figure 5.10). The second propositus had *RHCE(1)–D*

Table 5.11 Amino acid sequences Leu303 to Cys316 encoded by the 3′ end of exon 6 and the 5′ end of exon 7 of *RHD*, *RHCE*, and two hybrid genes encoding Evans. The important residues in Evans expression appear to be Val/Ile306 and Gly/Val314, but not Tyr/Cys310.

Gene	Exon 6													Exon 7		
RHD					306					310				314		
	...	L	I	S	V	G	G	A	K	Y	L	P		G	C	C ...
RHCE	...	–	–	–	I	–	–	–	–	C	–	–		V	–	– ...
RHD-CE Evans (AT & HD)	...	–	–	–	–	–	–	–	–	–	–	–		V	–	– ...
RHD-CE Evans (JD)	...	–	–	–	–	–	–	–	–	C	–	–		V	–	– ...

–, identical residue to that encoded by *RHD*.

linked (or possibly *in trans*) with *RHD–CE(10)* [449] (Figure 5.10). In both individuals, the *RHCE*-derived exon 1 encoded Arg41, characteristic of C^w (Section 5.12).

5.15.5 *DIV(C)*– and Riv (RH45)

Homozygosity for a haplotype denoted *DIV(C)*– was proposed to explain the reactions of red cells of a woman from the Ivory Coast, whose third child had fatal HDFN [487]. Her two children had the same weak expression of C as their mother. The following antigens are produced by *DIV(C)*–: partial D giving the reactions of a strong DIV antigen; apparently normal G; very depressed C; Go^a (a low frequency antigen always associated with DIVa, Section 5.6.4.3); three other low frequency antigens, Rh33, Riv, and FPTT [188,488]; and three high frequency antigens, Rh29, Nou (RH44), and Dav (RH47) [430,489,490].

No other *DIV(C)*– homozygote has been found, but the association with low frequency antigens has led to the identification of three propositi heterozygous for *DIV(C)*–, all of African ancestry [488]. Transcript and DNA analysis on three heterozygotes revealed that the *DIV(C)*– haplotype comprises *RHD*DIVa.2* linked to *RHCE–DIVa.2(2,3)–CE–D(5)–CE* (Figure 5.10) [491]. Exon 2 of the hybrid gene, derived from *RHD*DIVa.2* is probably responsible for the weak C, the junction of exon 3 from *RHD*DIVa.2* and exon 4 from *RHCE* for Riv, the junction of exon 4 from *RHCE* and exon 5 from *RHD* (or *RHD*DIVa.*2) for FPTT, and the junction of exon 5 from *RHD* and exon 6 from *RHCE* for Rh33 [491].

5.15.6 Anti-Hr_o (-RH17) and related antibodies

People with D–– and related phenotypes, who have been exposed to red cells of common phenotype by transfusion or pregnancy, usually have antibodies to high frequency Rh antigens.

Red cells and immune sera of D––, Dc–, and DC^w– people are mutually compatible, showing that there is no anti-C^w or -c component. All such sera are non-reactive with Rh_{null} cells. In tests with red cells of common Rh phenotypes, these sera appear to contain an antibody to a single high frequency determinant, Hr_o (RH17). In some of the sera, separable anti-e has also been identified [430,461,492].

Heterogeneity within anti-Hr_o specificity is demonstrated by the variable reactions of different anti-Hr_o with red cells of phenotypes representing homozygosity for certain rare Rh haplotypes, *DIV(C)*–, *D··*, r^G, R^{oHar}, and also with Rh_{mod} cells [430,451]. Adsorption and elution tests showed that those D–– sera reactive with DIV(C)– cells contained at least two antibodies to high frequency antigens, one that reacted with the DIV(C)– cells, named anti-Nou (anti-RH44), and one that did not [489,490]. Similar tests with D·· cells revealed an antibody, named anti-Dav (anti-RH47), in some D–– sera [430]. Anti-Dav reacted with cells of common Rh phenotype and with D·· cells. Other antibodies to high frequency antigens, non-reactive with Rh_{null} and D–– cells, have been identified in individuals with unusual Rh phenotypes, such as those homozygous for R^N (anti-Rh46), or for genes producing partial e antigens (anti-Hr, -Hr^B) [335,442]. As polyclonal anti-D represents a mixture of antibodies directed at numerous epitopes on different regions of the RhD protein (Section 5.6.3), so anti-Hr_o represents antibodies to epitopes on different regions of the RhCE protein. D–– cells lack the whole Hr_o mosaic and, when immunised, D–– people can make antibodies to different parts of the mosaic, collectively referred to as anti-Hr_o and including anti-Nou, -Dav, -Hr, and -Hr^B.

Anti-Hr_o in the sera of D––, Dc–, DC^w–, and DIV(C)– mothers has been responsible for severe, and often fatal, HDFN [461,477,485,487,493,494] (reviewed in [493,494]).

Monoclonal antibodies that behave as anti-Hr_o were produced in a mouse immunised with human red cells [444] and in a crab-eating macaque immunised with gorilla and human red cells [495]. These antibodies detect non-polymorphic epitopes on the RhCcEe protein, but not on RhD.

5.16 Rh-deficiency phenotypes: Rh_{null} and Rh_{mod}

The Rh_{null} phenotype, in which no Rh antigens can be detected on the red cells, was first described by Vos *et al.* [496] in 1961. Rh_{null} is very rare, as reflected by the high consanguinity rate among parents of Rh_{null} propositi. Two types of Rh_{null}, with an identical Rh phenotype, are distinguished on the basis of their inheritance and molecular genetics.

1 The amorph type, with apparent homozygosity for silent genes at *RHD* and *RHCE* loci, results from inactivating mutations in *RHCE* and a deletion of *RHD.*

2 The regulator type, in which the Rh genes are normal, but there is homozygosity (or compound heterozygosity) for inactivating mutations in *RHAG*, the gene encoding the Rh-associated glycoprotein (RhAG), without which Rh antigens are not expressed (Section 5.5.6).

Some mutations in *RHAG* give rise to low level expression of Rh antigens, a phenotype called Rh_{mod}.

5.16.1 Rh_{null} of the amorph type

The amorph type of Rh_{null} is extremely rare, with only five propositi reported: Japanese [497,498], German [499–501], Norwegian Lapp, Spanish [500,502] and Caucasian Brazilian [503]. Family analyses suggest that their unusual phenotype resulted from homozygosity for silent or amorph Rh genes and the symbol −−−/−−− has been used for the genotype. The parents and children of amorph Rh_{null} individuals are obligate heterozygotes for the amorph gene and, consequently, always appear, from serological results, to be homozygous for Rh.

Titration of anti-c and -e with red cells of 1803 German donors revealed four apparent heterozygotes for the amorph haplotype, verified by family studies [499]. A frequency of 0.0001–0.0002 was estimated for the amorph haplotype in Sweden [464].

The pertinent mutations have been resolved for three of the amorph Rh_{null} individuals (Table 5.12). In each there is no *RHD*, but homozygosity for a grossly intact *RHCE* gene containing an inactivating mutation. In three propositi, single nucleotide (exon 7) [503], dinucleotide

Table 5.12 Some mutations associated with Rh-deficiency phenotypes.

Rh_{null} amorph				
Name	Population	Mutation		References
DR	German	*RHCE*Ce*	TCA→C, frameshift after Ile322	[499,501]
	Japanese	*RHCE*ce*	Deletion TCTTC, frameshift after Leu26	[497,498]
DAA	Spanish	*RHCE*ce*	Intron 4 5′ splice site	[500,502]
	Brazilian	*RHCE*ce*	GGGG→GGG, frameshift after Gly321	[503]
Rh_{null} regulator				
Name	Population	Mutation		References
SF, JL	White South African	*RHAG*	CCTC→GA, frameshift after Tyr51	[83]
TB	Swiss	*RHAG*	Heterozygous A deletion, frameshift after Ala362 + no detectable transcript	[83]
HT	Japanese	*RHAG*	Val270Ile, Gly280Arg	[504]
WO	Japanese	*RHAG*	Gly380Val (partial exon 9 skipping)	[504]
YT	Australian	*RHAG*	Heterozygous Gly279Glu + intron 1 5′ splice site	[505–507]
TT	Japanese	*RHAG*	Intron 7 5′ splice site, frameshift after Thr315	[79]
AL	White American	*RHAG*	Intron 1 5′ splice site	[83,508]
AC	Spanish, Japanese	*RHAG*	Intron 6 3′ splice site, frameshift after Thr315	[508,509]
	Chinese	*RHAG*	Ser224Arg	[510]
KS		*RHAG*	Gly178Arg	[511]
Rh_{mod}				
Name	Population	Mutation		References
SM	Russian Jewish	*RHAG*	Met1Ile	[512]
VL	White American	*RHAG*	Ser79Asn	[83,513]
CB	French	*RHAG*	Heterozygous Asp399Tyr + ?	[514]
		RHAG	Heterozygous: Gly90Val; Gly187Asp	[515]
JRM	Japanese	*RHAG*	Ser227Leu	[76]
	Japanese	*RHAG*	AAC→AC, frameshift after Asn395	[516]

(exon 7) [500,501], and pentanucleotide (exon 1) [500] deletions cause shifts in the reading frame and premature termination of translation. If translated, the gene with the exon 1 mutation would produce a protein of only 31 amino acids. The genes with the exon 7 mutations would give rise to a 357- or 398-residue protein (compared with 417 residues in the normal protein), with completely changed C-terminal amino acid sequences, which, if produced, would be abnormally folded and unlikely to be inserted in the membrane. Alternatively, the protein might be inserted in the membrane, but not accessible to antibodies. The Spanish propositus had a mutation in the intron 4 donor splice site of *RHCE*, giving rise to aberrant transcripts [500].

5.16.2 Rh_{null} of the regulator type

The first Rh_{null} propositus, an Australian aboriginal woman, was found during an anthropological survey [496,518]. No close relative was available for testing. The second Rh_{null} propositus was a member of a large family [519]. The Rh groups showed that her rare phenotype resulted from inhibition of her Rh genes: her husband was dce/dce, yet their daughter was DCe/dce and must have received D and C from her Rh_{null} mother. Race and Sanger coined the term 'regulator' for this type of Rh_{null}. The regulator type of Rh_{null} can be recognised when a parent or child of the Rh_{null} propositus has both C and c, and/or both E and e. Family studies showed that the regulator locus is not part of the Rh locus.

Although Rh_{null} remains very rare, many propositi with the regulator type have been found in people of white European or Eastern Asian origin. No Rh_{null} of black African origin has been reported.

In 1996, Chérif-Zahar *et al.* [83] showed that Rh_{null} of the regulator type was associated with inactivating mutations in *RHAG*, making *RHAG* a prime candidate for the regulator locus. Other *RHAG* inactivating mutations in Rh_{null} individuals have confirmed the association (Table 5.12). Some Rh_{null} individuals are homozygous, others doubly heterozygous, for *RHCE*-inactivating mutations. In all cases except one, normal *RHD* and *RHCE* were present, the exception having no *RHD* [514]. As with the Rh proteins, it is possible that the predicted proteins, if translated, are not transported to the membrane or cannot be inserted into the membrane.

Some cases of Rh_{null} result from single or double missense mutations in *RHAG* (Table 5.12). The encoded amino acid substitutions are predicted to be in the seventh (Ser224Arg), ninth (Val270 Ile, Gly279Glu, and Gly280Val), and twelfth (Gly380Val) membrane-spanning domains. Why these apparently minor changes to the protein prevent expression of the Rh antigens is unclear. The missense mutation encoding Gly380Val is in the first nucleotide of exon 9 and also causes partial splicing of exon 9 [504].

In some families, heterozygosity for the regulator allele resulted in weakened expression of some Rh antigens [518–524].

5.16.3 Rh_{mod}

A phenotype associated with modified expression of antigens produced by both Rh haplotypes was called Rh_{mod} by Chown *et al.* [513]. Red cells with this phenotype would easily be mistaken for Rh_{null} if only limited testing were done. The rare phenotype was attributed to homozygosity for a modifier gene at a locus separate from Rh [513], subsequently shown by molecular analyses to be *RHAG* (Table 5.12) [83,514]. Parents of most Rh_{mod} propositi are consanguineous.

Rh_{mod} is a heterogeneous phenotype. Apart from G, the Rh antigens of the first propositus were only detected by adsorption/elution tests with selected sera [513]. The Rh antigens of the second propositus were much stronger and C, c, and G could be detected by direct testing [451,525]. The third propositus was originally called Rh_{null}, although D was revealed by adsorption and elution, the only Rh antigen to be detected [526]. Rh_{mod} cells are most easily distinguished from Rh_{null} by immune sera from some people with D–– and related phenotypes (anti-Hr_o) and by some anti-Rh29 [451]. Relatives of Rh_{mod} individuals, heterozygous for the modifier gene, may have reduced expression of Rh antigens [76,451,516,527]. Rh_{mod} cells of one Japanese individual were positive for Ol^a (RHAG1), an antigen of very low frequency also found in members of one Norwegian family with reduced expression of some Rh antigens and heterozygosity for the same mutation [76] (Section 5.20).

Five Rh_{mod} propositi have missense mutations in *RHAG*; three are homozygous [76,83,512], one doubly heterozygous [515], the other heterozygous with no mutation in the *trans* gene detected [514]. One mutation converts the translation-initiating methionine codon to isoleucine. It is probable that the small quantity of RhAG produced is translated from the ATG triplet that normally encodes Met8 [512]. Other amino acid substitutions are located on RhAG in the third membrane-spanning domain (Ser79Asn, Gly90Val) [83,515], the third cytoplasmic loop (Gly187Asp) [515], the extracellular vestibule area around the fourth external domain (Ser227Leu) [76], and the carboxy-terminal tail (Asp399Tyr) [514].

One Japanese Rh_{mod} was homozygous for a single nucleotide deletion in exon 9 of *RHAG* resulting in a new stop codon at positions 1384–1386 and a protein of 461 amino acids instead of the usual 409 [516]. All propositi had low quantities of RhAG in their red cell membranes.

5.16.4 Antibodies in the sera of Rh_{null} people

Anti-Rh29 (anti-'total Rh'), an antibody found in the serum of some immunised Rh_{null} individuals, reacts with red cells of all Rh phenotypes apart from Rh_{null} [499,521,524,528–530]. Anti-Rh29 has been made by people with Rh_{null} of both types, though not all Rh_{null} individuals make anti-Rh29 when immunised; two Rh_{null} sisters had no Rh antibody although they had a total of nine children. Other immunised Rh_{null} individuals are reported to have made anti-e [519] or -Hr_o [502]. Antibodies in the sera of two Rh_{null} donors who had received no known immunising stimulus behaved as anti-Hr_o, reacting with all red cell samples save those of Rh_{null} and D–– phenotypes [530,531].

Anti-Rh29 has been responsible for HDFN, managed successfully by repeated exchange transfusions with red cells of common Rh phenotypes [529,532] or with Rh_{null} red cells from family members [533]. Anti-Hr_o in an Rh_{null} woman was also responsible for HDFN [502]. An HTR caused by anti-Rh29 may have contributed to the death of an elderly Rh_{null} patient transfused with D– blood of common phenotype [534].

Many human autoantibodies are considered Rh-related because they do not react with Rh_{null} cells. Weiner and Vos [535] called these antibodies anti-pdl (anti-partially deleted), to distinguish them from anti-dl (anti-deleted), which react with all red cells, and anti-nl (anti-normal), which react with all red cells except for Rh_{null} and D–– cells.

5.16.5 Other antigens affected in Rh deficiency phenotypes

Affects of the Rh deficiency phenotypes extend beyond the Rh blood group system as a result of the complete or partial deficiency of the Rh proteins and RhAG from the band 3/Rh macrocomplex and possibly from the junctional complex (Section 5.5.7). Several red cell antigens are lacking, or at least show reduced expression, on Rh_{null} and Rh_{mod} red cells.

RhAG is not detected on Rh_{null} red cells of the regulator type and is expressed in reduced quantity on Rh_{mod} cells (Table 5.13). Indeed, as described above, it is the absence or altered conformation of RhAG that is primarily responsible for these phenotypes. RhAG is present on Rh_{null} cells of the amorph type, but in reduced quantity [83]. Duclos and DSLK antigen, located on RhAG [76] (Section 5.20), are also absent from most Rh_{null} cells.

Table 5.13 Estimated numbers of RhAG and CD47 molecules on Rh-deficiency and D–– cells [83,500,508].

	Number of molecules per cell (range)	
Phenotype	RhAG	CD47
Rh_{null} regulator	0	2900–3900
Rh_{null} amorph	44 000–58 000	2000–3000
Rh_{mod}	43 000	6600
D––	180 000–206 000	8000–12 000
D+	220 000–280 000	35 000–50 000

The high frequency antigens of the LW system, LW^a and LW^{ab}, are absent from all Rh_{null} cells and weakly expressed on Rh_{mod} cells (Chapter 16). Anti-Fy5 of the Duffy system behaves like anti-Fy3, except that it does not react with Rh-deficiency cells, none of which are Fy:–3 (Chapter 8). Rh-deficiency cells have reduced expression of glycophorin B and are often U– (Section 3.20). CD47, a glycoprotein on red cells with no blood group activity, is also present in reduced quantity on Rh-deficiency cells (Table 5.13).

Glycophorin A antigens, M, N, and En^a, are reported to be slightly enhanced in the regulator type of Rh_{null} (amorph type not mentioned) [536]. Elevation of i antigen on Rh-deficiency phenotype cells probably results from bone marrow stress caused by the associated anaemia [537]. Antibodies that reacted with Rh_{null} cells and with cells of other 'null' phenotypes, but not with cells of 'normal' phenotypes unless they had been papain-treated, were found in three patients with anaemia [538,539].

Knockout mice for the Rh gene (*Rhd*) had a complete loss of ICAM-4 (LW), but only moderate reduction in RhAG; *Rhag* knockout mice had a complete loss of Rh and ICAM-4, but no effect on CD47 [85].

5.16.6 Rh-deficiency syndrome

Rh_{null} red cells are morphologically and functionally abnormal. Most Rh_{null} and Rh_{mod} individuals have some degree of haemolytic anaemia, the severity of which varies from severe enough to merit splenectomy to a fully

compensated state requiring sophisticated tests to demonstrate shortened red cell survival. Typical symptoms of Rh-deficiency syndrome are the presence of stomatocytes (cup-shaped red cells) and some spherocytes, reduced survival of autologous red cells, increased red cell osmotic fragility, increased reticulocyte counts, increased fetal haemoglobin, enhanced i antigen strength, and reduced haemoglobin and haptoglobin levels (reviews in [514,540]).

Rh_{null} red cells have an abnormal organization of their membrane phospholipids [541], increased cation permeability, partially compensated by an increase in the number of K^+Na^+ pumps [542], reduced cation and water contents, and reduced membrane cholesterol content [528]. It is not known whether any of these defects accounts directly for the autohaemolyis in Rh-deficiency syndrome, but the haemolytic anaemia is alleviated by splenectomy, so whatever the ultimate cause of stomatocytosis in Rh-deficiency syndrome, it is the early sequestration of these abnormally shaped cells that is responsible for the anaemia [543]. Binding of CD47 on red cells to SIRPα on macrophages generates a negative signal that protects against phagocytosis of the red cells (Section 5.5.7), so reduced CD47 levels on Rh_{null} cells could be involved in their elimination, although enhanced phagocytosis of Rh_{null} red cells was not detected by a monocyte monolayer assay [544].

5.17 Low frequency Rh antigens and the antibodies that define them

5.17.1 Low frequency antigens

Twenty-four antigens of low frequency belong to the Rh system (Table 5.14). Presence of these antigens is associated with abnormal (usually partial or depressed) expression of one or more of the DCcEe antigens.

Table 5.14 Antibodies to low frequency Rh antigens.

Antigen		Red cell immune	'Naturally occurring'	Other antibodies present	HDFN	No. of examples	References
C^w	RH8	Yes	Yes	Yes	Yes	Many	[390,391,397,399–402]
C^x	RH9	Yes	Yes	Yes	Mild	Many	[404,405,407]
V	RH10	Yes	No	Yes	No	Many	[410,414,416]
E^w	RH11	Yes	No	No	Yes	Several	[329,330]
VS	RH20		Yes	Yes	No	Many	[411,414]
D^w	RH23		Yes	Yes*	No	Several	[223,545]
Go^a	RH30	Yes	Yes	Yes*	Yes	Several	[219,546]
Rh32	RH32	Yes	Yes	Yes*	Yes	Several	[443,547]
Rh33	RH33		Yes	Yes	No	Few	[191,548]
Rh35	RH35		Yes	Yes	No	1	[441]
Be^a	RH36	Yes	No	No	Yes	Several	[432,433,455–457]
Evans	RH37	Yes	Yes	Yes*	Yes	Several	[472]
Tar	RH40	Yes	Yes	Yes	Yes	Few	[235,236]
Rh42	RH42	Yes	No	No	Mild	2	[412]
Riv	RH45	Yes	No	Yes	Mild	1	[488]
JAL	RH48	Yes	Yes	Yes	Mild	Few	[426]
STEM	RH49	Yes	No	Yes	Mild	Several	[350]
FPTT	RH50		Yes	Yes	No	1	[188]
BARC	RH52			Yes	No	1	[227]
JAHK	RH53		Yes	Yes	No	Several	[377]
DAK	RH54	Yes		Yes	No	Several	[160]
LOCR	RH55	Yes		No	Mild	Several	[454]
CENR	RH56		Yes	Yes	No	Several	[431]
PARG	RH60	Yes		Yes	No	1	[701]

*Not always separable from other Rh specificities, see text.

Consequently, the low frequency antigens make useful markers for rare haplotypes. Although an oversimplification, as reference to the appropriate sections of this chapter reveals, the low frequency antigens can be loosely classified as follows:

- D^w, Go^a, Rh32, Rh33, Evans, Tar, FPTT, BARC, DAK – associated with partial D (Section 5.6.4);
- C^w, C^x – associated with abnormal C (Section 5.12);
- E^w – associated with abnormal E (Section 5.9.4);
- V, VS, Rh42, STEM – associated with abnormal e (Sections 5.9.5, 5.13);
- Rh32, Rh35, JAL, FPTT, DAK – associated with abnormal *DCe* (Section 5.14.1);
- CENR – associated with abnormal *DCC^{w}e* (Section 5.14.1.4);
- Rh33, JAL – associated with abnormal *Dce* (Section 5.14.2);
- Be^a, LOCR – associated with abnormal *dce* (Sections 5.14.2.3, 5.14.2.2);
- JAHK – associated with abnormal *dCe* (Section 5.11);
- Evans – associated with abnormal *D*–– (Section 5.15.2);
- Go^a, Rh33, Riv, FPTT – associated with *DIV(C)*– (Section 5.15.5).

It is not safe to assume that an antigen belongs to the Rh system simply because it is associated with abnormal expression of Rh antigens. The Ol(a+) members of a large family had weak expression of some Rh antigens while the Rh antigens of the Ol(a–) members were normal, yet the family showed that Ol^a is not inherited at the Rh locus [549] and Ol^a was subsequently shown to be encoded by *RHAG* [76] (Section 5.20.2). The low frequency antigen HOFM (700050) is associated with depressed C antigen in the only family in which it has been detected, but there is insufficient evidence for its elevation to the Rh system [550].

Most of the antigens listed in Table 5.14 are of low incidence in all populations tested, but the frequencies of some vary in different populations. C^w and VS would not be considered private antigens in white and black populations, respectively.

5.17.2 Speculation on the molecular basis of Rh32 and FPTT

The molecular backgrounds to some of the Rh low frequency antigens can be anticipated from the changes to the *RHD* and *RHCE* genes associated with their expression. These are described in other sections of this Chapter. Two antigens, Rh32 and FPTT, are each associated with two serologically very different phenotypes and these will be described in more detail here.

Rh32 is associated with the partial D antigen DBT, usually together with C and e (Section 5.6.4.8). R^N produces Rh32 together with slightly elevated D, weak C, and weak e (Section 5.14.1.1). DBT arises from an *RHD-CE-D* gene in which exons 5–7 (or 5–9) are *RHCE*-derived (Figure 5.11). R^N consists of *RHD* and *RHCE–D(4)–CE*. From comparison of the abnormal hybrid genes in the *DBT* and in the R^N haplotypes it appears that Rh32 could result from the conformation of an Rh polypeptide with the product of *RHD* exon 4 fused to the product of *RHCE* exon 5. Rh32 does not, however, simply arise from a unique linear amino acid sequence resulting from the gene rearrangements. A sequence of 21 amino acids spanning the junction of the products of exons 4 of *RHD* and exon 5 of *RHCE* is identical in the polypeptides encoded by the two genes. Rh32 probably results from interactions between *RHD*- and *RHCE*-specified amino acids within the extracellular vestibule: Met169, Met170, Ile172 from *RHD* and Gln233 from *RHCE* (Figure 5.11).

FPTT is associated with the rare R^{oHar} gene, which also produces a partial D antigen (DHAR), c, very weak e, and another low frequency antigen, Rh33 (Section 5.6.4.12). FPTT is also associated with DFR, another partial D antigen (Section 5.6.4.7). R^{oHar} is an *RHCE–D(5)–CE* gene, whereas DFR is produced by *RHD–CE–D* in which part of exon 4 is *RHCE*-derived. FPTT appears to be the opposite to Rh32, resulting from interactions within the extracellular vestibule: Leu169, Arg170, and Phe172 from exon 4 of *RHCE* and Glu233 from exon 5 of *RHD* (Figure 5.11).

5.17.3 Antibodies to low frequency antigens

Table 5.14 summarises published and unpublished information available to the author; other examples of these antibodies may well exist. Some antibodies have caused severe HDFN, but where antibodies are shown to have caused mild HDFN, in a few cases this may only represent a positive DAT on the baby's red cells. Anti-Go^a is implicated in a delayed HTR [551].

Although some specificities, such as anti-Go^a and anti-Rh32, are found as the sole antibody in some sera and may have caused HDFN, in other sera they occur together and are 'naturally occurring'. When these two specificities are found together they are generally not separable by adsorption. To take a specific example, the Tillett serum contained antibodies to many low incidence antigens, although Mrs Tillett had not been exposed to these antigens. Anti-Go^a, -Evans, and, sometimes, anti-Rh32 were present in Tillett serum, but those specificities could not

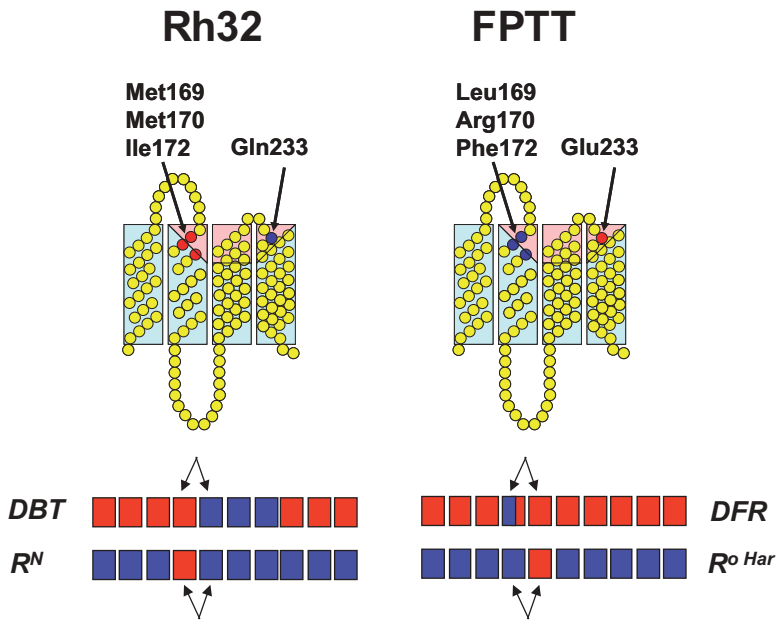


Figure 5.11 Molecular bases of the low frequency Rh antigens Rh32 and FPTT. Both low frequency antigens probably result from the interactions within the extracellular vestibule region involving amino acids encoded by *RHD* exon 4 and *RHCE* exon 5 for Rh32 and by *RHCE* exon 4 and *RHD* exon 5 for FPTT. Two-dimensional models represent part of the Rh proteins, including the extracellular vestibule region in pink.

be isolated from each other by adsorption and elution: adsorption with Rh:32 Go(a–) Evans– cells removed activity against Go(a+) and Evans+ cells, as well as against Rh:32 cells. Adsorption with Rh:32 cells of a later batch of Tillett serum, which no longer contained anti-Rh32, did not affect the strength of the anti-Go^a and -Evans, but adsorption with either Evans+ or Go(a+) cells removed all activity for those cells [157]. Presumably conformational similarities exist between Rh32, Evans, and Go^a determinants and some antibodies detect a common feature of these determinants whereas others can distinguish between them.

Some anti-D^w are specific for the product of *RHD*DVa*, in which the presence of Gln233 in an RhD protein appears to be the key factor (Section 5.6.4.4). Most anti-D^w, however, also react with Rh:32 cells, some reacting only weakly with the Rh:32 cells and some reacting as strongly with Rh:32 cells as with D^w+ cells [552]. Comparison of *RHD*DVa* genes, which produce D^w, with *RHD*DBT* and R^N genes, which produce Rh32 (Figures 5.8 and 5.10), reveals that all involve exon 5 encoding Gln233 (from *RHCE*) adjacent to exon 4 derived from *RHD*.

5.18 Rh antibodies

Most details on the specificities of Rh antibodies are to be found in the preceding sections, where the antigens they define are described. Provided here are more general comments on Rh antibodies, polyclonal and monoclonal, and on their clinical significance.

Rh antibodies are usually produced in response to red cell immunisation resulting from blood transfusion or pregnancy, although 'naturally occurring' Rh antibodies are occasionally encountered. Rh antibodies generally react optimally at 37°C, their reactivity being enhanced by protease treatment of the cells. Most Rh antibodies, in the absence of an enhancing medium, do not directly agglutinate untreated antigen-positive red cells.

Most Rh antibodies should be considered potential agents of HDFN and acute or delayed HTRs.

5.18.1 Anti-D

5.18.1.1 Alloanti-D

Anti-D are mostly IgG, but some sera contain an IgM component. Sera containing IgM anti-D usually

agglutinate saline suspended D+ cells, as do some sera containing relatively high concentrations of IgG anti-D alone. Most anti-D from hyperimmunised subjects also contain an IgA component [112]. IgG1 and IgG3 are the predominant anti-D subclasses; IgG1 is nearly always present and, in individuals who have received multiple immunisations, both subclasses are generally detected. IgG2 and IgG4 anti-D are also found occasionally [112,553,554]. 'Naturally occurring' IgG anti-D in the sera of untransfused men have been described [555,556], but these are rare.

The failure of almost all anti-D to activate complement is attributed to the distance between antigen sites, which prevents the collaboration between IgG molecules required for C1q binding [112]. D+ red cells that had been very heavily coated with anti-D bound up to 1600 molecules of C1q, yet the classical complement pathway was not activated [557]. One well-investigated anti-C+D (Ripley), however, would haemolyse D+ cells through the activation of complement [558,559] and a complement binding anti-D was found in the serum of a woman with a weak partial D [560].

Human anti-D was produced in mice with severe combined immunodeficiency (SCID) that had been reconstituted with peripheral blood mononuclear cells obtained from D– people who had recently been sensitised with D+ red cells [561]. When mononuclear cells were used from donors many years after sensitisation, no anti-D was produced in the mice even though anti-D was still present in the donor's plasma. It appears that long-lived or memory cells are not present in the peripheral blood of individuals who have not been recently boosted.

5.18.1.2 Monoclonal anti-D

The development of the mouse hybridoma technique in the late 1970s and 1980s led to the production of murine monoclonal antibodies to a host of blood group antigens, yet mouse monoclonal anti-D has not been made. Failure to make anti-D in mice stimulated attempts to produce human monoclonal antibodies, primarily to replace polyclonal antibodies as grouping reagents and for prophylactic use. The hybridoma technique of Köhler and Milstein was not very successful when applied to humans, but an alternative approach, that of immortalising human B lymphocytes by *in vitro* transformation with Epstein-Barr virus (EBV), did bring success.

Crawford *et al.* [562] were the first to clone EBV-transformed lymphoblastoid cells to produce a stable cell line secreting anti-D. Cell lines produced in this way are often unstable and difficult to grow in large-scale culture, so to overcome these problems EBV-transformed lymphoblastoid cells are usually fused with myeloma cells to produce a hybridoma [563]. CD40 activation of lymphocytes can been used to replace EBV transformation [564]. Greatest success in producing monoclonal anti-D has been achieved when lymphocytes from recently reboosted antibody makers were used. For reviews see [565–568].

Of the 52 cell lines secreting anti-D summarised by McCann *et al.* [567], 17 were IgM and 35 IgG; of the 33 IgG subclassed, 24 were IgG1, eight IgG3, and one IgG2. The predominance of IgG1 and IgG3 molecules reflects the antibody profile of the lymphocyte donors. EBV-transformation followed by fusion appeared to increase the probability of making IgM anti-D [567]. IgA monoclonal anti-D has been reported [569].

Reactions of monoclonal anti-D with red cells expressing partial D antigens have identified numerous epitopes on the RhD protein (Table 5.6 and Section 5.6.3). Anti-D monoclonals and blends of monoclonals are now almost exclusively used as Rh grouping reagents. IgM monoclonal anti-D reagents agglutinate all but the weakest of weak D samples, though most do not agglutinate DVI cells. Three monoclonal anti-D for detecting DVI antigen were produced by using DVI red cells for rosetting in the production and maintenance of transformed cell lines [229]. The potential use of monoclonal anti-D in HDFN prophylaxis is discussed in Section 5.18.1.4.

5.18.1.3 Anti-D genetics

Human immunoglobulin V-gene cDNA derived from peripheral blood lymphocytes can been amplified and cloned, and the V_H and V_L gene repertoires linked together at random to encode single chain Fv (scFv) antibody fragments. These synthetic scFv cDNAs are inserted into phage vectors so that they can be incorporated into filamentous phages. By linking the scFv cDNA to a gene for a phage coat protein the encoded scFv molecules are represented at the surface of the phage, which then behaves like an antibody. Phages containing genetic information for variable regions that bind to a specific antigen can then be isolated, by selecting with an appropriate solid phase antigen. Red cells can be used for this purpose. *Escherichia coli* can then be transduced with these genes, cloned, and the single chain variable region fragments secreted [570]. By this technique antibody fragments specific for D and E were produced from non-immunised donors, but these antibodies were of low affinity [571, 572]. Subsequently, similar phage repertoire cloning techniques have led to the production of Fab fragments

with D antigen binding characteristics identical to those of the parental antibodies [573,574]. Genetically engineered IgG anti-D molecules, secreted by insect or mammalian cell lines, have a functional Fc domain and behave normally in immunological functional assays [574,575].

Sequencing of the genes encoding the variable regions of the light and heavy chains (V_L and V_H) of monoclonal IgG anti-D, revealed an extremely restricted use of germline genes [269,576–583]. A list of the genetic characteristics of 113 monoclonal anti-D and of 56 non-D Rh antibodies are listed in a workshop report [584]. The germline V_H segments used in IgG anti-D are among the most cationic available in the human V_H repertoire [577]. This would explain the relatively high isoelectric point of anti-D, compared with that of serum IgG, which may be an important factor in binding to the D antigen, which is located close to the membrane lipids.

The IgM heavy chain variable region gene segment V4-34 is present in cold agglutinins with I and i specificity (Section 25.7.3). About 85% of IgM monoclonal anti-D are encoded by V4-34 and these antibodies agglutinate papain-treated D– red cells; some will agglutinate untreated D– cells at 4°C [585,586]. This cold agglutination is usually i-specific, but can be I-specific. V4-34 IgM monoclonal anti-D also exhibit tissue multireactivity, mostly directed against intracellular components, in particular cytoplasmic intermediate filament proteins [586,587]. Clearly the cold agglutinin characteristics of IgM anti-D must be a consideration in the development of reagents. The same non-polar hydrophobic amino acids in the V4-34 framework-1 sequence are critical for both anti-D and -i activity, so it is not possible to remove the cold agglutinin activity by site-directed mutagenesis, without also reducing anti-D activity [580]. Anti-i activity was also detected in a monoclonal anti-c blood grouping reagent [588].

5.18.1.4 Clinical significance of anti-D

Clinically, D is the most important red cell antigen after A and B. Anti-D has the potential to cause severe HTRs, so D+ red cells must never be transfused to patients with anti-D and red cells of donors and recipients must always be typed for D, except possibly in populations where the D– phenotype is extremely rare [589]. About 20–30% of D– patients who receive large volumes of D+ blood make anti-D [113–115], so ideally D+ red cells should not be transfused to D– patients, except in an emergency, and must never be transfused to D– girls and women of childbearing age. The same criteria should be applied to blood products that may be contaminated with red cells. Immunisation rates, however, are zero or close to zero in D– immunosuppressed patients transfused with D+ red cells [590].

Before the 1970s, HDFN caused by anti-D was a significant cause of fetal and neonatal morbidity and mortality. In 1970, the incidence of infant deaths and stillbirths from HDFN caused by anti-D in England and Wales was 1.2 per thousand births; by 1989 the figure had fallen to 0.02 per thousand births [591]. This remarkable fall in prevalence is predominantly the result of immunoprophylaxis with anti-D immunoglobulin, which prevents the production of maternal anti-D following D-incompatible pregnancies. The mechanism for this antibody-mediated immune suppression still remains unclear. Although red cell clearance may play a significant role, other important factors could be B-cell inhibition resulting from IgG-antigen complexes interacting with the inhibitory IgG receptor, FcγRIIB, and IgG-mediated disruption of antigen processing and presentation leading to reduced T-cell help and B-cell activation (reviewed in [592–594]). All D– women must receive anti-D immunoglobulin within 72 hours of delivery of a D+ baby, the dose of anti-D being related to the size of the transplacental haemorrhage. In addition, one of two injections of D– pregnant women with anti-D immunoglobulin at around 28–34 weeks' gestation reduces the rate of antenatal immunisation [595,596].

The severity of anti-D HDFN is highly variable. The most severely affected fetuses die *in utero* from about the 17th week of gestation onwards. In less severe cases, hydrops fetalis may occur. In severely affected infants who are born alive, jaundice may develop rapidly and lead to kernicterus, which can cause permanent cerebral damage [112]. Despite anti-D immunoglobulin prophylaxis, in England and Wales at least 500 fetuses develop haemolytic disease per year, and about 25–30 babies die from HDFN [596]. There have been three recent non-invasive innovations in the prevention and management of HDFN: routine antenatal anti-D prophylaxis (RAADP) mentioned above; Doppler ultrasonography of the middle cerebral artery, which correlates well with increasing levels of bilirubin in the amniotic fluid; and determination of fetal D genotype by analysis of cell-free fetal DNA obtained from the maternal plasma (Section 5.7.1) [596,597]. Preimplantation genetic diagnosis has been applied in the management of pregnancy in couples where the woman has high levels of anti-D and the man is heterozygous for D-positivity [303]. Following *in vitro* fertilisation, biopsy of single cells from early embryos

permits selection of an embryo lacking *RHD* for implantation, guaranteeing a D-negative pregnancy.

Analysis of the quantity, IgG subclass, and functional activity of anti-D can provide a guide to the potential severity of HDFN. Anti-D can be quantified by comparison with a standard in an AutoAnalyser or by flow cytometry. A recommendation for interpretation of anti-D levels is as follows: <4 IU/ml, HDFN unlikely, but continue to monitor; 4–15 IU/ml and >15 IU/ml, moderate and severe risk, respectively, refer to specialist unit [598]. IgG1 and IgG3 anti-D both cause HDFN, IgM, IgG2, and IgG4 do not [112]. IgG1 appears to be more important than IgG3 in the pathogenesis of fetal anaemia [599]. A variety of cellular functional assays model the *in vivo* destruction of antibody-coated red cells following interaction with Fcγ-receptors of the mononuclear phagocyte system [600]. All of these assays provide useful clinical information, but none is entirely reliable for predicting the severity of HDFN.

Anti-D immunoglobulin for the prevention of immunisation is in short supply. It is produced by immunising volunteers with red cells, creating the ethical dilemma of whether healthy individuals should be injected with blood products. For many years it appeared likely that monoclonal antibody and recombinant antibody technology, in which an almost infinite volume of immunoglobulin can be produced *in vitro* from one immunisation, would provide the answer to this problem, but no such product has become available. The difficulty is that clinical trials have been disappointing, with monoclonal antibodies generally less effective than polyclonal antibodies at clearance of D+ red cells. The reason for this is not clear, but could result from unnatural glycosylation of these antibody molecules [601,602].

Peptide immunotherapy, the use of peptides derived from the RhD protein to render D-specific helper T cells tolerant, is another potential approach to preventing anti-D HDFN. Immunodominant peptides from the RhD protein induce the proliferation from T-helper lymphocytes (Th cells) from alloimmunised donors *in vitro* [603]. In a humanised mouse model, an HLA-DR15 transgene conferred the ability to respond to immunisation with purified RhD protein. Treatment of these mice with the peptides administered to the nasal mucosa prevented antibody responses to RhD protein by inducing tolerance, suppressing both antibody production and T-cell activation [604].

Occasional unexpectedly mild cases of HDFN occur in the D+ fetuses of women with anti-D and a history of severe HDFN in previous pregnancies. This can occur as the result of maternal HLA antibodies blocking Fc receptors on fetal macrophages, protecting sensitised fetal red cells from destruction [605,606]. FcγRI-blocking antibodies could have a potential for the treatment of HDFN.

5.18.2 Anti-C, -c, -E, -e, and -G

The numerous complexities of the specificities of antibodies described as anti-C, -c, -E, and -e are described in Sections 5.8 and 5.9. These antibodies share many of the characteristics of anti-D. They are generally immune, mostly IgG, and predominantly IgG1, although IgG2, IgG3, and IgG4 have all been detected [554]. Antibodies of all these specificities have been involved in HTRs, particularly of the delayed type [112]. Anti-c is clinically the most important Rh antigen after anti-D and causes severe HDFN. In three series of studies, between 14 and 21% of c+ babies born to women with anti-c required exchange transfusion [607–610]. Anti-C, -E, -e, and -G have all caused HDFN, but the occurrence is rare and the outcome seldom severe [112]. The clinical significance of antibodies with compound CcEe specificities is described in Section 5.10. Anti-G may be mistaken for anti-C+D (Section 5.11). It is important that D– pregnant women with anti-G or anti-C+G receive anti-D immunoglobulin, to prevent them making anti-D. Unlike other Rh specificities, apparently 'naturally occurring' anti-E are not uncommon [353,354].

Intravascular HTRs have been associated with specific Rh antigens in the absence of any detectable Rh antibody [611]. Rh association was inferred because transfusion of red cells positive for a particular Rh antigen resulted in haemolysis, whereas transfusion of antigen-negative cells resulted in normal red cell survival. C, c, and e have been implicated in this type of reaction.

Human monoclonal antibodies to C, c, E, e, and G have been produced by cloning of EBV-transformed lymphoblastoid cell lines or by cloning of heterohybridomas produced from a fusion of EBV-transformed cells with mouse myeloma cells (see Section 5.18.1.2) [612–614]. Like anti-D (Section 5.18.1.3), antibodies to C, E, e, and G utilise *IGHV* genes restricted to the *IGHV3* superspecies [578,583,615].

5.18.3 'Enzyme-only' antibodies

Some Rh antibodies, often referred to as 'enzyme-only' antibodies, agglutinate red cells treated with protease enzymes, but are not detected by conventional antiglobulin tests with untreated cells. Although these antibodies are most often 'naturally occurring' anti-E [351–354,616], 'enzyme-only' anti-D, -C, -c, -e, and -ce have also been

identified [617–621]. 'Enzyme-only' antibodies are generally clinically insignificant and are not even detected when tests employing protease-treated red cells for antibody screening are avoided. There are rare exceptions, however. Examples of 'enzyme-only' anti-c, -e+ce, -E, and -C (which bound C3) caused HTRs [616–618,621]. One 'enzyme-only' anti-E became active by an indirect antiglobulin test during pregnancy and caused HDFN requiring exchange transfusion [622].

5.18.4 Rh autoantibodies

Rh antigens are the most common targets for warm autoantibodies [623]. The involvement of Rh antibodies in AIHA was first appreciated when autoantibodies with anti-e specificity were recognised [624]. Of the 'simple' Rh-specific autoantibodies, anti-e is the most common, but anti-c, -E, -D, and -C also occur, roughly in that order of prevalence [623,625,626]. These specificities occasionally occur alone, but more often adsorption tests with Rh-phenotyped red cells are necessary to determine the specificities present on the red cells and in the serum of AIHA patients. Some Rh antibodies with apparently simple specificities, dubbed 'mimicking antibodies' by Issitt *et al.* [627], can be totally adsorbed by 'antigen-negative' red cells, demonstrating a broader specificity. Cold type AIHA caused by complement activating IgM autoanti-D has been described [628].

Use of red cells with the rare Rh phenotypes Rh_{null} and D−− showed that antibodies to high incidence Rh antigens often occur as autoantibodies [535]. Rh-related antibodies may also be involved in some cases of drug-induced AIHA [112,623]. Loss or weakness of some Rh antigens has been reported in a few patients with AIHA [629].

Some D+ patients have developed anti-D and a positive DAT after transfusion of D+ blood [630–632]. The anti-D was transient, although an injection of D+ blood in one patient restimulated the antibody [631]. Autoanti-D may occur concurrently with alloanti-D in immunised individuals with partial D antigens [633,634] and, rarely, before the alloanti-D can be detected [635].

5.18.5 Transplant donor-derived Rh antibodies

Anti-D derived from donor lymphocytes in D+ recipients of solid organ transplants (kidney, liver, heart–lung, pancreas) has been responsible for haemolysis, sometimes severe [636–639]. The donor origin of such anti-D has been demonstrated by Gm grouping [640,641]. Anti-c, -E, and -e have also been detected in similar circumstances [636,637,642].

Anti-D has been observed in D+ recipients of D− bone marrow or peripheral blood stem cells [643–646], in one case not appearing until immunosuppression for graft-versus-host disease had been discontinued, 2 years after transplantation [645]. Anti-D, -E, and -G were detected in the serum of a DcE/dce patient 4 months after he received bone marrow from his sister, presumably the result of immunisation of the donor-derived lymphocytes by the patient's D+ E+ red cells [646].

5.19 Rh mosaics and acquired phenotype changes

Abnormal expression of some Rh antigens is occasionally observed in patients with myeloid leukaemias, polycythaemia, and other myeloproliferative disorders. In most cases these patients appear to be mosaics with two populations of red cells of different Rh phenotype [124,647–651], although a few have complete loss of certain Rh antigens [652–656]. One patient with myeloid metaplasia, previously known to be D+, was found to be D− and had made anti-D plus -C [653]. The strength of antigen expression and proportions of the two cell populations can vary over a period of time [647,650, 651,654].

There are also many examples known of apparently healthy people whose blood appears to contain two red cell populations, as judged by tests with Rh antisera, but have no sign of mosaicism in tests for other genetic markers [649,651,657]. The Rh mosaicism is not a transient condition and families of four propositi eliminated chimerism as a possible explanation. Screening with anti-D+C of blood from 552 individuals over 60 years old disclosed one Rh mosaic [657].

Three individuals with Rh mosaicism were also mosaics for another chromosome 1 marker, the Duffy blood group [651,658,659]. Jenkins and Marsh [658] found 30% D+ C+ Fy(a+) and 70% D− C− Fy(a−) red cell populations in a male blood donor. The results of testing his family showed that he could not be dce/dce: his father was DCe/DCe Fy(a+b−), his mother DCe/dce Fy(a−b+), and his sister DCe/dce Fy(a+b+). Extensive serological investigation led to the conclusion that the father had a homozygous dose of C and e. In another case, 30% of red cells were D+ C+ Fy(b+) and 70% D− C− Fy(b−), but no other sign of mosaicism was observed in the many

markers studied. The karyotype determined on lymphocyte and fibroblast cultures was normal [659].

A myelofibrosis patient with a mixture of D+ C+ and D– C– cells (father dce/dce, mother DCe/DCe) had an aberrant karyotype, a cytogenetic mixture with an abnormal population containing a balanced translocation involving chromosomes 1, 4, and 7 [652]. In most cases, however, no abnormality of chromosome 1, which contains the Rh genes, was observed.

A D+ woman became D– over a 3-year period, during which she was diagnosed with chronic myeloid leukaemia [655]. Reticulocyte transcript analysis revealed *RHD* with a deletion of 600G in exon 4, introducing a reading frameshift and premature stop codon, plus *RHCE*Ce* and *RHCE*ce*. The *RHD* mutation, which was present in neutrophils and cultured erythroblasts, but not lymphoid cells, probably resulted from a somatic mutation in a myeloid stem cell.

Körmöczi *et al.* [651] carried out a thorough analysis of individuals with Rh mosaicism or antigen loss (or spontaneous Rh phenotype splitting as they called it), three of whom had haematological diseases. Five individuals presented a stable mixed-field agglutination pattern over a period of time, two exhibited progressively diminishing proportions of D+ red cells, and two showed complete D antigen loss throughout the observation period. One had a mixture of D+ Fy(b+) and D– Fy(b–) red cells. In individuals with mixtures of C or E, the D+ red cells were C+ or E+ and the D– cells C– or E–. Genotyping of erythroid colonies cultured from single erythroid progenitors indicated loss of one complete haplotype (*DCe* or *DcE*) in the D– fraction. Further analyses of microsatellites on different tissues, sorted blood cell subsets, and erythropoietic progenitors indicated myeloid lineage-restricted loss of heterozygosity of variable chromosome 1 stretches encompassing the *RHD* and *RHCE* loci on the short arm and, in one case, even including the Duffy gene on the long arm. In most cases, the loss of heterozygosity probably arose from homologous recombination between chromatids of chromosome 1 prior to mitosis, rather than deletion of substantial segments of the chromosome, explaining the lack of any palpable cytogenetic abnormality.

5.20 The RHAG blood group system

The Rh-associated glycoprotein (RhAG) has long been recognised as a homologue of RhD and RhCE, and member of the Rh protein family closely associated with the Rh proteins in the red cell membrane (Section 5.5.6). In 2010, the recognition that three red cell surface antigens were located on RhAG (Figure 5.7) and encoded by *RHAG* led to the establishment of a new blood group system, RHAG [76] (system 30) (Table 5.15).

5.20.1 Duclos (RHAG1) and DSLK (RHAG3)

Duclos is a high frequency antigen, previously 901013. Apart from the red cells of Mme Duclos, the sole maker of anti-Duclos, the Duclos antigen is lacking only from those Rh_{null} or Rh_{mod} red cells that are also U–. Mme Duclos had an apparently normal DCe/dce phenotype, but a U antigen slightly weaker than normal [660]. A monoclonal antibody, MB-2D10, raised to human red cells, showed a Duclos-like specificity by reacting with all cells except those of Rh_{null} U– and Rh_{mod} U– phenotypes [661]; it differed from the Duclos antibody by reacting

Table 5.15 Antigens of the RHAG blood group system.

Antigen			Molecular basis*		
No.	Name	Frequency	Nucleotides	Exon	Amino acids
RHAG1	Duclos	High	316C (G)	2	Gln106 (Glu)
RHAG2	Ol[a]	Low	680C>T	5	Ser227Leu
RHAG3†	DSLK	High	490A (C)	3	Lys164 (Gln)
RHAG4†		Low	808G>A, 861G>A	6	Val270Ile, Ala280Ala

*Molecular basis of antigen-negative phenotype in parentheses.
†Provisional assignment.

with the red cells of Mme Duclos [662]. Immunoblotting demonstrated that the MB-2D10 epitope is located on RhAG [663]. Sequencing of DNA from archived serum revealed that Mme Duclos was homozygous for an *RHAG* mutation encoding Gln106Glu in the second extracellular loop of RhAG [76] (Figure 5.7). Human embryonic kidney (HEK) cells transfected with *RHAG* reacted with alloanti-Duclos, whereas HEK cells transfected with *RHAG* containing the Duclos mutation, did not.

DSLK is serologically similar to Duclos [76]. Although DSLK– red cells reacted with anti-Duclos and MB-2D10, they had aberrant expression of U and anti-DSLK reacted with all other red cells, including Rh_{null} U+, except Rh_{null} U– cells. The only DSLK– individual was homozygous for a mutation encoding Lys164Gln in the third external loop of RhAG (Figure 5.7).

Although Duclos-negative and DSLK-negative red cells have apparently normal expression of Rh antigens and RhAG, they have a profound weakening of U, in common with most Rh_{null} and Rh_{mod} phenotype red cells. The precise nature of the U antigen is not known, but it is considered to be dependent on an interaction between glycophorin B (GPB) and RhAG because the main causes for absence or reduced expression of U are either absence or gross alteration of GPB or absence or reduced expression of RhAG (Section 3.6). The Duclos- and DSLK-associated substitutions suggest that the interaction of RhAG with GPB involves the second and third extracellular loops of RhAG, with the increase in negative electrostatic potential on the RhAG surface disrupting the interaction with GPB [76].

5.20.2 Ol^a (RHAG2)

Ol^a (previously 700043) is an antigen of very low frequency described in a three-generation Norwegian family. Ol(a+) red cells in this family have weakened D, C, and E antigens, but recombination between the genes governing Rh and Ol^a expression demonstrated that Ol^a could not belong to the Rh system [549]. Two Ol(a+) members of the family were heterozygous for an *RHAG* mutation encoding Ser227Leu in the proposed extracellular vestibule and a Japanese Rh_{mod} individual with strong expression of Ol^a was homozygous for the same mutation [76].

In a three-dimensional model of Ol(a+) RhAG in a trimer with two molecules of RhD, the substituted residue is within 5 Å of the interface between the RhAG and RhD subunits (Figure 5.7). It is possible that the Ser227Leu substitution disrupts the formation of the RhAG-Rh complex, resulting in less Rh protein in the membrane or in an alteration of the molecular surface that forms the Rh epitopes [76].

5.20.3 An antigen provisionally numbered RHAG4

An antibody to an antigen of very low frequency, which caused severe HDFN, reacted with the red cells of the affected baby's father and two of his half-siblings, with evidence of weakened Rh antigen expression. The father was homozygous for two *RHAG* mutations: one silent; the other encoding Val270Ile in the ninth membrane-spanning domain of RhAG. The baby and the two of his half-siblings of the father were heterozygous for those mutations [664]. Val270Ile combined with Gly280Arg were previously described in one Rh_{null} individual [504]. It is possible that the Val270Ile mutation causes the expression of a novel epitope, possibly through improper folding of RhAG or improper quaternary association of RhAG with RhD/RhCE proteins.

5.21 Development and distribution of Rh antigens and RhAG

Rh antigens are readily detected on cord red cells; no surprise considering the part they play in HDFN. D, C, c, E, and e antigens have been detected on fetal red cells at the eighth week of gestation [665].

Rh antigens appear to be erythroid-specific. No D, C, c, E, or e antigen could be detected on granulocytes, lymphocytes, monocytes, or platelets by radioimmunoassay and fluorescent flow cytometry [666,667]. There is no evidence of Rh antigens on cells of other tissues.

Although RhAG is also generally considered erythroid-specific, *RHAG* transcripts have been detected in oesophageal squamous epithelium [668]. RhBG and RhCG, homologues of RhAG, are present in kidney, liver, skin, and testis [80,92,93] and probably in most types of epithelia [668,669], with RhBG and RhCG mainly located on the basolateral and apical membranes, respectively [669,670].

5.22 Functional aspects of the Rh and RhAG proteins

The topology of the Rh proteins in the cell membrane – polytopic, with cytoplasmic N- and C-termini – is characteristic of membrane transporters. RhAG bears even closer resemblance to red cell membrane transporters, as

it has a single *N*-glycan on one of its extracellular loops. There is substantial evidence that RhAG functions as a gas transporter, although the prime substrates – ammonia (NH_3), ammonium (NH_4^-), nitric oxide (NO), carbon dioxide (CO_2), and/or oxygen (O_2) – remain controversial. On the other hand, the RhD and RhCE proteins almost certainly are not transporters and their functions remain obscure.

5.22.1 RhAG: NH_3/NH_4^+ transporter?

NH_4^+ may be friend or foe. It is utilised by prokaryotes and lower eukaryotes as a source of nitrogen, whereas in mammals it is toxic and must be metabolised and excreted. RhAG shares between 20 and 27% sequence identity with proteins of a family of ammonium transporters, the Amt proteins, ubiquitous in lower organisms, including bacteria and yeast, and in plants [671]. These proteins are polytopic and generally have 11 membrane-spanning domains and an extracellular N-terminus [672].

Yeast (*Saccharomyces cerevisae*) cells have three membrane NH_4^+transporters, Mep1, Mep2, Mep3. Yeast cells lacking all three Mep proteins (triple-*mepΔ*) fail to grow in low levels (5 m*M*) of NH_4^+, though any one of the Mep proteins can restore growth. The growth defect in triple-*mepΔ* yeast cells was repaired by transfection with cDNA representing *RHAG* or *RHCG* [93]. Furthermore, transfection of yeast cells with *RHAG* or *RHCG* cDNA conferred resistance to a toxic concentration of methylammonium (CH_3NH_2), suggesting that the human proteins are involved in the export of the NH_4^+analogue.

Similar expression studies in yeast [673], *Xenopus* oocytes [674], and human HeLa cells [675] also indicated that RhAG facilitated the transfer of NH_3 though the membrane, either directly or as charged NH_4^+ (possibly in exchange for H^+), or both. Experiments on human normal or Rh_{null} red cells or resealed ghosts, that is, with or without RhAG, suggested that RhAG can act as a transporter of NH_3/NH_4^+ in its native cell [676,677].

Three-dimensional structural models based on the crystal structures of bacterial *E. coli* AmtB and *Nitrosomas europaea* NeRh50 suggest that human RhAG, RhBG, and RhCG lack the NH_4^+ binding site of AmtB. They have conserved the 'phenylalanine gate', a pair of phenylalanine residues (Phe120 and Phe225 in RhAG) that would only permit small molecules such as NH_3, CO_2, H_2O, O_2, or NO, to enter and pass through the conductance pore. Also conserved is the twin-histidine motif in the pore (His175 and His334 in RhAG), which is essential for substrate conductance. RhAG, therefore, appears to have structural characteristics compatible with conductance of neutral gases, including NH_3, but possibly not of the charged NH_4^+ ion [50–53,678].

NH_3 and CH_3NH_2 transport was severely impaired in red cells of *Rhag*$^{-/-}$ (knockout) mice, but only slightly impaired in those of mice lacking the only Rh gene, *Rhd* [85].

Why would red cells require an NH_3 or NH_4^+ transporter? NH_4^+ concentration is three times higher in red cells than in plasma and it has been suggested that RhAG promotes retention of NH_4^+ in red cells for transport to the liver or kidney and subsequent removal from the body, thus protecting against NH_4^+ toxicity in the brain and other organs [93,679]. Alternatively, it could function to minimise red cell volume changes that could occur when the red cell passes in and out of the high NH_3 concentration of the renal medulla [675].

5.22.2 RhAG: CO_2/O_2/NO channel?

Although there can be little doubt that RhAG can transport NH_3, its function in human red cells remains in question. CO_2 and NH_3 are both gases that are readily hydrated: CO_2 to HCO_3^-; NH_3 to NH_4^+. Results of experiments on the green alga *Chlamydomonas reinhardtii*, which is dependent on CO_2 for photosynthesis, indicated that proteins of the Rh family might function as channels for CO_2. Expression of the algal gene *RH1* was upregulated and downregulated by increased and decreased ambient CO_2 concentration, respectively [680]. *C. reinhardtii* lines lacking *RH1* mRNA and Rh1 protein as a result of RNAi interference, grow slowly even in high CO_2 environments, presumably because they fail to equilibrate CO_2 rapidly [681]. Rh_{null} human red cells have significantly reduced CO_2 permeability compared with normal cells. This reduction is similar to that observed with AQP1-null [Co(a–b–)] red cells, suggesting that RhAG and AQP1 share responsibility for passage of CO_2 through the red cell membrane, together being responsible for at least 50% of CO_2 permeability of the red cell membrane [682] (for further discussion on AQP1, see Section 15.8).

The primary function of the red cell is the transport of respiratory gases, so it is logical that some of the most abundant proteins in the red cell membrane are involved in the rapid transfer of these gases in and out of the cell. From the results of experiments on band 3-deficient red cells, Bruce *et al.* [95] developed the concept of the band 3 metabolon, involving a macrocomplex of membrane proteins, including the anion exchanger band 3, the Rh proteins, and RhAG (Section 10.7), plus various cytosolic proteins, including carbonic anhydrase II (CAII) and

deoxyhaemoglobin. It is proposed that RhAG transfers CO_2 through the membrane into the red cell, where it is hydrated to HCO_3^- by CAII. The highly soluble HCO_3^- is then exported from the cell by band 3 in exchange for Cl^-. If, as appears most likely, RhAG were a relatively non-specific channel for neutral small molecules, it could also function as a channel for O_2 to and from haemoglobin in the area around the metabolon. Another function of red cells is storage of nitric oxide and regulation of vasodilation through its release. Consequently, transport of NO though the membrane represents another potential function of RhAG [52,95]. The discussion in the section above on the suitability of the structure of RhAG as a gas channel for NH_3 would apply equally to CO_2, O_2, and NO.

From a thorough analysis of the evolution of Amt and Rh genes and proteins, Huang and Peng [94] propose that Amt proteins, ancient ancestors of the Rh proteins, evolved as NH_3/NH_4^+ transporters in primitive organisms, but that subsequent duplication and divergence of the genes driven in response to new selection pressures, led to the development of the Rh family of proteins as CO_2 channels, which expanded in vertebrates as the Amt proteins disappeared. Thus, the ability of RhAG to transport NH_3/NH_4^+ could reflect an ancestral characteristic, as opposed to the physiological function of rapid transfer of CO_2 through the red cell membrane.

5.22.3 RhAG and cation transport

Evidence for RhAG as a pore for monovalent cations comes from the association of heterozygosity for *RHAG* mutations with overhydrated stomatocytosis, a disorder characterised by abnormally shaped red cells that leak cations (Na^+ and K^+) at a rate 20–40 times greater than normal [75]. The mutations responsible encode Ile61Arg and Phe65Ser, which are likely to cause opening of the RhAG pore.

5.22.4 RhD and RhCE

The roles of RhD and RhCE are not known, but it is fairly certain that they do not function as transporters. They lack the amino acids conserved in other Rh-family proteins, including RhAG, that are considered essential to gas transport. The pair of Phe residues of the 'phenylalanine gate' are replaced by Met118 and Phe223 in RhD and Met118 and Val223 in RhCE; the twin-His motif is replaced by Tyr173 and Phe332 in both proteins. They could, however, be involved in gas movement by increasing the surface area-to-volume ratio of red cells [681]. It is feasible that RhD and RhCE play a role in facilitating the assembly of the band 3 macrocomplex [52]. RhD, RhCE, and RhAG might serve as independent attachments to the cytoskeletal network through interaction between their C-terminal tails and the adapter protein ankyrin-R and play a part in maintaining the stability of the Rh complex in the membrane [100]. Red cells lacking RhD, RhCE, and RhAG (Rh_{null}) or lacking RhD and RhCE, but 33–38% of normal RhAG levels (Rh_{null} of the amorph type) have abnormally shaped red cells (Section 5.16.6), whereas those lacking just RhD (D–) or RhCE (D––) are of normal shape.

5.23 Evolutionary aspects

Rh genes probably evolved from Amt genes in prokaryotes. They are widely distributed in vertebrates and invertebrates, but are not present in plants. In vertebrates Rh genes diversified and the Rh family expanded to four common paralogous clusters, represented by *RH* (*RHCE* and *RHD*), *RHAG*, *RHBG*, and *RHCG* in humans [94]. The degree of homology between *RHAG* and homologous human and some non-human genes is shown in Table 5.16. *RHAG* has a higher level of homology with its slime mould homologue, than with the *RH* genes [683].

The ancestral *RH* genes were formed, almost certainly, by duplication of an ancestral *RHAG* (or *RH50*) gene. Analyses of the numbers of synonymous and

Table 5.16 Percentage of identity between human *RHAG* and other genes of the human Rh family and *RHAG* homologues in other species [80].

Species	Gene	% identity to *RHAG*
Homo sapiens (man)	*RHAG*	100.0
	RHCG	50.9
	RHBG	49.9
	RHCE	33.0
	RHD	32.8
Mus musculus (mouse)	*Rhag*	76.5
Danio rerio (zebrafish)	*Rhg*	43.0
Drosophila melanogaster (fruit fly)	*Rhp*	41.4
Caenorhabditis elegans (nematode)	*Rhp-1*	35.9
	Rhp-2	43.0
Geodia cydonium (marine sponge)	*Rhg*	41.8
Dictyostelium discoideum (slime mould)	*RhgA*	34.5

non-synonymous substitutions in the *RH* and *RHAG* genes in man, macaque, mouse, and rat suggested that Darwinian selection had acted on both genes, but that *RHAG* is more conserved than the *RH* genes, having evolved 2–3 times more slowly [684,685]. This suggests that *RHAG* has greater functional significance than *RH*. Duplication of an *RHAG*-like gene to form an ancestral *RH* gene is estimated to have occurred around 510 million years ago, before divergence of jawless fish and jawed vertebrates [686]. *RH* gene homologues have been detected in all mammals studied [687].

Rh-related mRNA transcripts were isolated from the bone marrow of chimpanzee, gorilla, gibbon, crab-eating macaque (*Macaca fascicularis*), and rhesus monkey (*M. mulatta*), by reverse-transcriptase PCR using primers designed from the sequence of human *RH* genes [319,688]. The cDNA sequences demonstrated a high degree of homology to the human sequence and predicted proteins of 417 amino acids. Like most humans, chimpanzees and gorillas have at least two *RH* genes, although some chimpanzees have three or four genes; other primates, including orangutans and gibbons, old world and new world monkeys, and prosimians, have only one *RH* gene per haploid genome [689,690]. Consequently, the duplication of the ancestral *RH* gene that led to the evolution of *RHCE* and *RHD* in man must have occurred in the common ancestor of humans, chimpanzees, and gorillas, between 8 and 11 million years ago [691,692]. Based on the gene positions and orientation, *RHCE* appears to represent the ancestral gene [693].

A series of events are predicted to have generated the present Rh haplotypes [65,691]. Duplication of an ancestral *RHCE*, followed by divergence resulting from mutations and complex recombination events, generated genes resembling *RHD* and *RHCE*ce*. *Dce*, therefore, is the root of the human Rh system (Figure 5.12). Deletion or inactivation of *RHD* then created *dce*, non-reciprocal recombination of *RHD* sequences in the exon 2 region into *RHCE*ce* could have produced *DCe*, and a point mutation in the *RHCE*ce* would have produced *DcE*. In harmony with the thesis of Fisher and Race [694] from 1946, Carritt *et al*. [691] proposed that *dCe* arose from recombination between *DCe* and *dce*, *dcE* from recombination between *DcE* and *dce*, and *DCE* from recombination between *DCe* and *DcE*. The very rare haplotype *dCE* must have arisen from recombination between the uncommon haplotypes, *dCE* and *dcE*.

Deletion of *RHD*, to produce the D– haplotype common in Europe, must have occurred after duplication and diversification of the *RH* genes [70]. Selection in favour of a population in which both D+ and D– phenotypes are common is difficult to understand, considering the part they play in HDFN. Analysis of genomic data provided no evidence to support any effects of selection on the *RHD* deletion [695]. Anstee [696] has suggested that genetic drift and migration, rather than natural selection, could provide an explanation, with mixing of two populations, one the essentially D– Palaeolithic people from the Basque region and the other D+ Neolithic migrants [14]. The effect of earlier selective pressures in Africa cannot be dismissed, however, considering that three relatively common genetic mechanisms exist in Africa for deletion or inactivation of *RHD* (Section 5.6.1).

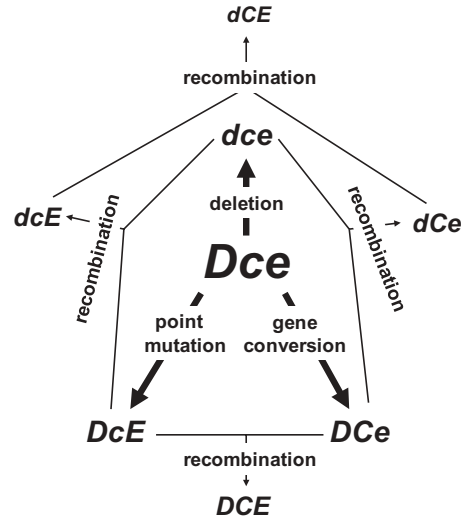


Figure 5.12 Scheme to show the proposed derivation of the eight Rh haplotypes from the ancestral haplotype, *Dce* (after [691]). The haplotype *dce* resulted from deletion of *RHD*, *DcE* from a point mutation within *RHCE*, and *DCe* from a gene conversion event between *RHD* and *RHCE*. The less common haplotypes *dcE*, *dCe*, and *DCE*, then arose from recombination events involving *dce*, *DcE*, and *DCe*. The very rare haplotype *dCE* resulted from recombination between *dcE* and *dCe*.

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6.1 Introduction

The Lutheran system consists of 20 antigens: LU1 to LU22 in the numerical notation, with two declared obsolete (Table 6.1). Four pairs of these antigens have allelic relationships and represent SNPs in the Lutheran gene, *LU*: Lu^a (LU1) and Lu^b (LU2); Lu6 and Lu9; Lu8 and Lu14; and Au^a (LU18) and Au^b (LU19). The null phenotype, Lu_{null} or Lu(a–b–), in which the red cells lack all Lutheran system antigens, results from homozygosity for inactivating in the Lutheran gene. Individuals with the Lu_{null} phenotype may make an antibody to the Lutheran glycoproteins (Lu-gps), anti-Lu3. Nucleotide changes in *LU* are associated with loss of eight other antigens of high frequency, which are also absent from Lu_{null} cells. The molecular bases for two other antigens of high frequency absent for Lu_{null} cells are unknown.

Red cells of the dominantly inherited In(Lu) and X-linked XS2 phenotypes have very low levels of Lutheran antigens, which are not usually detectable by agglutination methods. Both of these mod phenotypes are governed by genes encoding erythroid transcription factors: In(Lu) results from heterozygosity for inactivating mutations in *KLF1*, the gene for EKLF; XS2 from hemizygosity for a mutation in *GATA1*.

Lutheran antigens are located on two red cell membrane glycoproteins (CD239) of apparent MW 78 and 85 kDa, which belong to the immunoglobulin superfamily of receptors and adhesion molecules. The Lu-gps are ligands for the extracellular matrix glycoprotein, laminin. *LU* or *BCAM* is situated on chromosome 19q12-q13 and consists of 15 exons, with alternative splicing accounting for the two isoforms of the Lu-gps.

6.2 The Lutheran glycoproteins and the gene that encodes them

6.2.1 The Lutheran glycoproteins (Lu-gps)

Components of apparent MW 85 and 78 kDa were revealed by immunoblotting of red cell membranes with monoclonal anti-Lu^b or with alloanti-Lu^a, -Lu^b, -Lu3, -Lu4, -Lu6, -Lu8, -Lu12, -Au^a, or -Au^b [1–3]. The two components were not apparent when Lu_{null} or Lu_{mod} red cells were used.

Parsons *et al.* [4] purified the Lu-gps by immunoaffinity chromatography with a monoclonal antibody, BRIC 221. From the amino acid sequence obtained, they designed redundant oligonucleotide primers and used a PCR product to isolate a cDNA clone of 2417 bp from a human placental cDNA library. The predicted mature protein consists of 597 amino acids: 518 comprising an extracellular domain, 19 a single transmembrane domain, and 59 a cytoplasmic domain. This structure represents the 85 kDa isoform. Immunoprecipitation experiments with a rabbit antiserum prepared to an amino acid sequence of the cytoplasmic domain showed that the 78 kDa structure lacks part of the cytoplasmic domain [4]. The 78 kDa isoform had previously been identified as an epithelial cancer antigen and is often referred to by

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Table 6.1 Antigens of the Lutheran system.

Antigen				Molecular basis*		
No	Name	Frequency	Antithetical	Nucleotides	Exon	Amino acids
LU1	Lu^a	Polymorphic	Lu^b	230G>A	3	Arg77His
LU2	Lu^b	High	Lu^a	230G (A)	3	Arg77 (His)
LU3	Lu3	High		Various		
LU4	Lu4	High		1. 524G (A)	5	Arg175 (Gln)
				2. 524G (T)	5	Arg175 (Leu)
LU5	Lu5	High		326G (A)	3	Arg109 (His)
LU6	Lu6	High	Lu9	824C (T)	7	Ser275 (Phe)
LU7	Lu7	High		Not known		
LU8	Lu8	High	Lu14	611T (A)	6	Met204 (Lys)
LU9	Lu9	Low	Lu6	824C>T	7	Ser275Phe
LU11	Lu11	High		Not known		
LU12	Lu12	High		1. (99-104del)	2	1. (delArg34,Leu35)
				2. 419G (A)	3	2. Arg140 (Gln)
LU13	Lu13	High		1340C (T), 1742A (T)	11, 13	Ser447 (Leu), Gln581 (Leu)
LU14	Lu14	Low	Lu8	611T>A	6	Met204Lys
LU16	Lu16	High		679C (T)	6	Arg227 (Cys)
LU17	Lu17	High		340G (A)	3	Glu114 (Lys)
LU18	Au^a	Polymorphic	Au^b	1615A (G)	12	Thr539 (Ala)
LU19	Au^b	Polymorphic	Au^a	1615A>G	12	Thr539Ala
LU20	Lu20	High		905C (T)	7	Thr302 (Met)
LU21	Lu21	High		282C (G)	3	Asp94 (Glu)
LU22	LURC	High		223C (T)	3	Arg75 (Cys)

Obsolete: LU10, previously Singleton; LU15, AnWj (now 901009).
*Molecular basis of antigen-negative phenotype in parentheses.

its earlier name BCAM [5], or as Lu(v13) [6]. The 85 kDa isoform is 5–10-fold more abundant on red cells than the 78 kDa isoform [7].

6.2.2 The Lu-glycoproteins belong to the immunoglobulin superfamily (IgSF)

The immunoglobulin superfamily (IgSF) is a large collection of glycoproteins, abundant on leucocytes, but also present on other cells, which contain repeating extracellular domains with sequence homology to immunoglobulin variable (V), constant (C1 or C2), or intermediate (I) domains. Each IgSF domain consists of approximately 100 amino acids and is structured into two β-sheets stabilised by a conserved disulphide bond (Figure 6.1). IgSF glycoproteins mostly function as receptors and adhesion molecules, and may be involved in signal transduction [8].

The extracellular domain of the Lu-gps is organised into five IgSF domains, V-C1-I-I-I, with a distinctive bend and flexible junction between domains 2 and 3 [4,5,9,10] (Figure 6.2). There are five potential *N*-glycosylation sites, one in the third domain and the other four in the fourth domain. At least five other IgSF glycoproteins are present in the red cell surface membrane: the Scianna, LW, and Ok blood group glycoproteins (Chapters 13, 16, and 22), CD47 (Chapter 5), and CD58 (LFA-3).

Many of the Lutheran antigens were mapped to an IgSF domain through the expression of *LU* cDNA deletion mutants in K562 erythroleukaemia cells, each construct encoding Lu-gp lacking one, two, three, or four of the IgSF domains [11]. Subsequently the location of most of the Lutheran antigens has been identified by DNA sequencing [11–15] (Table 6.1, Figure 6.2).

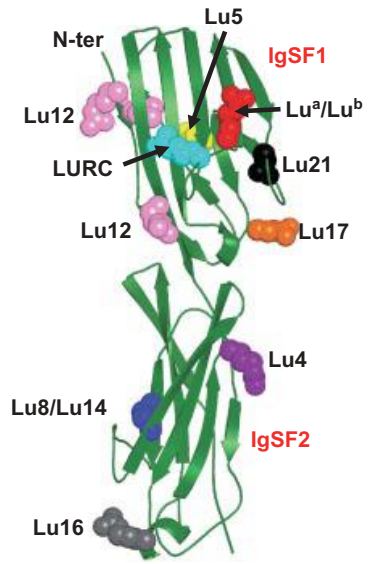


Figure 6.1 Structure of the two N-terminal domains of the Lu-gps (IgSF1 and IgSF2), showing the location of the amino acids associated with some Lutheran antigens [9]. (Thanks to Nicholas Burton for providing the structure.)

Table 6.2 Exon/intron organisation of *LU* [11,12].

Exon	Domain encoded	Exon size (bp)	Intron size (kb)
1	5′ UT + leader	105	2.0
2	1 IgSF (V)	122	0.7
3	1 IgSF (V)	229	0.09
4	2 IgSF (V)	71	0.5
5	2 IgSF (V)	95	0.09
6	2 IgSF (V)	185	0.53
7	3 IgSF (C2)	137	0.31
8	3 IgSF (C2)	157	3.5
9	4 IgSF (C2)	116	0.1
10	4 IgSF (C2)	142	0.17
11	5 IgSF (C2)	137	0.15
12	5 IgSF (C2)	145	0.09
13	TM + 19 residues cyt	145	0.97
14	cyt (85 kDa isoform)	118	0.09
15	1 residue cyt (85 kDa isoform)	498	

TM, transmembrane; cyt, cytoplasmic.

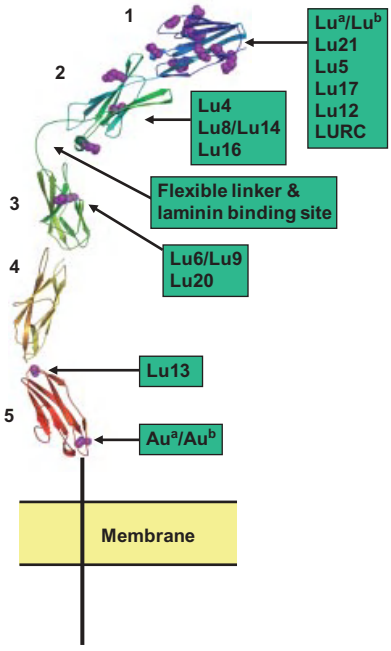


Figure 6.2 Structure of the Lu-gps, showing five IgSF domains and the location of the Lutheran antigens on those domains. (Thanks to Nicholas Burton for providing the structure.)

6.2.3 Organisation of the *LU* gene

The *LU* (*BCAM*) gene is 12.5 kb organised into 15 exons (Table 6.2). Exon 1 encodes the signal peptide; exons 2–12 the five IgSF domains (two exons per domain except domain 2, which is encoded by exons 4–6); exon 13 the transmembrane domain and the cytoplasmic domain common to both isoforms; exons 14 and 15 the C-terminal 40 amino acids of the larger isoform [11,12]. Two *LU* transcripts have been isolated, one of 2.5 kb encoding the larger Lu-gp isoform (85 kDa) and one of 4.0 kb encoding the smaller (78 kDa) isoform [6]. The two transcripts differ as a result of alternative splicing of intron 13. In the 2.5 kb transcript intron 13 has been removed by splicing and exons 14 and 15 encode the C-terminal 40 amino acids of the larger isoform. In the 4.0 kb transcript intron 13 remains, explaining the larger size of the intron. The 5′ end of the intron contains a UGA translation stop codon, so the unspliced intron 13 and exons 14 and 15 are not translated and the protein product has a cytoplasmic domain consisting only of the 19 amino acids encoded by exon 13 [12].

The 5′ flanking region of *LU* does not contain TATA or CAAT boxes, but showed an organisation typical of ubiquitous genes with several potential binding sites for the Sp1 transcription factor. The region between –673 and

–764 upstream of the coding region contains binding sequences for GATA and CACCC or Sp1 transcription factors [12].

6.2.4 Linkage and chromosome location

In 1951, when the Lutheran locus was shown to be genetically linked to the locus controlling ABH secretion (*FUT2*, believed at that time to be the Lewis blood group locus), blood groups were involved in the first recognised human autosomal linkage and, consequently, the first demonstration of recombination due to crossing-over in humans [16,17]. Linkage analysis revealed that *LU–FUT2* was linked to the gene for the third component of complement (*C3*) and indirectly linked to the Lewis gene (*FUT3*), so when *C3* was assigned to chromosome 19 by somatic cell hybridisation *LU* was also assigned to chromosome 19 [18,19]. *LU* (*BCAM*) is located at 19q12-q13 as part of a linkage group that also includes the H gene (*FUT1*) and the LW blood group gene (*ICAM4*).

6.3 Lu^a and Lu^b (LU1 and LU2)

The first Lutheran antibody, anti-Lu^a, was described in 1945 by Callender *et al.* [20] and in more detail the following year by Callender and Race [21]. Ten years later the antithetical antibody, anti-Lu^b, was described by Cutbush and Chanarin [22].

6.3.1 Molecular basis for Lu^a and Lu^b

The Lu^a/Lu^b polymorphism results from a single base change in exon 3 of *LU*, encoding an amino acid substitution in the first IgSF domain of the Lu-gps: *LU*A* His77; *LU*B* Arg 77 [11,12] (Table 6.1). The importance of this amino acid substitution in Lu^a or Lu^b expression was confirmed by *in vitro* site-directed mutation [12]. The nucleotide change is associated with an *Aci*I restriction-site polymorphism.

6.3.2 Frequencies and inheritance of Lu^a and Lu^b

Lu^a is widely distributed amongst Europeans, Africans, and North Americans with a frequency of around 8%, but is very rare or absent from all other indigenous populations studied [23]. Typical frequencies were obtained from tests with anti-Lu^a and -Lu^b on about 1500 white Canadians: Lu^a 6.9%; Lu^b 99.9%; Lu(a–b+) 93.1%; Lu(a+b+) 6.8%; Lu(a+b–) 0.1%; *LU*A* 3.5%; *LU*B* 96.5% [24,25]. Of 922 Chinese in Taiwan tested with anti-Lu^a and -Lu^b, all were Lu(a–b+) [26].

Table 6.3 Lutheran genotype frequencies on four populations of American blood donors, obtained by testing on the BeadChip array [29].

		Genotypes		
Ethnic group	No. tested	*LU*A/A*	*LU*A/B*	*LU*B/B*
Caucasians	1243	0	0.05	0.95
African Americans	690	0	0.06	0.94
Hispanic	119	0.01	0.02	0.97
Asian	51	0	0.02	0.98

In two large surveys, in which donor red cells were screened with anti-Lu^b and all negatives tested with anti-Lu^a, the following results were obtained: South of England, approximately 250 000 tested, 230 Lu(a+b–), 72 Lu(a–b–) [27]; South Wales, 75 614 tested, 39 Lu(a+b–), 15 Lu(a–b–) [28]. In a predominantly European population, therefore, roughly one in 1000 is Lu(b–); approximately two thirds of these being Lu(a+b–) and one third Lu(a–b–) (see Section 6.8.2.9).

Table 6.3 shows *LU*A/B* genotype frequencies in four ethnic groups of Americans determined by SNP testing [29]. All of 1102 Chinese were genotyped as homozygous for *LU*B* [30].

6.3.3 Variation in antigenic strength

The Lutheran antigens are very variable in strength. Lu^a on red cells from different families may vary quantitatively, but the antigenic strength remains roughly constant within the family. Occasionally adsorption and elution tests are required to detect weak Lu^b on Lu(a+b+) cells. There is also heterogeneity of Lutheran antigen strength between individual red cells within a person, which accounts for the characteristic mixed-field agglutination patterns often seen with Lutheran antisera, especially anti-Lu^a [21,22], and the wide range of survival times of Lu(b+) cells introduced into an Lu(a+b–) person with anti-Lu^b [31]. The abundance of Lu^b antigens on red cells, as determined by Scatchard analysis with purified monoclonal anti-Lu^b, is relatively low and shows wide variation. The number of Lu^b sites was estimated at 1640–4070 on Lu(a–b+) cells and 850–1820 on Lu(a+b+) cells [32].

Red cells from cord samples and from infants in the first year of life have markedly weakened expression of

Lu^a and Lu^b compared with those from adults. Ten of 155 cord blood samples had the phenotype Lu(a–b–), which is very rare in adults [33]. Adult levels of Lu^a and Lu^b antigenic expression are reached by the age of 15 [34].

6.3.4 Anti-Lu^a and -Lu^b

6.3.4.1 Anti-Lu^a

The first example of anti-Lu^a was found in a multiply transfused patient, together with anti-c, anti-C^w, anti-Kp^c, and anti-N [21]. The antibody had been stimulated by transfusion of blood from a donor named Lutheran. Anti-Lu^a has been reported after pregnancy and/or transfusion, and often appears together with other antibodies, especially red cell reactive HLA antibodies (anti-Bg) [35]. Anti-Lu^a may also be 'naturally occurring' [36,37], and in some cases a 'naturally occurring' antibody may be augmented by transfusion [38,39]. Anti-Lu^a suitable for grouping reagents is uncommon.

Lu^a antibodies are usually IgM, but, like other Lutheran-system antibodies, often have IgG and IgA components [40]. Anti-Lu^a often agglutinate Lu(a+) red cells directly, with a thermal optimum well below 37°C. Some also react in an antiglobulin test, and a few, predominantly IgG examples, are reactive only by an antiglobulin test.

A single-chain variable-fragment (scFv) with Lu^a specificity has been produced by phage display and recombinant DNA technology, and a monoclonal anti-Lu^a constructed [41].

6.3.4.2 Anti-Lu^b

Anti-Lu^b is relatively rare, often found as a single antibody. It has been stimulated by transfusion and by pregnancy; 'naturally occurring' examples have not been found.

Anti-Lu^b are often optimally active in the antiglobulin test, but directly agglutinating anti-Lu^b have been described, many with a temperature optimum of about 20°C. Most anti-Lu^b are mixtures of IgG and IgM, although IgA may also be present [40]. IgG anti-Lu^b may be predominantly IgG1, although IgG2 and IgG4 may be present [42].

Two monoclonal anti-Lu^b (BRIC 108 and LM342/767.31) have been produced from mice immunised with Lu(b+) red cells [1,43], although adsorption and elution tests demonstrated some binding of BRIC 108 to Lu(a+b–) cells [44].

6.3.4.3 Clinical significance of anti-Lu^a and -Lu^b

On the rare occasions that Lutheran antibodies are implicated in HTRs, they are almost always mild and delayed [36,40,45], although there could be exceptions [46] (see Section 6.4.2). Radiolabelled Lu(a+) red cells injected into a patient with anti-Lu^a survived normally [47]. Similar survival tests in patients with anti-Lu^b showed that at least a proportion of injected Lu(b+) cells could be removed fairly rapidly [31,48]. Least incompatible red cells are usually suitable for transfusion, but, if possible, antigen-negative red cells should be selected for strong examples of the antibody.

No case of HDFN caused by anti-Lu^a or -Lu^b and requiring any treatment other than phototherapy is reported, although raised bilirubin or a positive DAT may be detected [40]. One explanation for this could be poor development of Lutheran antigens on neonatal red cells (Section 6.3.3), but there is another possible explanation. Babies of mothers with high-titre IgG1 anti-Lu^b or -Lu6 had no sign of HDFN, their red cells gave negative DATs, and Lutheran antibody could not be detected in their sera [49]. Maternal IgG1 usually becomes concentrated in the fetal circulation by active placental transfer. As Lu-gp is present on placental tissue [4], it is possible that Lutheran antibodies are adsorbed by placental cells, preventing their transfer to the fetus.

6.4 Other Lutheran antigens and antibodies

In addition to Lu^a and Lu^b, the Lutheran system contains 17 other antigens: three pairs of antithetical antigens – Lu6 and Lu9, Lu8 and Lu14, and Au^a and Au^b – plus 11 antigens of high frequency (Table 6.1). Recombination as a result of crossing-over has never been observed within the Lutheran system. All Lutheran antigens are absent from Lu_{null} cells and absent from or expressed very weakly on In(Lu) cells. Like Lu^a, Lu^b, and Lu3, Lu4, Lu5, Lu6, Lu7, Lu8, Lu12, Lu13, Lu14, Au^a, Au^b, and Lu20 have been shown, by immunoblotting and/or by flow cytometry with K562 cells transfected with *LU* cDNA, to be located on the Lu-gps [2,3,11,50,51]. Nucleotide changes in the *LU* gene encoding amino acid changes in the Lu-gps have been shown to be associated with absence of all of the high frequency antigens or presence of the low frequency antigens, with the exception of Lu7 and Lu11 (Table 6.1). Lu11 has not been shown to be inherited and has not been shown to be located on the Lu-gps or encoded by the *LU* gene, and so should be referred to as a para-Lutheran antigen. Lu3 and anti-Lu3 will be discussed in Section 6.7.

With the exception of anti-Lu8, antibodies to none of the antigens described in this section have been incriminated in a serious HTR or in HDFN. All of the antibodies have been produced in Lu(a–b+) individuals, with the exception of anti-Lu16.

6.4.1 Lu6 (LU6) and Lu9 (LU9)

Lu6 and Lu9, Lutheran antigens of high and low frequency, respectively, have an antithetical relationship, and represent a SNP and *Cfo*I restriction polymorphism in *LU* encoding an amino acid substitution in the third IgSF domain [13] (Table 6.1).

The original anti-Lu9 was found, together with anti-Lu^a, in the serum of a white woman (Mrs Mull) [52]. The anti-Lu9 was responsible for a weak DAT with the red cells of her three babies. Red cells of the husband of Mrs Mull were Lu(a+b+) and Lu:9. Study of his family showed that Lu9 expression was controlled by the *LU* locus, although it did not represent an allele of *LU*A* and *LU*B*. The only other example of anti-Lu9 was found in a multitransfused woman and tests on 200 red cell samples unearthed another Lu:9 sample (0.5%) [53]. Tests with the original anti-Lu9 suggested a higher frequency of 1.7% [52], but that figure may be inaccurate as the serum also contained anti-HLA-B7 (-Bg^a) [35].

Red cells of the original Lu:–6 propositus, and those of her two Lu:–6 siblings, were strongly Lu:9, suggesting homozygosity [54]. All other Lu:–6 individuals (who are not Lu_{null}) have also been Lu:9.

In vivo red cell survival studies, macrophage binding assays, and transfusion of Lu:6 cells to patients with anti-Lu6 have suggested that anti-Lu6 is not usually of any clinical significance, but similar assays in an elderly woman suggested that her IgG1 anti-Lu6 was clinically significant; she was transfused with Lu_{null} cells, which had normal or near normal survival [55]. An Iranian Jewish woman with anti-Lu6 was transfused with two units of Lu:6 red cells with no apparent haemolysis, but after the transfusion a monocyte-based functional assay became positive, although a ^{51}Cr survival test was negative [56]. Red cells of the baby of a woman with high-titre IgG1 anti-Lu6 gave a negative DAT and no anti-Lu6 could be detected in the baby's serum, suggesting that the antibody was unable to cross the placenta [49] (see Section 6.3.4.3).

6.4.2 Lu8 (LU8) and Lu14 (LU14)

Lu8 and Lu14, Lutheran antigens of high and low frequency, respectively, have an antithetical relationship, and represent a SNP and *Fat*I and *Nla*III restriction polymorphisms in *LU*, encoding an amino acid substitution in the second IgSF domain [13] (Table 6.1).

The original anti-Lu8 was reported in 1972 as an antibody to a high frequency antigen absent from Lu_{null} cells [57]. Two Lu8 antibodies have been implicated in acute HTRs [46,58]; one gave positive results in monocyte monolayer assays [58].

An antibody in the serum of a multiply transfused dialysis patient, reported in 1977, reacted with red cells of 14 of 580 (2.4%) random white donors and also reacted strongly with three examples of Lu:–8 red cells and was numbered anti-Lu14 [59]. The apparent antithetical relationship between Lu8 and Lu14 was supported by family studies. Lu14 also appeared to have a higher frequency in Lu(a–b+) than in Lu(a+b+) samples, suggestive of allelic association [59]. Many other examples of anti-Lu14 have been found since [35,60], which included an IgG anti-Lu14 that was apparently 'naturally occurring'. Monoclonal anti-Lu^b gave consistently higher titration scores with Lu:14 cells than with Lu:–14 cells [61]. The frequency of Lu14 in 610 Danish donors and 600 English donors was 1.5% and 1.8%, respectively. Genomic testing for Lu8 on 11 418 Austrian donors revealed six (0.05%) negatives and 488 (4.3%) heterozygous positives [62].

6.4.3 Au^a (LU18) and Au^b (LU19), the Auberger antigens

Au^a and Au^b represent a SNP in exon 12 of *LU* encoding an amino acid substitution in the fifth IgSF domain (Table 6.1) [11].

For many years the Auberger antigens were considered to represent a system independent of Lutheran, mainly because of results on one family, which showed recombination between *LU*A* and the gene for Au^a [63]. When the family was retested for Au^a and tested for Au^b, errors in the original testing were discovered and the family now supported linkage between Auberger and Lutheran [64]. Family studies confirmed that Auberger and Lutheran antigens are controlled by a single gene [65].

The first anti-Au^a was identified in 1961 in the serum of a multitransfused woman, which also contained anti-E, -K, -Fy^b, and -Bg (-HLA) [66]. Only two other examples of anti-Au^a are reported, also in sera containing multiple antibodies to red cell antigens [67]. Anti-Au^b was not found until 1989 [68] and three further examples were soon found [69]. All four sera containing anti-Au^b also contained anti-Lu^a.

Au^a has an incidence of between 80 and 90% in European populations [66,67]. Au^b has an incidence of about

50% in a European population and 68% in an African American population [68]. Genotyping in Chinese predicted antigen frequencies for Au^a and Au^b of 98% and 24%, respectively [30].

6.4.4 Lu4 (LU4)

The first Lu:–4 propositus, a white woman who had made anti-Lu4, had Lu:–4 siblings [70]. All of about 2700 predominantly white donors were Lu:4. The second example of anti-Lu4 was identified in a prenatal patient with no known history of transfusion or previous pregnancy [71].

Homozygosity for two different mutations within the codon for Arg175, encoding either Gln or Leu, are responsible for the Lu:–4 phenotype in the two propositi [13,71] (Table 6.1). A monoclonal antibody produced in a mouse immunised with Lu-gp bound an epitope on IgSF2, but did not bind if Arg175 was substituted by either Asn or Ala [72].

6.4.5 Lu5 (LU5)

Anti-Lu5, -Lu6, and -Lu7, were initially found at an AABB 'wet' workshop [54]. At least 10 examples of anti-Lu5 have been identified and have been found in both black and white people [35,54,73,74]. Two of the Lu:–5 propositi had an Lu:–5 sibling [73,74]. None of 423 mostly white donors was Lu:–5 [54]. Two Lu:–5 individuals were homozygous for the same missense mutation in *LU* [13] (Table 6.1).

Results of a chemiluminescent functional assay suggested that one anti-Lu5 might cause increased clearance of transfused Lu:5 red cells [74].

6.4.6 Lu7 (LU7)

The original anti-Lu7 was found in an Lu:–7 woman with an Lu:–7 brother [54]. An IgG3 antibody in an Lu:–7 Latino woman was assumed to be another example of anti-Lu7, but Lu:–7 cells were not available for confirmation [51]. Her baby's red cells did not give a positive DAT. None of 285 mostly Caucasian donors was Lu:–7 [54].

Lu7 is located on the fourth IgSF domain of the Lu-gps [11].

6.4.7 Lu11 (LU11), a para-Lutheran antigen

The first example of anti-Lu11, an IgM antibody, was present in the serum of a white woman [75]. At least two other examples have been identified since [35]. There is no evidence that Lu11 is inherited. All of 500 predominantly white donors were Lu:11 [75].

6.4.8 Lu12 (LU12)

The first example of anti-Lu12 was produced by women of Polish and Ukrainian extraction, whose red cells were Lu(a–), but had only weak expression of Lu^b [76]. Red cells of her father, which had a weak Lu12 antigen, and of her Lu:–12 sister, were also Lu(a–b+^{w}) and, like the cells of the propositus, had only weak expression of other high frequency Lutheran antigens. Red cells from all except one of 1050 Canadian donors reacted strongly with anti-Lu12; those from the exceptional donor reacted weakly and were Lu(a–b+^{w}). The second example of anti-Lu12 was found in an Lu:–12 white woman with two Lu:–12 siblings [77]. An *in vivo* red cell survival test suggested that this antibody had the potential to cause accelerated destruction of transfused Lu:12 cells.

Lu:–12 had two different molecular backgrounds in two unrelated individuals: a six-nucleotide deletion in exon 2 encoding and a missense mutation in exon 3 (Table 6.1). Although the mutations are in different exons, when mapped to a three-dimensional schematic presentation of the Lu-gp the amino acid changes appeared to be located in close spatial proximity because of the protein folding [13]. Lu12 and Lu^b are both located on the first IgSF domain, so the weakening of Lu^b could result from conformational changes in the first domain, or could result from reduced expression of the Lu-gps associated with loss of Lu12.

6.4.9 Lu13 (LU13)

The original anti-Lu13 was unpublished. A second anti-Lu13 was found in a Finnish woman, but anti-Lu13 was not available for testing her red cells [78]. A family in which three of five siblings were Lu:–13 has been mentioned briefly [79].

Two unrelated Lu:–13 individuals have the same two missense mutations encoding amino acid substitutions: one in the region of the interface between IgSF domains 4 and 5; the other in the cytoplasmic domain [13] (Table 6.1). Each also had a silent mutation in the codon for Ser557. One of the individuals was homozygous for all three changes, but the other was apparently heterozygous, suggesting the presence of a null allele *in trans*.

6.4.10 Lu16 (LU16)

Anti-Lu16 was found together with anti-Lu^b in the sera of four Lu(a+b–) black women [80,81]. Two were homozygous for a missense mutation in exon 6 of *LU* (Table 6.1), in addition to the sequence in exon 1 associated with Lu^a expression [13,81]. In one case the anti-Lu16 plus anti-Lu^b was present in an untransfused

woman during her first pregnancy. There were no indications of HDFN at delivery [81].

6.4.11 Lu17 (LU17)

The only example of anti-Lu17 was found in an Italian woman [82], who was homozygous for a missense mutation in exon 3 of *LU* [13] (Table 6.1). *In vivo* studies suggested that anti-Lu17 might be capable of causing a modest reduction in survival of transfused Lu:17 red cells [83].

6.4.12 Lu20 (LU20)

Anti-Lu20 was identified in the serum of an Israeli thalassaemia patient [50], who was homozygous for a missense mutation in exon 7 of *LU* [13] (Table 6.1). The serum also contained anti-C, -K, and -Fy^b.

6.4.13 LU21

Anti-LU21 was identified in the serum of a Caucasian woman of Israeli origin during her second pregnancy [14]. There was no evidence of HDFN in her second, third, or fourth pregnancies. A MAIEA assay suggested that LU21 was located on IgSF1, the same domain as Lu^b, and the woman was subsequently shown to be homozygous for a mutation encoding an amino acid substitution in IgSF1 [14] (Table 6.1).

6.4.14 LURC (LU22)

LURC appears to be a high frequency antigen involving both Lu^b (Arg77) and Arg75 in IgSF1 [15]. Anti-LURC was produced by a woman with Lu(a+b+^{w}) red cells who was heterozygous at codons 75 and 77. It appears that Cys75, Arg77 results in weak Lu^b and no LURC, whereas Arg75, His77, results in Lu^a and no LURC. The common sequence, Arg75, Arg77 produces both Lu^b and LURC. Val96Ile in IgSF2, encoded by 586G>A in exon 5, also present in the LURC– propositus, is very conservative and would probably cause no conformational rearrangements. This change has been associated previously with Lu^a phenotype with no evidence of antibody formation [84].

6.5 Recombinant Lutheran antigens

Lutheran antigens have been used as models for the application of recombinant proteins in antibody identification [11,85–87]. Recombinant proteins containing all or some of the IgSF domains of the Lutheran protein have been expressed in eukaryote or prokaryote cells. The purified protein was then used in agglutination inhibition tests, attached to polystyrene plates for detection by an ELISA procedure, or coupled to superparamagnetic particles for detection in a particle gel immunoassay. Alloanti-Lu^a or -Lu^b were detected with high sensitivity and specificity.

6.6 Effects of enzymes and reducing agents on Lutheran antigens

Lutheran antigens are destroyed by treatment of red cells with trypsin or α-chymotrypsin; papain has little effect. Sulphydryl reducing agents, such as AET and DTT, break inter- and intra-polypeptide chain disulphide bonds resulting in the unfolding of the protein. Red cells treated with 6% AET or 200mM DTT at pH8.0 did not react with most Lutheran antibodies tested, including many examples of anti-Lu^a and -Lu^b [1,88,89]. This is to be expected, considering that the Lutheran antigens are located in the disulphide-bonded IgSF domains of the Lu-gps.

6.7 Lu_{null} and anti-Lu3 (LU3)

Lu_{null} phenotype is extremely rare and has a recessive mode of inheritance. Lu_{null} cells lack all Lutheran system antigens. Individuals with the Lu_{null} phenotype may make an antibody, anti-Lu3, which reacts with all red cells apart from those with the Lu_{null} phenotype. Lu_{null} red cells have normal expression of those antigens, such as AnWj, that are expressed very weakly on In(Lu) red cells.

Lu_{null} phenotype was first found in 1963 in an English woman [90], followed by three Lu_{null} members of a Canadian family [91] and two in a Japanese family [92]. The presence of anti-Lu3 in the serum of an African American woman with Lu(a–b–) red cells suggested Lu_{null}, but no family study was possible [93].

The molecular background of Lu_{null} has been identified in five individuals. All are either homozygous or doubly heterozygous for inactivating mutations in the *LU* gene.

1 An English woman [90]. Heterozygosity for two inactivated alleles: one a nonsense mutation 691C>T in exon 6, encoding Arg231Stop (*LU*02N.01*), the other a deletion of exons 3 and 4 (*LU*02N.02*) [94].

2 A Japanese blood donor. Homozygosity for a nonsense mutation 711C>A in exon 6 encoding Cys237Stop

(*LU*02N.03*) [94]. His parents were heterozygous for the mutation.

3 A German woman of Czech origin. Homozygosity for a nonsense mutation 361C>T in exon 3 encoding Arg-121Stop (*LU*02N.04*) [94].

4 A Japanese blood donor. Homozygosity for a 27 kb deletion encompassing exons 3 to 15 of *LU* [95].

5 A pregnant Dutch Caucasian woman. Homozygosity for a dinucleotide insertion, 123GG, in exon 2, converting 42Glu-Val-Met to 42Gly-Arg-Stop [96].

6.7.1 Anti-Lu3 (-LU3)

All Lu_{null} propositi have been found following the detection of an antibody to a high frequency antigen, anti-Lu3. Anti-Lu3 has a single specificity and reacts equally strongly with Lu(a+b–), Lu(a+b+), and Lu(a–b+) cells. Adsorption with Lu(a+b–) cells will remove the activity for Lu(a–b+) cells and *vice versa* [90,92]. Lu3 is present on all red cells that express any Lutheran antigen.

An antibody resembling anti-Lu3 was detected in an African American woman whose red cells behaved like Lu_{null} and were AnWj+ (excluding In(Lu), Section 6.8.2.4), but reacted very weakly with anti-Lu^b, as revealed by an adsorption/elution test. An emergency transfusion with Lu(b+) red cells resulted in no indications of haemolysis and a DAT remained negative [97].

Two patients with ovarian cancer and DAT-positive red cells had potent Lu3-like autoantibodies, one of which was responsible for an HTR [98,99]. The red cells were Lu(a–b+) in one patient and apparently Lu(a–b–) Lu:–3 in the other, probably the result of blocking of the Lutheran antigen sites by the antibody.

Two murine monoclonal antibodies (BRIC 221 and BRIC 224) behave serologically like anti-Lu3. They react with all red cells except Lu_{null} cells [4].

6.8 Lu_{mod} : the In(Lu) phenotype

In(Lu) was the name given for a rare, dominant suppressor of the Lutheran antigens [100] and has subsequently also been used to describe the phenotype. Red cells of most individuals with an *In(Lu)* gene appear to be Lu(a–b–) and Lu_{null} by agglutination tests, but will bind selected Lutheran antibodies, as determined by adsorption and elution tests. The first family showed that the In(Lu) phenotype was dominantly inherited through three generations [101]. Unlike true Lu_{null} individuals, these propositi have usually been found in searches of random donors. Fifty-two propositi of families with dominant Lu_{mod} had 63 Lu_{mod} and 61 not-Lu_{mod} siblings [2,27,102,103]. An analysis of Lu_{mod} x not-Lu_{mod} matings revealed 64 Lu_{mod} and 61 not-Lu_{mod} children. Both counts are very close to the 1:1 ratio expected for dominant inheritance.

Adsorption and elution tests with anti-Lu^a and -Lu^b permitted the determination of the true Lutheran genotype in some In(Lu) members of families, demonstrating recombination between *In(Lu)* and *LU* and, therefore, that *In(Lu)* is not inherited at the *LU* locus [27,28,100].

With two possible exceptions [104], no Lutheran-system antibody has been detected in the serum of any person with an *In(Lu)* gene, because of the weak expression of Lutheran system antigens on the red cells of most In(Lu) individuals. Sera of at least 12 In(Lu) women with not-Lu_{null} children have been tested [27,105].

6.8.1 The molecular genetic background to In(Lu)

Erythroid Krüppel-like factor (EKLF) is an erythroid-specific transcription factor with three zinc fingers homologous to those found in the Krüppel family of transcription factors [106]. EKLF binds the sequence CCACACCCT and *KLF1*, the gene encoding EKLF, is only expressed in bone marrow and spleen [107]. EKLF functions synergistically with the major erythroid transcription factor, GATA-1, and with the ubiquitous Krüppel protein Sp1, to activate transcription of erythroid genes [108]. EKLF is essential for terminal differentiation of erythroid cells and EKLF absence in mice leads to embryonic death from severe anaemia [108,109].

In 2008, Singleton *et al.* [109] demonstrated that In(Lu) resulted from heterozygosity for mutations in *KLF1*, in the presence of a normal *KLF1* allele (Table 6.4). Expression profiles from erythroblasts cultured from In(Lu) individuals revealed a reduced expression of erythroid genes in general, including the genes for the following blood groups: Colton, Lutheran, Ok, Indian (CD44), Duffy, Scianna, MN (glycophorin A), and Diego (band 3). Expression of the Lutheran gene throughout *ex vivo* erythropoiesis was very low in In(Lu) cells, compared with control cells. Singleton *et al.* [109] suggest that this dramatic reduction in Lutheran expression in In(Lu) cells may be related to its transcriptional activation being at a later stage in erythropoiesis than other erythroid-specific genes. No individual homozygous for *In(Lu)* has been found; no surprise considering the embryonic lethality of this condition in mice.

Table 6.4 Mutations in *KLF1* associated with In(Lu).

ISBT symbol	Mutation	Exon	Encoded protein change	References
*KLF1*BGM 01*	–124T>C		Promoter	[109]
*KLF1*BGM 11*	90G>A	2	Trp30stop	[104]
*KLF1*BGM 12*	304T>C	2	Ser102Pro	[104]
*KLF1*BGM 02*	380T>C	2	Leu127stop	[109]
	517–519delC	2	Pro173Pro fs stop64	[110]
	551–556GGACCG>A	2	Gly184Glu fs stop167	[110]
*KLF1*BGM 03*	569delC	2	Pro190Leu fs stop47	[109]
	637C>T	2	Glu213stop	[110]
	802C>T	2	Arg268stop	[110]
	809C>A†	2	Ser270stop	[111,112]
*KLF1*BGM 04*	874A>T	2	Lys292X	[109]
	889T>C	2	Leu300Pro	[110]
*KLF1*BGM 05*	895C>T	2	His299Tyr	[109]
	902inT	2	Arg301Leu fs stop52	[110]
	947G>A	3	Cys316Tyr	[110]
*KLF1*BGM 06*	954dupG	3	Arg319Glu fs stop34	[109,112]
	968C>G	3	Ser323Trp	[110]
	977T>G†	3	Leu326Arg	[112]
*KLF1*BGM 07*	983G>T	3	Arg328Leu	[109]
*KLF1*BGM 08*	983G>A	3	Arg328His	[109]
*KLF1*BGM 09*	991C>G	3	Arg331Gly	[109]
	991C>T	3	Arg331Trp	[110]
	994A>G	3	Lys332Glu	[112]

†Deduced from amino acid changes.

6.8.2 Other effects of *In(Lu)*

Despite a general effect on expression of erythroid genes by *In(Lu)* [109], there are a number of phenotypic effects of *In(Lu)* in addition to suppression of all high frequency Lutheran antigens.

6.8.2.1 P1 antigen

The effect of *In(Lu)* on expression of P1 antigen is less obvious than that on Lutheran antigens. Amongst 236 members of 41 In(Lu) families the distribution of P_1 and P_2 among the In(Lu) members was significantly different from that observed in the not-In(Lu) members and in the general population [27,103,113] (Figure 6.3). The 36 In(Lu) P_1 people may have possessed a strong $P1^+$ allele, or been homozygous for $P1^+$, or both. There is no evidence that P1 antigen is suppressed in recessive Lu_{null} individuals [113]. Three families in which P_2 In(Lu) and P_2 Lu(a–b+) parents have a P_1 Lu(a–b+) child confirm the effect of *In(Lu)* on P1 [27,28,114].

6.8.2.2 i antigen

The monomorphic i antigen is also suppressed by *In(Lu)*, as judged by selected anti-i [27,113,114]. Red cells of neonates have a strong i antigen and this is not dramatically suppressed in red cells of babies with an *In(Lu)* gene [115]. The i antigen was of normal strength in two recessive Lu_{null} people [113]. I antigen is unaffected by *In(Lu)* [113].

6.8.2.3 CD44 and antigens of the Indian system

The CD44 glycoprotein, which is present on a variety of tissues including red cells, carries the antigens of the Indian system (Chapter 21). Expression of CD44, and consequently of the three high frequency Indian antigens, is suppressed by *In(Lu)*, although these determinants are still easily detected on In(Lu) cells [116–118]. *In(Lu)* has little or no effect on leucocyte or serum expression of CD44 [119], reflecting the erythroid-specific influence of

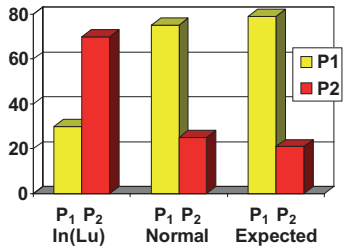


Figure 6.3 Suppression of P1 by *In(Lu)* in propositi and relatives from 41 families [27,103,113], showing the proportion of P_1:P_2 in In(Lu) and normal Lu phenotype family members, and the expected ratio in a normal population.

EKLF. CD44 and In^b are expressed normally on Lu_{null} cells of the recessive type [117,120].

6.8.2.4 AnWj antigen

AnWj is an antigen of very high frequency, which is probably associated with the CD44 glycoprotein (Chapter 21). It is not expressed, or at least is expressed only very weakly, on red cells of individuals with an *In(Lu)* gene. AnWj was initially given the number LU15, but this became obsolete when AnWj was found to be expressed normally on recessive Lu_{null} cells [121]. The one family showing inheritance of AnWj demonstrated recombination between the genes for AnWj and Lu^a [122].

6.8.2.5 Other antigens suppressed by *In(Lu)*

Analysis of a series of families suggested that In(Lu) red cells are more often weak for the Knops system antigens, Kn^a, McC^a, Sl^a, and Yk^a, and for Cs^a antigen, than are red cells from the general population [123]. Antigens of the Knops system reside on the red cell C3/C4 receptor, CR1 (CD35) (Chapter 20).

MER2 is a red cell polymorphism on CD151 (Chapter 23). Strength of expression of MER2 is variable. When MER2 antibodies were titrated with red cells from members of a large three-generation Sardinian family with the *In(Lu)* gene, lower scores were obtained with red cells of nine members with In(Lu) phenotype, compared with those of 12 Lu(a–b+) members [88].

Equine anti-lymphocyte globulin reacts with red cells, but reacts less strongly with In(Lu) cells than cells of common Lutheran type or Lu_{null} cells [124].

Agglutination with concanavalin A lectin of red cells of all five In(Lu) individuals from two families was reduced, compared with cells of the other family members [125]. Band 3, the red cell anion exchanger (Chapter 10), is the major concanavalin A binding protein, so this reduction in binding was interpreted as suggesting an abnormality in glycosylation of band 3 in In(Lu) cells.

6.8.2.6 CD75

CD75 represents a cluster of monoclonal antibodies reacting with lactosaminyl sequences, either sialylated (CD75s) or non-sialylated (CD75) [126]. These antibodies are unique as their reactivity with red cells is enhanced by the presence of an *In(Lu)* gene. Guy and Green [127] showed by haemagglutination tests and radiobinding assays that there was a substantial increase in expression of CD75 on In(Lu) red cells compared with cells of common Lutheran type. Lu_{null} red cells have normal CD75 expression, but Lu_{mod} cells of the X-linked type (Section 6.8.3) are CD75-negative [128]. Protease treatment of red cells did not abolish the CD75 determinant. One CD75 monoclonal antibody reacted with sialidase-treated red cells, but two others did not [128], so both CD75 and CD75s appear to be enhanced by *In(Lu)*. It is not obvious how mutations in *KLF1* could enhance expression of these carbohydrate antigens, apart from removing some masking effect, possibly excessive sialylation, in erythroid cells.

6.8.2.7 Abnormal red cell morphology and electrolyte metabolism associated with *In(Lu)*

Individuals with an *In(Lu)* gene are generally healthy with no obvious anaemia or reticulocytosis, although there may be some association with a degree of acanthocytosis [125,129] and with increased fetal haemoglobin levels [111,112]. Autologous *in vivo* survival of In(Lu) red cells is normal [129].

Osmotic fragility of In(Lu) cells is normal, although incubation of these cells in plasma for 24 hours at 37°C resulted in significant resistance to osmotic lysis compared with cells of common Lutheran type, in which osmotic fragility increases [125]. Before incubation, In(Lu) and control cells had similar concentrations of Na^+ and K^+ ions; during incubation, In(Lu) cells, but not control cells, lost K^+ and, to a lesser extent, gained Na^+ ions. This reduction in total cation content in In(Lu) red cells could explain their relative resistance to osmotic lysis. Significant haemolysis of In(Lu) cells was observed within a few days of storage at 4°C in modified Alsever's solution [129]. This haemolysis could be reduced by the addition of glucose or ATP.

6.8.2.8 Variable effects of *In(Lu)*

The typical phenotype of individuals with an *In(Lu)* gene is Lu(a–b–) with Lutheran system and AnWj antigens only detectable by extremely sensitive methods and P1, i, In^b and some other antigens depressed to a lesser extent. In some families the effect is less extreme, and weakly expressed Lutheran system antigens and AnWj can be detected by direct testing [54,130]. In the person with the *KLF1* promoter sequence mutation, −124TC (Table 6.4), expression of the Lutheran antigens and of In^b was weaker than on normal red cells, but stronger than on most In(Lu) cells, suggesting production of some EKLF [109].

6.8.2.9 Frequency of Lu(a–b–) and In(Lu) phenotypes

In(Lu) is by far the least rare type of Lu(a–b–) phenotype. Analysis of the families of 50 Lu(a–b–) propositi demonstrated that 41 were inherited in a dominant fashion; the genetical background of the other nine could not be determined, but serological tests suggested that most of these were also of In(Lu) type [27,28]. Several large surveys in England and Wales have shown that the incidence of Lu(a–b–), as determined by testing with either anti-Lu3 or with anti-Lu^b and testing the negatives with anti-Lu^a, varies between 0.005 and 0.032% [27,28,90,103] (Table 6.5).

6.8.3 Lu_{mod} of the X-linked type

Five members of a large Australian family had an Lu_{mod} phenotype with serological features characteristic of both In(Lu) and Lu_{null} [134]. The red cells were Lu(a–b–) and lacked the other Lutheran antigens by agglutination tests, yet anti-Lu^b could be adsorbed and eluted from the cells. The cells were AnWj+ and appeared to have slightly enhanced i antigen. They also had weak P1 antigens, although this may have resulted from the presence of a weak $P1^+$ gene in the family. The mode of inheritance of Lu_{mod} in this family showed the features of a recessive X-borne inhibitor gene. All the Lu_{mod} members were males and, although the Lu_{mod} phenotype occurred in successive generations, there was no example of transmission of the phenotype from parent to child. The regulator locus was called *XS*: *XS1* common allele; *XS2* rare inhibitor allele.

The zinc-finger transcription factor GATA-1 plays a central role in erythroid and megakaryocyte development through its interaction with multiple proteins, including EKLF [108]. It is encoded by a gene (*GATA1*) on the short arm of the X-chromosome at Xp11.23. *GATA1* hemizygous male knock-out mice embryos die from severe anaemia, with erythropoiesis arrested at a proerythroblast-like stage [135]. A variety of missense mutations in *GATA1* are associated with macrothrombocytopenia and anaemia and several acquired mutations are associated with malignancies, especially in children with Down syndrome [136].

The X-chromosome location, plus the recognition that the *In(Lu)* gene is the transcription factor EKLF, led Singleton *et al.* [137] to investigate *GATA1* in the Australian family with X-linked suppression of Lutheran. In *GATA1* of the Lu_{mod} propositus they found 1240T>C converting the termination codon (TGA) to an arginine codon (CGA), predicting a GATA-1 protein with an extraneous 41 amino acids at the carboxy terminus. The sister of the propositus, who had normal Lutheran antigens, had the common *GATA1* sequence. The effects of the *GATA1*

Table 6.5 Frequency of Lu_{null} and Lu_{mod} in several populations.

Population	No. tested	No. of $Lu_{null/mod}$	Incidence of $Lu_{null/mod}$	Antibodies used for screening	References
S. London, UK	~250 000	79	~0.0003	Anti-Lu^b (-Lu^a)	[27]
Sheffield, UK	18 069	1	0.0001	Anti-Lu3	[90]
Cambridge, UK	3197	1	0.0003	Not stated	[103]
S. Wales	75 614	15	0.0002	Anti-Lu^b (-Lu^a)	[28]
Houston, USA	42 000	8	0.0002	Anti-CD44*	[125]
Portland, USA	2400	3	0.0012	Anti-AnWj*	[131]
Detroit, USA; African Americans	7314	2	0.0003	Not stated	[132]
Taiwan Chinese	1922	1	0.0005	Anti-Lu^b, -Lu^a	[26,133]

*Only In(Lu) phenotype detected.

mutation in the propositus appear to have gone further than his unusual blood group phenotype: he had a haemoglobin level slightly below normal (122 g/L) and a low platelet count (86×10^9/L) with occasional macrothrombocytes and a history of bruising.

6.9 Acquired Lu(a–b–) phenotypes

A bizarre case is reported of an autoimmune thrombocytopenic purpura (AITP) patient with an antibody resembling anti-Ku, whose red cells had temporarily lost their Kell system antigens [138]. These red cells had normal expression of Lu^a, Lu^b, and LW^a. One year later the Kell antigens had returned to normal and the anti-Ku-like had disappeared, but now the cells lacked Lutheran antigens and the patient had produced an antibody resembling anti-Lu3. Expression of LW^a was also extremely depressed. Lu(a–b+) red cells of another patient with AITP became Lu(a–b–), but retained normal AnWj and LW expression [139]. This patient also had an antibody Lu3-like.

6.10 Distribution, functions, and disease associations

6.10.1 Distribution of the Lu-glycoproteins

Lu^b was not detected on lymphocytes, granulocytes, monocytes, platelets, or the erythroleukaemic cell lines K562 and HEL [1,140,141]. The Lu-gps are widely distributed. They are under developmental control in the liver, with a high level of expression on fetal hepatic epithelial cells during the first trimester. They are also present in placenta, in arterial walls of a variety of adult tissues, including tongue, trachea, oesophagus, skin, cervix, ileum, colon, stomach, and gall bladder, and in the basement membrane region of superficial epithelia and around mucous glands [4]. Both *LU* transcripts were detected in all tissues analysed, with the 2.5 kb transcript, encoding the larger (85 kDa) isoform, predominant except in a colon carcinoma cell line [6].

6.10.2 Functional aspects

The Lu-gps are members of the immunoglobulin superfamily of adhesion molecules, receptors, and signal transducers with five (V-C1-I-I-I) IgSF domains (Figure 6.2). Laminin is a family of extracellular matrix glycoproteins abundant in basement membranes and also present in vascular endothelia. They are heterotrimers composed of three genetically distinct chains, α, β, and γ. At least 12 laminin isoforms exist, derived from combinations of five different α chains, three β chains, and three γ chains [142,143]. Lu-gps bind specifically and with high affinity to the laminin-type globular domains 1-3 of the α5 chains of the two isoforms of laminin that contain α5 chains (LN-511 and -521) [144,145]. Lu_{null} red cells, which have no Lu-gps but normal expression of the other putative laminin binding protein CD44, bind no laminin [146]. Both isoforms of the Lu-gp have the same laminin binding capacity [147,148]. Transfection of human and murine erythroleukaemia cell lines with *LU* cDNA induced binding of solubilised and immobilised laminin [144,146,147]. The laminin-binding site is formed by Asp312 and a surrounding group of negatively charged residues in the region of the flexible linker between IgSF domains 2 and 3 of the Lu-gps (Figure 6.2) [9,10], although Arg175 in IgSF2, the key residue for Lu4 expression (Section 6.4.4), was crucial for binding of a monoclonal antibody that inhibits laminin binding [72]. *N*-glycosylation is not involved in laminin binding [146].

The cytoplasmic domain of the 85 kDa isoform, but not the 78 kDa isoform, contains an SH3 binding motif and five potential phosphorylation sites [4]. Adhesion events involving laminin appear to be controlled, at least in the 85 kDa isoform, by phosphorylation of serines 596, 598, and 621 [7] (see Section 6.10.3). Both isoforms interact directly with αI-spectrin, the main component of the red cell cytoskeleton, through Arg-Lys at positions 573–574 of their cytoplasmic tails [144,149–151]. This interaction with spectrin appears to modulate the adhesive activity of the Lu-gps as disruption of the interaction resulted in weakened linkage to the cytoskeleton and enhanced adhesion of red cells to laminin [150,151]. Phosphorylation of the cytoplasmic tail might weaken its interaction with spectrin, enabling the freely floating transmembrane molecules to cluster and generate a larger adhesive force. Lu-gps in kidney epithelial cells bind non-erythroid αII-spectrin through Arg573–Lys574, mediating actin reorganisation during cell adhesion and spreading on LN-511/521 [152].

During erythropoiesis *ex vivo*, the Lu-gps appear on the erythroid cells at about the orthochromatic erythroblast stage [153,154]. This late appearance correlates with binding of the cells to laminin [147]. The presence of LN-511/521 on the subendothelium basement membrane of bone marrow sinusoids has led to speculation that the Lu-gps are involved in facilitating movement of maturing erythroid cells from the erythroblastic islands

of the bone marrow (see Section 16.8), across the sinusoidal endothelium, to the peripheral circulation [144,153]. No obvious pathology, however, is associated with Lu_{null} or Lu_{mod} phenotypes, in which erythroid cells lack or have very low expression of the Lu-gps.

A mouse gene encoding a protein with 72% identity to human Lu gp binds LN-511/521 [144,155], but murine red cells do not express Lu-gps [156]. Lutheran-null mice are healthy and develop normally, yet, despite apparently normal renal function, 90% of their glomeruli exhibited abnormalities [156].

6.10.3 Disease associations

The IgSF gps expressing Lutheran and LW (Chapter 16) blood group activity are overexpressed on SS red cells in sickle cell disease: SS red cells express about 67% more Lu-gp than normal cells and bind increased quantities of laminin [146–148]. Enhanced binding of the Lu-gps to LN-511/521 on the endothelia of inflamed or damaged blood vessels could contribute to blockage of the vessels and the painful episodes of vaso-occlusion often suffered by sickle cell patients [157]. Although LN-511 and -521 are usually considered unique ligands of the Lu-gps, the integrin α4β1 or very late antigen-4 (VLA-4) on SS reticulocytes and peripheral blood mononuclear cells may bind Lu-gps on mature red cells and on endothelial cells, contributing to the vaso-occlusion [158,159].

Only SS red cells bind laminin and resist high shear-stress forces, despite the presence of Lu-gps on normal (AA) red cells [146,147]. Protein kinase A (PKA)- or Rap-1-dependent phosphorylation of Lu-gp Ser621 in SS red cells, stimulated by the physiological stress mediator epinephrine through the β2-adrenergic receptor, could induce conformational changes to the external domains of these proteins, modulating their attraction to their corresponding ligands on endothelial cells [7,157,160,161]. Hydroxyurea, a drug that reduces the frequency and severity of vaso-occlusive crises in sickle cell disease, increased levels of Lu-gp on SS red cells, but decreased their adhesion to laminin by inhibiting phosphorylation [162].

Red cells of two hereditary spherocytosis patients with 40% spectrin deficiency demonstrated enhanced adhesion to laminin under physiological flow conditions [151]. This adhesion, which was completely abolished by soluble Lu-gp, was brought about by impaired interaction between the Lu-gps and the cytoskeleton, rather than by phosphorylation (see Section 6.10.2).

Polycythemia vera (PV) is a chronic myeloproliferative disease in which clonal proliferation of multipotent haemopoietic cells results in an increase in the red cell mass. It is usually associated with a somatic mutation in the gene for JAK2 tyrosine kinase (Val671Phe) and is often complicated by thrombotic events [163]. The Lu-gps are phosphorylated in PV, but not in normal cells under the same conditions [164]. Expression of recombinant JAK2 containing the PV mutation in an erythroid cell line potentiated Lu-gp phosphorylation. As phosphorylation of the Lu-gps increases red cell adhesion, this increased red cell adhesiveness could be a factor promoting thrombosis in PV [164].

B-CAM, the 78 kDa isoform of the Lu-gps, is upregulated following malignant transformation of a variety of cell types [5,157]. For example, it is strongly induced in basal cell carcinomas and squamous cell carcinomas, two of the most frequent human malignancies [165]. Furthermore, laminin was also upregulated in sites surrounding the tumour nests, suggesting that interaction between the Lu-gp and laminin may be involved in progression of epithelial skin cancers.

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7 Kell and Kx Blood Group Systems

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7.1 Introduction

Kell was the first of many blood group systems disclosed by the antiglobulin test [1]. When Allen *et al.* [2] described the fourth Kell system antigen, Kp^b, they concluded prophetically, 'There is, probably, much still to be learned about the Kell blood group system'. There are now 35 antigens in the Kell system (Table 7.1) and Kell is closely related to the Kx system.

There are seven sets of antigens in the Kell system with allelic relationships: K and k; Kp^a, Kp^b, and Kp^c; Js^a and Js^b; K11 and K17 (Wk^a); KEL14 and KEL24; KEL25 and KEL28; KEL31 and KEL38. There are an additional 17 high frequency antigens and three low frequency antigens. All have been shown to be associated with nucleotide changes in *KEL*, except KEL13, which has been shown to be on the Kell-glycoprotein, and Km, which requires the presence of Xk (see below). Recombination as a result of crossing-over has never been observed within *KEL*.

None of the Kell antigens are expressed on cells of the Kell-null phenotype, K_o, which arises from homozygosity for *KEL* inactivating mutations. Ku antigen is present on all cells save those of the K_o phenotype. In K_{mod}, which also arises from *KEL* mutations, all antigens of the Kell system are expressed weakly (Section 7.7).

Several rare phenotypes occur in which all or most of the high frequency Kell antigens are expressed only weakly. Some are due to epistasis, such as the McLeod phenotype and depressed Kell associated with some Gerbich negative phenotypes, and some arise from interactions within the *KEL* gene. In patients with Kell-related autoantibodies, the depressed Kell phenotype may be acquired and transient (Section 7.9).

The Kell antigens are located on CD238, a red cell transmembrane glycoprotein of apparent MW 93 kDa, a metalloendopeptidase that processes endothelin-3 (Section 7.2).

The *KEL* gene is situated on chromosome 7q33 and consists of 19 exons.

McLeod syndrome is a form of neuroacanthocytosis, which includes an abnormal Kell red cell phenotype. McLeod phenotype red cells have depressed Kell antigens and lack the high frequency antigen Kx. The inheritance of Kx is controlled by an X-borne gene, *XK*, and represents a blood group system (the Kx system) independent of Kell. The Xk protein and Kell glycoprotein are linked by a disulphide bond. The Kx system is described in this chapter (Section 7.13) because of its phenotypic and biochemical associations with Kell.

The numerical antigen notation is generally used in this chapter, except for those antigens more commonly known by their traditional symbols: K, k, Kp^a, Kp^b, Kp^c, Ku, Js^a, Js^b, Ul^a, and Km.

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Table 7.1 Antigens of the Kell system.

Antigen				Molecular basis*		
No.	Name	Frequency	Antithetical	Nucleotides†	Exon	Amino acids
KEL1	K	Polymorphic	k	578C>T	6	Thr193Met
KEL2	k	High	K	578C (T)	6	Thr193 (Met)
KEL3	Kp^a	Polymorphic	Kp^b (Kp^c)	841C>T	8	Arg281Trp
KEL4	Kp^b	High	Kp^a	1. 841C (T)	8	Arg281 (Trp)
			Kp^c	2. 842G (A)	8	Arg281 (Gln)
KEL5	Ku	High		Various		Various
KEL6	Js^a	Polymorphic	Js^b	1790T>C	17	Leu597Pro
KEL7	Js^b	High	Js^a	1790T(C)	17	Leu597 (Pro)
KEL10	Ul^a	Low		1481A>T	13	Glu494Val
KEL11	K11 (Côté)	High	KEL17	905T (C)	8	Val302 (Ala)
KEL12	K12 (Boc)	High		1523A (G)	15	His548 (Arg)
KEL13	K13	High		986T (C)	9	Leu329 (Pro)
KEL14	K14 (San)	High	KEL24	1. 539G (C)	6	Arg180 (Pro)
				2. 538C (T)	6	Arg180 (Cys)
				3. 539G (A)	6	Arg180 (His)
KEL16	'k-like'	High		Not known		
KEL17	K17 (Wk^a)	Low	KEL11	905T>C	8	Val302Ala
KEL18	K18	High		1. 388C (T)	4	Arg130 (Trp)
				2. 389G (A)		Arg130 (Gln)
KEL19	K19 (Sub)	High		1475G (A)	13	Arg492 (Gln)
KEL20	Km	High				Absence of Xk
KEL21	Kp^c	Low	Kp^b (Kp^a)	842G>A	8	Arg281Gln
KEL22	K22	High		965C (T)	9	Ala322 (Val)
KEL23	K23	Low		1145A>G	10	Gln382Arg
KEL24	K24 (Cls)	Low	KEL14	539G>C	6	Arg180Pro
KEL25	VLAN	Low	KEL28	743G>A	8	Arg248Gln
KEL26	TOU	High		1217G (A)	11	Arg406 (Gln)
KEL27	RAZ	High		745G (A)	8	Glu249 (Lys)
KEL28	VONG	Low	KEL25	742C>T	8	Arg248Trp
KEL29	KALT	High		1868G (A)	17	Arg623 (Lys)
KEL30	KTIM	High		913G (A)	8	Asp305 (Asn)
KEL31	KYO	Low	KEL38	875G>A	8	Arg292Gln
KEL32	KUCI	High		1271C (T)	11	Ala424 (Val)
KEL33	KANT	High		1283G (T)	11	Arg428 (Leu)
KEL34	KASH	High		758A (G)	8	Tyr253 (Cys)
KEL35	KELP	High		708G (T), 2024G (A)	8,18	Leu260 (Phe), Arg675 (Gln)
KEL36	KETI	High		1391C (T)	12	Thr464 (Ile)
KEL37	KUHL	High		877C>T	8	Arg293 (Trp)
KEL38	KYOR	High	KEL31	875G (A)	8	Arg292 (Gln)

*Molecular basis of antigen-negative phenotype in parentheses.
†1 is the first nucleotide of the translation-initiating codon, which is 120 bp downstream of the traditional position for the first nucleotide in early reports.
Obsolete: KEL8, previously Kw; KEL9, previously KL; KEL15, previously Kx (now XK1).

7.2 The Kell glycoprotein and the gene that encodes it

7.2.1 The Kell glycoprotein

A glycoprotein of apparent MW 93 kDa was isolated from detergent-solubilised red cell membranes in the 1980s by immunoprecipitation with antibodies to Kell-system antigens: anti-K, -k, -Js^b, or -KEL22 [3–5]. Treatment of the Kell glycoprotein with an *N*-glycanase reduced the apparent MW by about 15 kDa, whereas *O*-glycanase had little effect. The Kell glycoprotein is phosphorylated, but not palmitoylated [6,7]. Kell antibodies do not generally react with isolated Kell glycoprotein by immunoblotting, though mouse monoclonal and rabbit antibodies, produced by immunising animals with purified Kell glycoprotein, detect the 93 kDa Kell glycoprotein on immunoblots [3,8]. No Kell glycoprotein was detected on blots of K_o cells or isolated from K_o cells by immunoprecipitation with a variety of polyclonal and monoclonal Kell antibodies.

Based on the amino acid sequence of a tryptic peptide, primers were synthesised and a specific oligonucleotide probe prepared by the polymerase chain reaction. Lee *et al.* [9] used this probe to screen a human bone marrow cDNA library and a clone was isolated with an open reading frame encoding a 732 amino acid polypeptide. Rabbit antibody prepared to a synthetic 30 amino acid peptide derived from the cDNA sequence bound to Kell glycoprotein on an immunoblot.

Hydropathy analysis indicated a type II membrane protein with a single hydrophobic membrane-spanning region, a highly hydrophilic N-terminal cytoplasmic domain of 47 amino acids (or 28 amino acids if the codon for Met 20 is used for translation initiation), and a large, 665-amino acid, C-terminal extracellular domain (Figure 7.1). The N-terminal methionine residue is probably cleaved from the mature protein. The extracellular domain has six Asn-Xxx-Ser/Thr putative *N*-glycosylation sites (positions 94, 115, 191, 345, 627, and 724), though Asn724 is unlikely to be glycosylated as residue 725 is proline, which usually inhibits glycosylation. There are15 extracellular cysteine residues, suggesting the presence of seven intramolecular disulphide bonds, resulting in extensive folding of the molecule. The Kell protein has structural and sequence homology with a family of zinc-binding endopeptidases (for functional aspects see Section 7.12) and has been modelled, based on the crystal structure of the external domains of neutral endopeptidase 24.11 (NEP) and endothelin-converting enzyme 1 (ECE-1) [10,11]. The Kell-glycoprotein has two globular extracellular domains, consisting mostly of α-helical segments. The domain closest to the membrane contains the N- and C-terminal sequences and the enzyme-active site; the outer domain contains almost all of the amino acid sites responsible for Kell-system alloantigenicity [10].

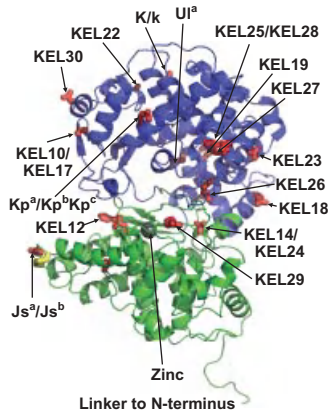


Figure 7.1 Homology model for Kell ectodomain, with the highly conserved peptidase domain in green and the variable distal membrane domain in blue. The linker region is the site of attachment to the transmembrane domain. Positions associated with Kell antigens are labelled. Modified from [11].

Kell glycoprotein is closely associated in the membrane with the Xk protein and a 120 kDa heterodimer can be isolated by immunoprecipitation under non-reducing conditions [12]. The two proteins are linked by disulphide bonding between Cys72 of Kell and Cys347 of Xk [13] (Section 7.13.2). The Kell-Xk heterodimer is part of the 'junctional' or 4.1R red cell membrane complex that contains band 3, Rh proteins, and glycophorin C, and is linked to the spectrin-actin junction of the cytoskeleton through protein 4.1R and p55 [14] (see Section 10.7 and Figure 10.2).

With Fab fragments of three monoclonal antibodies directed at epitopes on the Kell glycoprotein, figures of 4000–8000 sites per red cell were obtained, but Fab fragments from a fourth antibody gave a figure of 18 000 sites per cell [15]. The number of K antigen sites per red cell has been estimated as 4000–6200 on K+ k– cells and 2500–3500 on K+ k+ cells by use of radioiodinated polyclonal and monoclonal anti-K [16,17].

7.2.2 Organisation of the *KEL gene*

KEL spans about 21.5 kb organised into 19 exons of coding sequence [18] (Table 7.2). Exon 1 encodes a possible translation initiating methionine residue and Sp1 and GATA-1 binding sites. The exon 1 region is involved

Table 7.2 Exon/intron organisation of *KEL*.

Exon	Codons	3′ intron size kb	Comments
1	5′UT Met1	0.34	
2	2–27	0.29	Cytoplasmic
3	28–74	0.26	Transmembrane
4	75–133	~2.6	KEL18
5	134–175	0.33	
6	176–224	~3.2	KEL14/KEL24, K/k
7	225–245	0.093	
8	246–308	0.23	KEL25/KEL28, KEL27, KEL34, KEL35, $Kp^a/Kp^b/Kp^c$, KEL31, KUHL, KEL11/KEL17, KEL30
9	309–358	~1.3	KEL22, KEL13
10	359–401	~6	KEL23
11	402–438	~1.6	KEL26, KEL32, KEL33
12	439–471	0.24	KEL36
13	472–497	0.44	KEL19, Ul^a
14	498–531	0.19	
15	532–568	0.15	KEL12
16	569–590	0.23	HELLH
17	591–647	0.35	Js^a/Js^b, KEL29
18	648–679	~1.3	KEL35
19	680–732 3′UT		

UT, untranslated; HELLH, consensus sequence for zinc neutral endopeptidases.

in negative regulation of the promoter in non-erythroid tissue [19]. Exon 2 encodes the cytoplasmic domain and a second possible translation initiation site at Met20, exon 3 the membrane-spanning domain, and exons 4–19 the large extracellular domain. The 5′ flanking region to nucleotide −176 contains two GATA-1 binding sites and a CACCC box [18].

7.2.3 Linkage and chromosome location

KEL was located on 7q33 through linkage to *PIP*, the gene for prolactin-inducible protein [20], indirect linkage to the cystic fibrosis gene (*CFTR*) [21], and *in situ* hybridisation with cDNA encoding the Kell protein [22,23]. An analysis of 31 families informative for segregation of Yt blood group gene and *KEL* revealed loose linkage between these loci, with maximum likelihood of a recombination fraction of 0.26 [24].

7.3 K and k (KEL1 and KEL2)

In 1946, in the first report on the applications of the direct antiglobulin test, Coombs, Mourant, and Race [1] described an antibody of new specificity. This antibody, originally called anti-Kell and subsequently anti-K or anti-KEL1, reacted with the red cells of the husband and two children of the antibody producer and with about 7% of random blood samples [25].

Three years later, Levine *et al.* [26] described anti-Cellano, an antibody antithetical to anti-K. As *k* had already been used to represent the common allele of *K*, the symbol k was subsequently adopted for the product of that gene, despite K and k being products of co-dominant alleles.

Kell antigens are well developed at birth. K was found in fetuses of 10–11 weeks gestation and k at 6–7 weeks [27].

7.3.1 The molecular basis of the K/k polymorphism

The k/K polymorphism results from a C578T transition within exon 6 of the *KEL* gene, which gives rise to an amino acid substitution in the Kell glycoprotein: Met193 in K and Thr193 in k [28,29]. A *Bsm*I (*Bsa*MI) restriction site is present in *KEL*01* (*K*), but not *KEL*02* (*k*) [28] (Table 7.3). In the *KEL*02* product, Asn-Arg-Thr193 is a

Table 7.3 Sequences from nucleotides 571–582 of *KEL*, encoding amino acids 191–194, in four alleles encoding k, K, and two variants.

Allele						Antigen
				578		
k	*KEL*02*	AAC	CGA	A**C**G	CTG	
		Asn[N]	Arg	**Thr**	Leu	k
K	*KEL*02*	AAC	C<u>GA</u>	<u>ATG</u>	<u>C</u>TG	
		Asn	Arg	**Met**	Leu	K
	*KEL*01.2* [30]	AAC	CGA	**TC**G	CTG	
		Asn[N*]	Arg	**Ser**	Leu	weak K, possibly k
	*KEL*01M.01* [31]	AAC	CGA	A**G**G	CTG	
		Asn*	Arg	**Arg**	Leu	very weak K
				193		

N, *N*-glycosylation. **N***, probable *N*-glycosylation. *, probable no *N*-glycosylation. Underlining in *K* allele sequence shows *Bsm*I restriction site.

consensus sequence for *N*-glycosylation of Asn191, whereas Asn-Arg-Met in the product of *KEL*01* is not. Immunoblotting revealed that the K and k proteins are of apparent MW 110 and 115 kDa, respectively, supporting the suggestion that Asn191 is glycosylated in the latter, but not in the former [32,33]. Enzymatic deglycosylation of the Kell glycoprotein on red cells did not affect binding of either anti-K or -k to cells of the appropriate phenotype, demonstrating that k expression is not dependent on *N*-glycosylation of Asn191 [33].

7.3.2 Frequencies of K and k

In tests on nearly 10 000 English blood donors (mostly white), 9.02% were K+ [34]. From this figure the following gene and genotype frequencies have been calculated: *K* 0.0462; *k* 0.9538; *K/K* 0.0021; *K/k* 0.0881; *k/k* 0.9097 (assuming *k* is the only allele of *K*). K is much less common in Africans and extremely rare in eastern Asia and in Native Americans [35] (Table 7.4). K achieves its highest level among people of the Arabian and Sinai peninsulas, where up to 25% may be K+.

The k antigen has a high incidence in all populations. From the gene frequencies given above it can be estimated that the incidence of K+ k– would be 1 in 476. The incidence of k– was found to be one in 549 London blood donors [50].

Table 7.5 shows genotype frequencies in Austrian blood donors obtained by genotyping with allele-specific primers [51].

7.3.3 K/k genotyping

A variety of methods, involving SNP testing in exon 6 of *KEL*, are available for predicting K/k phenotypes for DNA. All these tests may give a false prediction if a Kell null or mod allele or the *K* variant allele *KEL*01.2* is present (Sections 7.3.4 and 7.7).

Anti-K is a relatively common cause of severe HDFN (Section 7.3.5.2), so in pregnant women with anti-K it is advantageous to predict fetal K phenotype. This can be done from fetal DNA in maternal plasma, thus avoiding invasive procedures such as amniocentesis or chorionic villus sampling (see Section 5.7.1). Finning *et al.* [52] achieved 98.6% accuracy by real-time quantitative PCR, involving the application of locked nucleic acids to prevent mispriming of the *KEL*01*-specific primer on the *KEL*02* allele. This technology is now employed in England to provide a routine service to pregnant women with anti-K, with a level of accuracy of around 99.6% [53]. A method for fetal *K* detection incorporating matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) gave 94% accuracy [54].

K genotyping has also been applied to preimplantation genetic diagnosis (PGD). In K– women with a K+ k+ partner, half of the embryos derived from *in vitro* fertilisation (IVF) will have a paternal *KEL*01* allele and half will not. K genotyping of DNA from individual blastomeres obtained by IVF was used for PGD to ensure implantation of a K– embryo in two women with anti-K,

Table 7.4 Frequency of some Kell system antigens and deduced gene frequencies.

Antigen	Population	No. tested	Positive (%)	Gene frequency	References
K	English	9875	9.02	0.0462	[34]
	Parisians	81 962	8.55	0.0437	[36]
	Finns	5000	4.10	0.0207	[37]
	African Americans	4079	1.50	0.0075	[38]
	Japanese	14 541	0.02	0.0001	[39]
Kp^a	White people	18 934	2.28	0.0114	[34,40–42]
Kp^c	Japanese (Osaka)	4442	0.32	0.0016	[*]
	Japanese (Miyagi)	5974	0.18	0.0009	[43]
Js^a	African Americans	1298	15.87	0.0828	[38,44,45]
	Black Africans	593	15.68	0.0818	[46]
Ul^a	Finns	2620	2.6	0.0131	[37]
	English	5000	0	0.0000	[37]
	Swedes	501	0.2	0.0011	[37]
	Chinese	12	1 pos.		[37]
	Japanese	8000	0.46	0.0023	[47]
KEL17	English	11 044	0.29	0.0015	[48]
KEL31	Japanese	400	1.5	0.0075	[49]

*H. Yamaguchi, Y. Okubo, T. Seno, unpublished observations.

Table 7.5 Results of genotyping tests on about 11 000 Austrian blood donors [51].

	*KEL*02* *k*	*KEL*04* *Kp^b*	*KEL*07* *Js^b*	*KEL*11* *K^{11}*
Homozygous positive (%)	91.98	98.31	99.99	99.61
Heterozygous positive (%)	7.94	1.67	0.01	0.39
Negative (%)	0.08	0.02	0	0

both of whom had previously lost babies as a result of fatal HDFN [55].

7.3.4 Unusual K and k expression

Red cells of two unrelated Swiss-German blood donors, and the mother of one of them, reacted with most polyclonal and monoclonal anti-K, albeit slightly less strongly than with normal K+ cells, but gave significantly weak or negative reactions with some anti-K reagents [30]. Like *KEL*02* homozygotes, they were homozygous for 578C, but were also heterozygous for 577A>T, encoding Ser193 (*KEL*01.02*) (Table 7.3). Ser193 would be expected to support *N*-glycosylation of Asn191. It is somewhat surprising that Ser193 would be responsible for K expression, particularly as flow cytometric analyses suggested a homozygous dose of k. Consequently the *KEL*01.02* allele appears to produce both variant K and k antigens. Poole *et al.* [30] suggest that anti-k detect an epitope that is not substantially altered by a Thr193Ser substitution, whereas anti-K recognise a distinct conformational epitope created by any substitution of Thr193. A third unrelated example of this variant was found in an American Caucasian [56]. Despite encoding K, *KEL*01.02* would be recognised as a *KEL*02* allele by most genotyping systems.

Weak K is produced by the very rare *KEL*01* alleles that also produce Kp^a [57] (Section 7.4). Whereas this K could be missed by some serological methods, it would be predicted by genotyping.

The term K_{mod} was used for four individuals with very weak K that could only be detected by adsorption and elution, no k, and weak expression of high frequency Kell-system antigens [31]. All four were homozygous for a *KEL* 578C>G encoding Thr193Arg (*KEL*01M.01*) (Table 7.3). Weakness of other Kell antigens was due to reduced quantity of Kell glycoprotein. Heterozygosity for

other K_{mod} alleles on a *KEL*01* background has also been responsible for weakened expression of K.

A K+ woman had an antibody resembling anti-K in the serum. Her red cells and those of her daughter expressed a weak K antigen, which did not react with her K-like antibody [58]. Weak expression of K has also occurred in the McLeod [59] and Gerbich-negative [60] phenotypes (Sections 7.10 and 7.13).

During a terminal episode of sepsis, red cells of a patient previously known to be K– k+ became K+, as did K– transfused cells [61]. Post-mortem blood samples contained a Gram-positive organism, *Streptococcus faecium*. K– red cells incubated with a culture containing disrupted *S. faecium* were converted to K+.

Weakness of k in K+ k+ individuals, associated with a missense mutation in the *KEL* gene, is likely to result from heterozygosity for a K_{mod} allele and *KEL*01*, the weakness of the other high frequency Kell antigens being masked by normal strength antigens being produced by the *KEL*01* allele (Section 7.7.2).

7.3.5 Anti-K

7.3.5.1 Alloanti-K

Anti-K is the most common immune red cell antibody outside of the ABO and Rh systems; about two-thirds of non-Rh red cell immune antibodies are anti-K [62]. Giblett [63] estimated the relative potency of antigens in stimulating the formation of antibodies and, excluding ABO and D, K attained the highest score with a relative potency of twice that for c, about 20 times that for Fy^a, and over 100 times that for S. Anti-K is often found in sera containing antibodies to high incidence Kell system antigens. Seventy-five percent of people with IgG autoantibodies related to the Kell system also have alloanti-K in their serum [64]. Conflicting results were obtained in two studies comparing HLA Class II genotypes with anti-K production. In one, anti-K alloimmunisation was not restricted by HLA-DRB1 genotype [65], whereas in the other study frequencies of *HLA-DRB1*11* and *HLA-DRB1*13* were significantly higher in patients with anti-K, than in those without [66].

Anti-K, like other Kell system antibodies, are generally IgG, and predominantly IgG1 [67]. Although IgG anti-K may occasionally agglutinate K+ red cells directly, the antiglobulin test is usually the method of choice. Anti-K often react poorly in low ionic strength solutions (LISS) [68,69] and fewer molecules of anti-K bind to red cells in LISS than in normal strength saline [70]. Problems in detecting anti-K have also been encountered in automated systems [71].

A few examples of apparent non-red cell immune anti-K have been described in untransfused, healthy, male blood donors [72,73]. Microbial infection has been associated with the presence of IgM or IgA anti-K [74–78]. *Escherichia coli*, *Enterococcus faecalis*, *Morganella morganii*, and mycobacterium, responsible for pulmonary tuberculosis, have been implicated. In some cases cell-free preparations from these stool cultures inhibited IgM anti-K and K antigens were detected on the bacterial cells [74,79].

Human monoclonal anti-K are generally used as grouping reagents. Murine monoclonal anti-K have been produced by immunising mice with plasmids encoding K, followed by a boost injection of plasmid-transfected cells [80].

7.3.5.2 Clinical significance of anti-K

All Kell-system antibodies must be considered potentially clinically significant and, where possible, antigen-negative red cells should be selected for transfusion. Anti-K can be responsible for severe and fatal HTRs.

Anti-K can cause severe HDFN [62,81]. In one series of tests [82], maternal anti-K was detected in 127 of 127 076 pregnancies (0.1%); 13 of the pregnancies with maternal anti-K produced a K+ baby, five (38%) of whom were severely affected with HDFN. K immunisation usually results from transfusion. In a Dutch survey, 83% of women with anti-K had a history of red cell transfusion [83]. It is common practice, therefore, for girls and premenopausal women to be transfused only with K– red cells. In addition, first trimester screening for red cell antibodies in the Netherlands resulted in an improvement from 61 to 100% survival of severely affected fetuses in K alloimmunised pregnancies [84].

Unlike RhD (Section 5.18.1.4), no prophylaxis is available for the prevention of K alloimmunisation during pregnancy and at delivery. A 15-residue peptide representing the K protein, with Met179 at the C-terminus, was identified as the major helper T-cell epitope in the alloresponse to K [85]. Administration of this peptide via a suppressive route, such as the nasal mucosa, may have the potential to reduce or prevent K alloantibody production in susceptible women.

The pathogenesis of anti-K HDFN differs from that caused by anti-D. Severity of the anti-K disease is harder to predict than the anti-D disease. There is very little correlation between anti-K titre and severity of disease [62], though severe HDFN due to anti-K of titre less than 32 is extremely rare [86]. Anti-K HDFN is associated with

lower concentrations of amniotic fluid bilirubin than in anti-D HDFN of equivalent severity and post-natal hyperbilirubinaemia is not prominent in babies with anaemia caused by anti-K [82,87,88]. There is also reduced reticulocytosis and erythroblastosis in the anti-K disease, compared with anti-D HDFN. These symptoms suggest that anti-K HDFN is associated with a lower degree of haemolysis and the fetal anaemia appears to result predominantly from a suppression of erythropoiesis [87,88]. Kell glycoprotein appears on erythroid progenitors very early in erythropoiesis, whereas the Rh proteins are late to appear [89,90]. Vaughan *et al.* [91] found that *in vitro* growth of K+ erythroid blast-forming units (BFU-E) and colony-forming units (CFU-E) was specifically inhibited by monoclonal and polyclonal anti-K. They speculated that the Kell glycoprotein, an endopeptidase (Section 7.13), might be involved in regulating the growth and differentiation of erythroid progenitors, possibly by modulating peptide growth factors on the cell surface. Consequently, binding of anti-K to the Kell glycoprotein might impede its enzymatic activity and suppress erythropoiesis. Unfortunately, this theory does not take into account the K_o phenotype, in which no Kell glycoprotein is present on the surface of erythroid cells, yet erythropoiesis is apparently normal. It is more likely, therefore, that anti-K suppresses erythropoiesis through the immune destruction of early erythroid progenitors. Early erythroid progenitors cultured from $CD34^+$ cells derived from K+ neonates expressed K and elicited a strong response from monocytes in a functional assay in the presence of anti-K; no response was obtained with anti-D because Rh antigens do not appear on erythroid cells until much later, when they have become haemoglobinised erythroblasts [92]. Anti-k and anti-Kp^b also inhibit BFU-E growth *in vitro* [93].

In addition to inhibiting erythropoiesis, Kell antibodies also inhibit *in vitro* proliferation of granulocyte-monocyte and megakaryocyte progenitors (CFU-GM and CFU-MK) [75,76]. Pronounced thrombocytopenia, leukopenia, and neutropenia have been detected in cases of HDFN caused by anti-K [94,95].

7.3.5.3 'Mimicking' autoanti-K

Autoantibodies that appear to have K specificity, but which can be adsorbed and eluted from K– cells, have been detected in the serum and red cell eluates of K– patients [96–98]. These antibodies caused strong DATs and were not associated with any weakening of high frequency Kell-system antigens.

7.3.6 Anti-k

Less than 2 in 1000 people are k– and capable of making anti-k, yet many examples of this rare antibody have been described [99]. Most anti-k are IgG (often IgG1 [67]) and work best by the antiglobulin test, but cold agglutinating IgM anti-k are known [100,101]. Anti-k has been responsible for HTRs [99,102] and HDFN [99,102–104]. The characteristics of fetal anaemia (reticulocytopenia and normal bilirubin levels) caused by anti-k are similar to those due to anti-K, suggestive of suppression of erythropoiesis [104] (Section 7.3.5.2)

IgG1 and IgG2a monoclonal anti-k, which could not be adsorbed and eluted from k– red cells, have been raised in mice [105–107]. One antibody which behaved like anti-k, but did not react with k+ KEL:–22 red cells [108] (k and KEL22 are probably spatially related [109], Figure 7.1). Some murine monoclonal antibodies react with red cells of all Kell phenotypes except K_o, but react more strongly with K– k+ and K+ k+ cells than with K+ k– cells and may behave as anti-k at an appropriate dilution [80,107,110].

7.4 Kp^a, Kp^b, and Kp^c (KEL3, KEL4, and KEL21)

In 1957, Allen and Lewis [40] described anti-Kp^a and its probable antithetical antibody anti-Kp^b. Kell became a complex blood group system in the following year when Kp^a and Kp^b alleles were shown to be linked to *K* and *k* [2]. Family evidence confirmed this very close linkage; K+ Kp(a+) people never receive both *K* and Kp^a from the same parent and never pass them on to the same child. Despite numerous studies of families with K+ Kp(a+) propositi, the KKp^a allele was never been found. However, in 2009 gene sequencing demonstrated that two K+ k+ Kp(a+b+) unrelated Caucasians with very weak K antigens were heterozygous for KKp^a (*KEL*01.03*) and kKp^b (*KEL*02.04*) [57]. The suppressive effect of Kp^a on other Kell antigens expressed on the same molecule is described in Section 7.7.3.

Tests with anti-Kp^a on just under 19000 white people from Europe and North America [34,40–42], showed 2.28% to be Kp(a+), a gene frequency of 0.0114 for Kp^a (Table 7.4). A Kp^a gene frequency of 0.0086 in Austrian blood donors was obtained by molecular genotyping [51] (Table 7.5). Only 1.21% of K+ people are Kp(a+) [34]. Although about 9% of white people are K+, only 2.7% of Kp(a+) mostly white people from Boston were K+ [40].

Kp^a appears to be extremely rare in other ethnic groups. Kp^b is a public antigen in all populations.

In 1979, Yamaguchi *et al.* [111] found that the red cells of a Japanese blood donor were Kp(a–b–) with otherwise unremarkable Kell antigens. Her red cells reacted with the serum containing anti-Levay [112], an antibody to the first inherited private red cell antigen, originally reported in 1945 [113,114]. Study of the informative family of the Japanese propositus proved that Levay is the product of *Kp^c*, a third Kp allele. In one Japanese family, two Kp(a–b–c+) members appeared to be heterozygous for *Kp^c* and a Kell-null gene *K^o* [43]. The incidence of Kp^c in Japan is shown in Table 7.4. Other than the original Levay-positive propositus and her family, only one Kp(c+) individual has been found outside Japan: a Kp(a+b–c+) Spanish American with anti-Kp^b.

Kp^a and *Kp^c* differ from the common allele, *Kp^b*, by single nucleotide changes at adjacent sites within the same codon in exon 8 [115]. *Kp^b* has CGG encoding Arg281; *Kp^a* has TGG, Trp281 and *Kp^c* has CAG, Gln281. The *Kp^a* and *Kp^c* mutations introduce *Nla*III and *Pvu*II restriction sites, respectively. Site-directed mutagenesis experiments confirmed that the single base change is responsible for the Kp^a/Kp^b polymorphism [29].

7.4.1 Anti-Kp^a

The first (Penney) appeared to be 'naturally occurring' but, as with most anti-Kp^a, reacted best by the antiglobulin test [34,40]. Anti-Kp^a can cause delayed HTRs; only one case is reported as severe [116].Anti-Kp^a very rarely causes severe HDFN, but there are reports of a requirement for neonatal transfusion [117,118], one case of hydrops fetalis [119], and one neonate who presented with purpura, respiratory failure, severe liver dysfunction, hyperbilirubinaemia, and anaemia [120]. In one case, symptoms are described as consistent with suppression of erythropoiesis in addition to immune red cell destruction [118]. Owing to the relative rarity of Kp^a, anti-Kp^a, and serious clinical sequelae of incompatible transfusion, Kp(a+) red cells are not required in antibody screening panels [121].

Murine monoclonal anti-Kp^a have been produced by immunising mice with plasmid DNA followed by a boost injection of plasmid-transfected cells [80].

7.4.2 Anti-Kp^b

The first anti-Kp^b (Rautenberg) was found during routine crossmatching [2]; the serum also contained anti-K, as do some other examples [34]. Although anti-Kp^b is usually IgG (IgG1 + IgG4 [67]), two anti-Kp^b that appear to be 'naturally occurring' and did not react by the antiglobulin test have been mentioned [34].

Serious HDFN due to anti-Kp^b is very rare, but two cases are reported where obstetric intervention and, in one case, transfusion, were required [122,123]. Both mothers had been transfused during childhood. *In vivo* red cell survival studies and monocyte monolayer assays predict that anti-Kp^b has the potential to cause reduced survival of transfused Kp(b+) cells [124,125]. Anti-Kp^b has been responsible for a delayed HTR [124], although Kp(b+) units of blood have been administered to patients with anti-Kp^b, with no indications of transfusion reaction or reduced red cell survival [126,127].

Autoanti-Kp^b has been responsible for AIHA [128–130], in one case in a 12-week-old infant [129]. One autoanti-Kp^b was pure IgM [131]. Autoantibodies to Kell-system antigens are often associated with weakened expression of Kell (Section 7.9).

A murine monoclonal antibody (BRIC 203) defined an epitope shared by Kp^b and Kp^c, but not Kp^a [15]. A human single-chain Fv (scFv) antibody fragment specific for Kp^b has been isolated from a V gene phage-display library derived from non-immunised donors [132].

7.4.3 Anti-Kp^c

The first anti-Kp^c, which was called anti-Levay for 34 years, was made in response to transfusion in a patient who also made the first examples of anti-Lu^a, -C^w, and human anti-N [113,114]. Several more anti-Kp^c have been found since, all immune and all in Japanese.

7.5 Js^a and Js^b (KEL6 and KEL7)

Js^a, a new antigen present on the red cells of about 20% of African Americans, but in none of 500 white people, was first described in 1958 [44,133]. Anti-Js^b was found in 1963 in the serum of a Js(a+) black woman with four Js(a+) children [134,135]. This antibody failed to react with the red cells of 13 of 1269 black donors. Twelve of the 13 were tested with anti-Js^a and all were positive. The antibody did not react with the Js(a+) red cells of two sisters, believed to be homozygous for *Js^a* because all of their 10 children were Js(a+).

The first hint that Js^a and Js^b might belong to the Kell system came from the observation that cells of the Kell-null phenotype (K$_o$) were Js(a–b–) [38]. A search of 4000 black donors revealed six K+ Js(a+) propositi and the

subsequent family studies suggested control of Js^a and Js^b at the *KEL* locus. This was confirmed by four large Brazilian families with K+ Js(a+) propositi [136].

Js^a is almost completely confined to people of African origin [35]. The incidence of Js^a among African Americans is about 16%, giving a frequency of 8% for the Js^a gene (Table 7.4). Js^a is very rare in white people. Of 11 000 African Americans tested with anti-Js^b, 34 were Js(b–) [137]. The Js(a+b–) phenotype has not been reported in a person of non-African origin. Genetic testing revealed Js^a (*KEL*06*) frequencies of 8.18% and 11.68% in Afro-Caribbean donors and from donors originating from Ngazidja (an island off the east coast of Africa), respectively [138].

An 1790T>C SNP in exon 17 encoding a single amino acid substitution in the Kell glycoprotein is responsible for the Js^b/Js^a polymorphism: Leu597for Js^b; Pro597 for Js^a [139] (Table 7.1). This has been confirmed by site-directed mutagenesis experiments [29]. An *Mnl*I restriction site is eliminated in the Js^a allele. The Leu597Pro substitution is between two cysteine residues and could affect disulphide bonding and, consequently, folding of the molecule. In a A>G1899 synonymous SNP at Leu633 codon, about 80% of Js^b alleles have 1899A and 20% G1899 [140].

7.5.1 Anti-Js^a

Anti-Js^a generally react best by the antiglobulin test and are red cell immune in origin. An apparently 'naturally occurring' IgM anti-Js^a in a Japanese woman directly agglutinated Js(a+) cells [141].

Anti-Js^a has been responsible for HDFN, including hydrops in one case [142–144]. Two anti-Js^a, barely detectable by routine serological tests, caused delayed HTRs [145,146].

7.5.2 Anti-Js^b

All examples of anti-Js^b have been found in black people. They generally work best by the antiglobulin test. Anti-Js^b has caused severe HDFN resulting in fatal hydrops fetalis [147,148]. The poor predictive value of anti-Js^b titres is typical of Kell-system antibodies [149]. The mother of a hydropic baby received a transfusion of 275 ml Js(b+) red cells and suffered no symptoms of an HTR, although the survival of the Js(b+) cells was substantially reduced [147]. Anti-Js^b has been responsible for a delayed HTR [150]; multiple transfusions of Js(b+) red cells to transfusion-dependent patients with anti-Js^b have resulted in no adverse reactions [151].

Autoanti-Js^b, enhanced by polyethylene glycol, was detected in the serum of a Js(a–b+) renal patient whose red cells gave a weakly positive DAT [152].

A potent murine monoclonal IgG anti-Js^b was raised by immunising a mouse with a murine erythroleukaemia (MEL) cell line expressing recombinant human Kell glycoprotein [153]. Human-mouse chimeric IgM antibodies have been engineered from this clone, in order to produce a directly agglutinating reagent [154].

7.6 Other Kell-system antigens

In addition to the Kell polymorphisms – K/k, $Kp^a/Kp^b/Kp^c$, and Js^a/Js^b – a number of other Kell-system antigens are known, all of either high frequency or low frequency (Table 7.1). They are absent from K_o cells and expressed either weakly or not at all on McLeod phenotype cells. All have been allocated to the Kell system through family evidence, location of the antigen on the Kell glycoprotein by immunochemical means, and/or association of antigen presence or absence with a sequence change in the *KEL* gene. Ku and Km are described in Sections 7.7 and 7.13.3, respectively.

7.6.1 Ul^a (KEL10)

An incompatible crossmatch revealed an antibody, named anti-Ul^a, that reacted with the red cells of 2.6% of Helsinki blood donors, but is rare in most other populations [37]. Three families with K+ Ul(a+) members had demonstrated that Ul^a belongs to the Kell system [155]. An antibody antithetical to anti-Ul^a has not been found. Ul^a is often considered a predominantly Finnish characteristic, but 0.46% of Japanese [47] and one of 12 Chinese [37] were Ul(a+) (Table 7.4).

Ul^a results from a mutation encoding a Glu494Val substitution and acquiring an *Acc*I restriction site [115].

Anti-Ul^a is very rare. No anti-Ul^a was detected in the serum of 19 Ul(a–) mothers of Ul(a+) children [37]. One case of HDFN caused by anti-Ul^a is reported [156].

7.6.2 KEL11 and KEL17 (Wk^a)

The original anti-KEL11, found, in the serum of a French Canadian woman (Mrs Côté), reacted with all red cells tested except for her own, those of two siblings, and K_o phenotype cells, and reacted extremely weakly with McLeod phenotype cells [157]. Thus Côté serum appeared to contain an antibody recognising a new high frequency antigen related to the Kell system.

Anti-Wk^a reacted with red cells of 0.3% of English blood donors (Table 7.4), but with those of only 0.1% of K+ donors [48]. None of 1000 Kp(a+) donors was Wk(a+). The families of five K+ Wk(a+) donors showed that Wk^a was always inherited with k; there was no recombinant and 13 non-recombinants. KEL:–11 red cells were found to be Wk(a+) and the allelic status of *KEL11* and *Wk^a* was confirmed by family studies [48,158]. As KEL11 has never been called Wk^b, the numerical notation for Wk^a, KEL17, will now be used here.

*KEL*11* and *KEL*17* differ by a single nucleotide, creating an *Msc*I restriction site in the *KEL*17* allele. *KEL*11* encodes Val302; *KEL*17* encodes Ala302 [115].

Anti-KEL11 and -KEL17 are rare antibodies. A patient with anti-KEL11 was transfused with 11 units of KEL:11 red cells with no adverse clinical outcome [159]. ^{51}Cr-labelled KEL:11 cells survived normally and there was no increase in reactive monocytes in a monocyte monolayer assay. One example of anti-KEL11 was implicated in severe fetal anaemia, despite results in a chemiluminescent functional assay suggesting that the antibody was not clinically significant [160].

7.6.3 KEL12

Five examples of anti-KEL12 and four KEL:–12 propositi are reported [161–164]. All are white (although one was originally described as black [165]). Two of the propositi each had a KEL:–12 sibling [163,164]. One of the KEL:–12 propositi and her KEL:–12 sister had both been transfused and both had anti-KEL12 [163]. Two of the propositi were transfused with KEL:12 blood with no evidence of *in vivo* destruction [163,164].

Two unrelated KEL:–12 individuals had a mutation encoding His548Arg and abolishing an *Nla*III restriction site [32].

7.6.4 KEL13

The only reported KEL:–13 propositus was a much transfused man of Italian parentage [166]. His red cells and those of his KEL:–13 sister displayed weakened expression of k, Kp^b, Js^b, Ku, and KEL12 and gave an enhanced score with anti-Kx (typical of cells from a K^o heterozygote), suggesting that the KEL:–13 siblings have a K^o allele. This was confirmed by molecular testing, which revealed heterozygosity for one allele encoding Leu329Pro and the other containing a nonsense mutation, Gln532stop [167]. KEL:–13 can be considered a K_{mod} phenotype (*KEL*02M.03*). In transfected human embryonic kidney cells, fewer Leu329Pro mutant Kell proteins were transported to the cell surface, compared with control cells [167].

7.6.5 KEL14 and KEL24

The original anti-KEL14 was found in the serum of a white woman [161,168], and KEL14 was shown to be an inherited character retrospectively when Dp, a previously described public antigen [169], was found to be KEL14 [170]. The KEL:–14 propositus, a white woman with consanguineous parents, had four KEL:14 and two KEL:–14 siblings [169].

IgG and IgM murine and IgG human monoclonal anti-KEL14 have been produced [105,108].

An antibody in the serum of a white woman, which reacted with the red cells of her baby and several of the baby's relatives, appeared to be antithetical to anti-KEL14 and was numbered anti-KEL24 [171]. Anti-KEL24 reacted with all three KEL:–14 samples tested, but with none of 700 other red cell samples, and gave a higher titre with KEL:–14,24 cells than with KEL:14,24 cells.

Two unrelated KEL:–14,24 individuals had a mutation introducing a *Hae*III restriction site [172]: KEL14 represents Arg180; KEL24, Pro180. Two other mutations in two unrelated KEL:–14 Japanese encoded Arg180Cys and Arg180His [173], and the Arg180Cys mutation was also found in a KEL:–14 patient of Middle Eastern descent with anti-KEL14 [174] (Table 7.1).

7.6.6 KEL18

The only three reported KEL:–18 individuals were white and had anti-KEL18 [175–177]. Despite being serologically identical, the first two unrelated propositi had different single base mutations in the same codon, encoding different amino acid substitutions: Arg130Trp and Arg130Gln [32]. The two mutations created *Eco*571 and *Taq*II restriction sites, respectively. No example of KEL:–18 was revealed by tests on 54 450 blood donors [178].

In vivo survival studies and mononuclear phagocyte assays predicted that the original anti-KEL18 would not cause an acute HTR, but that transfusion therapy with KEL:18 red cells would be ineffective in all but an emergency [178]. One anti-KEL18 caused mild HDFN, necessitating phototherapy for hyperbilirubinaemia [177].

7.6.7 KEL19

The first anti-KEL19 was found in a KEL:–19 woman with a KEL:–19 brother and two KEL:19 sisters [179]. The second anti-KEL19, identified in the serum of a

black man, caused a delayed HTR, eliminating four units of incompatible blood [180]. None of 10 757 donors tested with anti-KEL19 was KEL:–19 [180]. Two unrelated KEL:–19 individuals had a mutation encoding Arg492Gln [32].

7.6.8 KEL22

Two examples of anti-KEL22 were found in the sera of unrelated Israeli women of Iranian Jewish origin, with a total of three KEL:–22 siblings [181,182]. Three unrelated KEL:–22 individuals had a mutation encoding Ala322Val [32].

Anti-KEL22 in the second KEL:–22 propositus was responsible for mild HDFN in her fourth and fifth children and severe HDFN in her sixth child, requiring exchange transfusion with the mother's washed red cells [182,183]. The IgG isotype was IgG1 during the fourth and fifth pregnancies and IgG1 plus IgG3 during the sixth.

7.6.9 KEL23

An antibody in the serum of a white woman of Italian ancestry reacted with red cells of her two children, her husband, and his mother, but with none of 2100 reference samples [184]. The antibody precipitated Kell glycoprotein from the husband's red cells and the antigen was designated KEL23. Red cells lacking high frequency Kell antigens were all KEL:–23. Two KEL:23 family members were heterozygous for a mutation encoding Gln382Arg and creating a *Bcn*I restriction site [32].

Anti-KEL23 caused a strongly positive DAT on the red cells of the third baby of the propositus, but did not cause HDFN [184].

7.6.10 KEL25 (VLAN) and KEL28 (VONG)

KEL25 and KEL28 are low frequency antigens representing mutations in the same codon encoding Arg248Gln and Arg248Trp, respectively [185,186]. Both antigens were initially shown to be located on the Kell glycoprotein by MAIEA analyses [186,187]. KEL25 was detected on the red cells of a Dutch blood donor when they were crossmatched with the serum of a patient of unknown transfusion history [187]. Two sisters and a niece of the donor were also KEL:25. Anti-KEL25 consisted of IgG1 and IgG2 isotypes and directly agglutinated KEL:25 red cells. None of 1068 donors was KEL:25. Anti-KEL28 was responsible for fetal anaemia, suggestive of suppressed erythropoiesis, in a family of ethnic Chinese from Timor [186].

7.6.11 KEL26 (TOU)

KEL26 is an antigen of high frequency absent from K_o cells and shown to be located on the Kell glycoprotein by a MAIEA analysis [188]. Two examples of anti-KEL26 have been identified, one in a Native American man and the other in a Latino woman. Neither had been transfused, but the woman had been pregnant twice. A mutation was detected, encoding Arg406Gln in three KEL:–26 samples from two families [32]. A monocyte monolayer assay suggested that the original anti-KEL26 was not clinically significant [188].

7.6.12 KEL27 (RAZ)

KEL27 is a high frequency antigen, not present on K_o cells, expressed weakly on McLeod phenotype cells, and located on the Kell glycoprotein as determined by a MAIEA assay [189]. Anti-KEL27 was found in a Kenyan Indian woman, the only KEL:–27 person known. She is homozygous for a mutation encoding Glu249Lys [185].

7.6.13 KEL29 (KALT)

Anti-KEL29 was found in a Mexican with a history of pregnancies, but no transfusion. She was homozygous for a mutation encoding Arg623Lys and deleting a *Tfi*I restriction site. KEL29 is the most C-terminal of the Kell antigens on the Kell glycoprotein and is unique for a Kell-system antigen because it is destroyed by trypsin treatment of intact red cells [190]. Consequently, the most N-terminal trypsin cleavage site must be between Leu597 (Js^b, trypsin-resistant) and Arg623.

Red cells of the baby of the propositus gave a positive DAT, but no treatment for HDFN was required.

7.6.14 KEL30 (KTIM)

Anti-KEL30 was made by a white American, with a history of pregnancies and transfusion. KEL:–30 resulted from homozygosity for a mutation encoding Asp305Asn eliminating a *Taq*I recognition site [190].

7.6.15 KEL31 (KYO), KEL38 (KYOR), and KEL37 (KUHL)

Anti-KEL31 was found through routine antibody screening of Japanese blood donors, but no further example of anti-KEL31 was found in 100 000 donors [49]. Six of 400 Japanese donors were KEL:31. KEL31 was located on the Kell glycoprotein by a MAIEA analysis and results from an Arg292Gln substitution. Two KEL31 Japanese patients who were homozygous for the mutation encoding Arg-292Gln had antibodies to KEL38, the antigen antithetical to KEL31 [191].

The only two known KEL:–37 individuals are an Asian woman and her sister with apparently normal Kell phenotypes, except that the red cells of the sister were KEL:–31 [192]. Both sisters are homozygous for a mutation encoding Arg293Trp, in the codon adjacent to that responsible for KEL:–31. Despite the propositus being Kp(a–b+), the antibody initially presented as an alloanti-Kp^b, but 4 years later reacted strongly with Kp(b–) red cells, but not with K_o cells.

7.6.16 KEL32 (KUCI) and KEL33 (KANT)

KEL32 and KEL33 are serologically related Kell antigens of high frequency. Anti-KEL32 was produced by a previously transfused African American woman with a KEL:–32 sibling [193]. Anti-KEL33 was produced by an individual of European origin. KEL:–32 red cells are also KEL:–33, but KEL:–33 red cells appear to be KEL:32 [193]. KEL:–32 results from homozygosity for a mutation encoding Ala424Val. The KEL:–33 individual was heterozygous for a mutation encoding Arg428Leu and a Kell-null mutation encoding Arg406stop. The close proximity of the amino acid changes responsible for the KEL:–32 and KEL:–33 phenotypes probably explains the partial serological compatibility.

7.6.17 KEL34 (KASH)

The antibody defining KEL34 is non-reactive with the red cells of the antibody maker, a woman of Pakistani origin, and with those of her brother, both of whom have a K_{mod} phenotype and are homozygous for a *KEL* allele encoding a Tyr253Cys substitution [194]. The additional cysteine could disrupt disulphide bonding, causing misfolding in the Kell glycoprotein and the K_{mod} phenotype.

7.6.18 KEL35 (KELP)

A pregnant woman with an antibody to a high frequency Kell-system antigen was homozygous for two *KEL* mutations, encoding two amino acid substitutions: Leu260Phe, which is surface exposed and most likely the cause of the KEL:–35 phenotype, and Tyr253Cys, which is buried a short distance from the surface (6Å) and could affect the conformation of a nearby surface-exposed loop [195]. KEL:–35 red cells are also KEL:–12, but no explanation is provided by the protein model.

7.6.19 KEL36 (KETI)

Anti-KEL36 was found in a British patient homozygous for a *KEL* allele encoding Thr464Ile [196]. Anti-KEL36 was non-reactive with two examples of K_o red cells and with K_{mod} KEL:–34 cells.

7.7 The Kell-null and Kell-mod phenotypes and anti-Ku (-KEL5)

7.7.1 K_o, the Kell null phenotype

In the same year as the discovery of Kp^a and Kp^b, Chown *et al.* [197] found a new Kell phenotype, K– k– Kp(a–b–), in two sisters. The consanguineous parents and two other sisters were of the common Kell phenotype K– k+ Kp(a–b+). The propositus had made an antibody that reacted with all but K– k– Kp(a–b–) cells. This antibody was used to search for another example of the new phenotype [198,199]: the 3122nd blood tested did not react and was also K– k– Kp(a–b–), and the rare phenotype was named K_o [2]. Family studies showed that K_o results from apparent homozygosity for an amorph gene at the *KEL* locus [34]. In several families heterozygosity for a silent gene producing no K or k explains abnormal inheritance.

K_o phenotype results from homozygosity or compound heterozygosity for a variety of mutations, including numerous nonsense mutations, single nucleotide insertions or deletions, splice site mutations, and several missense mutations [200–205]. The presence of null alleles has explained unexpected *KEL*01/02* genotyping results in individuals with K+ k– red cells and occasionally individuals with K– k+ cells. Many null and mod alleles are listed in the dbRBC and ISBT databases [206,207]. The Kell-glycoprotein has a large, extracellular C-terminal domain, so many of the mutations that cause early termination of mRNA translation might be expected to produce a truncated protein that could be detected by immunoblotting with Kell antibodies, but there is no evidence for this [3,8,201]. One possible explanation involves nonsense-mediated mRNA decay, which clears eukaryotic cells mRNA molecules containing premature termination codons [203,205]. Ser363Asn and Ser676Asn mutants, expressed in human embryonic kidney cells, were retained in a pre-Golgi compartment and not transported to the cell surface [201]. Arg128stop mutations, homozygous in two African Americans, were present in Js^a (*KEL*06*) alleles [201].

K_o red cells lack expression of all Kell antigens, including, by definition, Ku and Km. The strength of Kx antigen detected on the surface of intact K_o red cells is reported to be enhanced [208], yet the quantity of Kx protein is reduced [209] (Section 7.13.2). K_o red cells demonstrate no morphological abnormality [210] or unusual

expression of antigens belonging to other blood group systems, except Kx.

Only one K_o was found from testing 16 518 white donors with the serum of the original K_o propositus [42]. Several studies provided only one example of K_o from 24 953 white people [34]. These results suggest a frequency of about 0.007 for K^o alleles in white people. One K_o was found among 14 541 Japanese, suggesting a similar frequency [39].

7.7.2 K_{mod} phenotype

Marsh and Redman [211] introduced K_{mod} as an umbrella term to describe phenotypes in which Kell antigens are expressed very weakly, often requiring adsorption/elution tests for detection, and in which Kx antigen expression is elevated. K_{mod} cells have reduced quantity of the Kell glycoprotein. Because some K_{mod} red cells have very weak expression of Kell antigens, the distinction between K_o and K_{mod}, in some cases, may be dependent on the serological reagents and methods used.

K_{mod} usually arises from homozygosity for a missense mutation in the *KEL* gene, or heterozygosity with another such mutation or a null mutation [167,204,206,207]. KEL:–13 (Leu329Pro) [167] and KEL:–34 (Tyr253Cys) [194] (Section 7.6.17) could also be considered K_{mod} phenotypes. A synonymous mutation within the Gly573 codon (*KEL*02M.04*), 16 bp downstream of the 3′ splice site of exon 16, caused exon 16 skipping and introduction of a premature splice site [212]. The weak expression of Kell antigens probably results from some normal splicing events. Homozygosity for a Ser363Asn mutation (*KEL*02M.01)* resulted in a K_{mod} phenotype [167], whereas heterozygosity for the same mutation and for a null allele (Arg192stop or intron 3 splice site mutations) gave rise to K_o phenotype [201]. Based on transfection studies with HEH cells, the majority of the Ser363Asn and Tyr677Cys (*KEL*02M.02)* mutant proteins are degraded intracellularly and not transported to the cell surface, whereas Leu329Pro (*KEL*02M.03)* and Gly703Arg (*KEL*02M.04)* proteins are degraded to a lesser extent, but more so than in controls [167,201].

In Austria, genotyping of 401 apparent K+ k– samples revealed *KEL*01/02* (578C/T) heterozygosity in 14 (3.5%) cases: nine were genuinely k– and six of these had null splice site or nonsense mutations, whereas in the other three no *KEL* mutation was detected; in four of the remaining five, k could be detected by adsorption and elution and all four had mod mutations; and the other one had weak k and was heterozygous for a Kp^a allele (Section 7.3) [204].

7.7.3 The Kp^a effect

In the original description of Kp^a, Allen and Lewis [40] noted some difficulty in k typing some K+ Kp(a+) family members, now known to result from weakening of k and other Kell antigens owing to a reduced quantity of Kell-glycoprotein with the Arg281Trp substitution responsible for Kp^a expression [29]. The Kp^a effect is only recognised under certain conditions:

1 when an alternative allele, such as *K*, is present on the opposite chromosome;

2 when there is a K^o gene *in trans* [213,214]; or

3 with difficulty, when there is homozygosity for Kp^a. Kp^c, a low incidence allele of Kp^a, does not appear to produce a similar effect when *in trans* with K^o [43].

Expression of cDNA constructs in human embryonic kidney cells showed that the Kp^a mutation causes retention of most of the Kell glycoprotein in a pre-Golgi compartment owing to differential processing, suggesting aberrant transport of the Kell glycoprotein to the cell surface [29].

7.7.4 Anti-Ku (-KEL5)

Anti-Ku is the typical antibody of immunized K_o individuals and detects an antigen present on all red cells apart from those of the K_o phenotype. It appears to be a single specificity and cannot be separated into components of other Kell specificity by adsorption and elution [215]. Exceptional K_o individuals with anti-Kp^b or -k have been reported [34,216].

Anti-Ku has been responsible for severe and fatal HTRs [197,217,218] and for HDFN characterised by fetal anaemia [219]. Monocyte monolayer assays on 11 examples of anti-Ku suggested a high potential for causing HTRs and HDFN [220].

Some K_{mod} individuals make an antibody that resembles anti-Ku, but differs from anti-Ku by being nonreactive with the weakly Ku+ K_{mod} cells of the antibody maker. Antibodies made by different K_{mod} individuals are often not mutually compatible, because K_{mod} arises from a variety of different amino acid substitutions [167]. Anti-KEL13, -KEL34, and even -Kp^b could be considered as Ku-like antibodies in individuals with K_{mod} phenotypes.

7.8 Kell depression in Gerbich-negative phenotypes

The phenomenon of Kell depression associated with some Gerbich-negative phenotypes was first recognised

in a K+ woman and her brother with the rare Ge:–2,–3 phenotype [60]. Both showed weakened expression of K, k, and Kp^b, with about half the number of K antigen sites of K+ k+ Ge:2,3 cells [16]. Nine of 11 red cell samples from Ge:–2,–3 people showed at least some degree of weakening of Kell antigens [221], as did red cells of the Ge:–2,–3,–4 Leach phenotype [222,223]. All six Ge:–2,3 samples had normal expression of Kell antigens [221]. Red cells with the K_o, K_{mod}, and McLeod phenotypes have normal expression of Gerbich antigens. The biochemical nature of the phenotypic association between Gerbich and Kell is not understood, but studies on mice suggest that the Kell glycoprotein belongs to the same membrane complex as glycophorin C, which expresses Gerbich antigens [14].

7.9 Acquired and transient depressed Kell phenotypes

In 1972, Seyfried *et al.* [128] described the case of a boy with severe AIHA whose red cells gave a weakly positive DAT and had weak expression of k, Kp^b, Js^b, and Ku. His serum contained a potent anti-Kp^b responsible for an HTR. Within 16 weeks of the start of the investigation, his positive DAT had virtually disappeared, there was no sign of the anti-Kp^b, and his Kell antigens were back to normal strength. Similar examples of Kell-related autoantibodies associated with weak Kell antigens have been described since [224–228].

Anti-Kp^b was responsible for a positive DAT on the cells of a patient who was genetically Kp(a+b–) [226]. Her k and Js^b antigens were weakly expressed, but she had strong Kp^a. Nine months later the DAT was negative, the anti-Kp^b undetectable, her k and Js^b back to the strength expected for Kp(a+b–) cells, and own anti-Kp^b from the initial study no longer reacted with her cells.

A patient with autoimmune idiopathic thrombocytopenic purpura (AITP) had a potent antibody to a high frequency Kell antigen. His red cells gave a negative DAT and displayed profound depression of Kell system antigens. Transfused cells also lost their Kell antigens. Five months later the antibody had disappeared and the patient's Kell antigens had returned to normal. An environmental agent, possibly of microbial origin, may have been responsible [229]. Another similar case in an AITP patient is reported [230]; in remission his Kell antibody disappeared and his Kell antigens returned to normal, but his red cells lost their Lutheran antigens during a subsequent relapse (see Section 6.9).

Another patient with AITP and with IgM anti-Kp^b was typed as K– k– Kp(b–), but Js(b+), with a positive DAT with anti-IgM, but not with anti-IgG [131]. When the anti-Kp^b disappeared, his red cells were K– k+ Kp(b+) Js(b+), with no DAT. Boscoe *et al.* [131] suggest that there was no reduction in expression of Kell glycoprotein in this patient, but that his apparent Kp(b–) phenotype resulted from blocking of the Kp^b epitopes by the IgM autoantibody, and that the large IgM molecules also blocked k epitopes by steric hindrance. This is consistent with the relatively close proximity of k and Kp^b as predicted by a three-dimensional model of the Kell glycoprotein [10] and by a competitive MAIEA assay [109].

The possibility that Kell glycoprotein is expressed on megakaryocytes [94] might provide an explanation for the ITP in these patients.

7.10 Effects of enzymes and reducing agents on Kell antigens

Treatment of red cells with the proteases papain, ficin, or trypsin does not reduce expression of Kell antigens (with the exception of KEL29 [190] and KEL38 [191], Sections 7.6.13 and 7.6.15, which are trypsin-sensitive); the effects of α-chymotrypsin and pronase are variable [231]. Treatment of red cells with a mixture of trypsin and chymotrypsin, with trypsin followed by chymotrypsin, or *vice versa*, abolishes activity of Kell antigens [231,232], but some Kell-related monoclonal antibodies continue to agglutinate red cells treated in this way [15].

The Kell glycoprotein has 15 cysteine residues in its extracellular domain and thiol reducing agents, which dissociate disulphide bonds between cysteine residues, destroy Kell antigens on intact red cells. Kell antigens are conformational and are destroyed by 100–200 mM DTT and by 6% AET at pH 8 [233,234]. Js^a and Js^b are inactivated by substantially lower concentrations of DTT (<2 mM) [233]. Two cysteine residues flank the position of the amino acid substitution associated with the Js^a/Js^b polymorphism (residue 597) and probably form part of the Js^a and Js^b epitopes [139]. Like natural K_o cells, artificial K_o cells produced by AET treatment of red cells have enhanced expression of Kx antigen [234]. AET treatment of red cells is not a reliable way of identifying Kell-system antibodies as AET destroys many other red cell antigens,

including the Lutheran, Yt, Dombrock, LW, Knops, and JMH antigens.

7.11 Kell antigens on other cells and in other species

7.11.1 Other cells and other tissues

No Kell-system antigens or Kell glycoprotein has been detected on human lymphocytes, granulocytes, monocytes, or platelets, by flow cytometry with several Kell-related monoclonal antibodies [235] or by immunoblotting with a monoclonal antibody to purified Kell glycoprotein [8]. Cells of the human erythroleukaemic line, K562, do not express Kell antigens, unless induced to synthesise haemoglobin by hemin [235,236]. Kell mRNA transcripts were detected in haemopoietic tissue, bone marrow, and fetal liver, and in peripheral blood leucocytes [237]. There is also indirect evidence that Kell glycoprotein could be present on progenitors of granulocytes, monocytes, and megakaryocytes [93,94] (Section 7.3.5.2).

KEL mRNA transcripts were found to be about equally abundant in erythroid tissues and testis, and were detected in lesser amounts in lymph node, brain, colon, spleen, skeletal muscle, and several other tissues. Immunoblotting and immunohistochemistry revealed Kell glycoprotein in testis, lymphoid tissues, and skeletal muscle [19,237]. Kell glycoprotein was co-isolated with Kx protein in skeletal muscle [237].

7.11.2 Evolutionary aspects

Chimpanzees (*Pan troglodyte*) have the Kell phenotype that is common in humans, except that they are Js(a+b–) [238]. Antigens k, Kp^b, and Js^a are present on the red cells of gorilla and gibbon, K and Js^a on those of old world monkeys, and k on those of new world monkeys [239].

KEL and its mouse homologue, *Kel*, share 80% sequence identity; their glycoprotein shares 74% amino acid sequence identity [240]. The mouse Kell glycoprotein is disulphide-linked to Xk protein.

7.12 Functional aspects

Kell protein shares a pentameric zinc-binding motif, His-Glu-Xxx-Xxx-His (HEXXH, HELLH in Kell, Figure 7.1), with zinc-dependent endopeptidases [9]. Closest homology is with the neprilysin (M13) family, which includes neutral endopeptidases 24.11 (NEP or CD10) and two endothelium-converting enzymes (ECE-1 and ECE-2) [241]. These enzymes process a variety of biologically active peptides. ECE-1 and ECE-2 cleave big endothelin (ET)-1, big ET-2, and big ET-3, inactive peptides of about 40 amino acids, to create 21-amino-acid peptides with vasoconstrictor activity, ET-1, ET-2, and ET-3. A *KEL* cDNA construct lacking the regions encoding the cytoplasmic and membrane-spanning domains was expressed in insect (sf9) cells and a truncated Kell glycoprotein was secreted. This secreted glycoprotein cleaved big ET-3 at Trp21-Ile22 to produce ET-3. It could also process ET-1 and ET-2 from big ET-1 and big ET-2, but to a much lesser extent [242]. Secreted Kell glycoprotein was inactivated by mutating the HELLH motif, essential for catalytic activity, to HGLLH. Red cells of normal Kell phenotype, but not those of the K_o phenotype, were also capable of processing ET-3. Clapéron *et al.* [33] claimed that Kell-glycoprotein expressing K is enzymatically inactive, but this is disputed by Sha *et al.* [243], who found that the proteins expressing either K or Js^a had similar activity to the common form. ET-3 is a biologically active peptide with multiple roles, so the function of the Kell glycoprotein remains unclear. *Kel* knockout mice, like K_o humans, have no obvious abnormalities, but minor effects suggested possible physiological functions of Kell glycoprotein in heart, red cell ion transport, neovascularisation in tumours, and motor function [244].

Kell glycoprotein appears on erythroid progenitor cells at an early stage of erythropoiesis, before glycophorin A or band 3 [89,90]. When K562 erythroleukaemia cell line is stimulated with hemin to develop as an erythroid line it expresses Kell but no NEP, but when stimulated into megakaryocyte development with phorbol ester it expresses NEP but no Kell [245]. Kell may, therefore, play a role in erythropoiesis (but see Section 7.3.5.2).

7.13 The Kx blood group system

The Kx system consists of one antigen, Kx (XK1 or 019001), located on the Xk protein, which is encoded by an X-linked gene, *XK*. Absence of Kx from red cells results in severe reduction in expression of Kell antigens, the McLeod phenotype.

7.13.1 The Xk protein and the gene that encodes it

Xk, a red cell membrane protein of apparent MW 37 kDa, was isolated from red cells by immunoprecipitation with

human alloanti-Kx [246]. Xk protein is not glycosylated [12,247]; it is phosphorylated and palmitoylated [6,7].

Patients with McLeod syndrome (MLS) lack Kx and occasionally have interstitial deletions of chromosome Xp21. Ho *et al.* [247] assembled a cosmid contig of 360 kb covering the region between genes flanking *XK*. They then hybridised the cosmids with genomic DNA from patients with MLS and identified the breakpoints of a deletion of about 50 kb. Genomic fragments that spanned deletion endpoints were used to screen human cDNA libraries and a consensus full-length transcript for a candidate *XK* gene was derived from seven cDNA clones. The open reading frame predicted a 444 amino acid polypeptide with no *N*-glycosylation site and a calculated MW of 50 913 Da. Rabbit antibodies raised to synthetic peptides with sequences corresponding to the *XK* cDNA clone bound to the 37 kDa Xk protein on immunoblots of membrane proteins derived from red cells of common phenotype, but not to blots of proteins from McLeod phenotype red cells [209]. The N-terminal 22 amino acids of the protein were in accordance with the corresponding nucleotide sequence of the cDNA clone [12].

Hydropathy analysis of the amino acid sequence of Xk suggested a protein that spans the membrane 10 times, with internal N- and C-termini [247] (Figure 7.2). The predicted topographical arrangement is similar to that of members of a family of proteins that co-transport a neurotransmitter together with Na^+ and Cl^- ions [248], the amino acid sequence bearing closest resemblance to an Na^+-dependent glutamate transporter [247]. Xk also shares similarity with ced-8, a protein of the nematode *Caenorhabditidis elegans*, which is involved in the regulation of apoptosis [249].

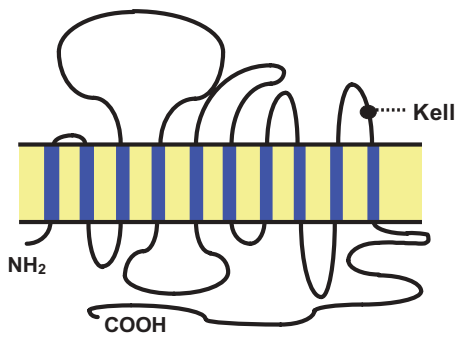


Figure 7.2 Diagrammatic representation of the topology of the Xk protein in the red cell membrane, showing cytoplasmic termini, 10 membrane-spanning domains, and (•) Cys347, which is linked by a disulphide bond with Cys72 of the Kell glycoprotein.

XK is organised into three exons, encoding amino acids 1–82, 83–168, and 169–444, respectively [249]. *XK* mRNA showed widespread distribution, with high levels of expression detected in fetal liver and in adult skeletal muscle, brain, and heart [249]. Immunohistochemical analysis showed Xk in human type II muscle fibres [250] and, in the rat, Xk is widespread in the brain, but its neuronal location is cytoplasmic and not at the cell surface [251]. Kx was not detected on neutrophils [252].

A mouse homologue of *XK* encodes a protein with 82% identity to human Xk protein with 10 predicted membrane-spanning domains [253].

7.13.2 Association of Xk protein and Kell glycoprotein

When immunoprecipitation experiments were performed under non-reducing conditions with monoclonal anti-K and K+ red cells, a disulphide-bonded heterodimer comprising the 93 kDa Kell glycoprotein and the 37 kDa Xk protein was detected [12]. Rabbit antibody raised to the purified complex reacted with both components on immunoblots and precipitated the Xk protein from K_o red cells, but not from McLeod phenotype cells.

Precipitation with a rabbit antibody raised to a peptide representing the second extracellular loop of Xk (Figure 7.2) isolated the Kell-Xk complex from COS-1 mammalian cells co-transfected with *KEL* and *XK* cDNA. Conversion of cysteine residues to serine by site-directed mutagenesis demonstrated that Cys347 on the fifth extracellular loop of Xk is linked by a single disulphide bond to Cys72 of the Kell glycoprotein [13]. Time-course studies on the *KEL* and *XK* transfected COS-1 cells demonstrated that the Kell-Xk complex is assembled in the endoplasmic reticulum and transported to the cell surface [254].

The relative quantity of Xk protein in K_o is lower than in cells of common Kell type [201,209]. In contrast, Kx antigen, as determined by serological techniques with alloanti-Kx, is expressed more strongly on K_o cells than on cells of common Kell type [208], suggesting that the presence of the Kell glycoprotein may impair access of the anti-Kx to the Xk protein in intact cells. Unlike Kell system antigens, Kx is not denatured by the disulphide-bond reducing agents DTT and AET. On the contrary, red cells treated with the appropriate concentrations of these chemicals resemble K_o cells and have enhanced serological expression of Kx [234,255].

Both *KEL* and *XK* transcripts were detected in early stages of human erythropoiesis, but in mice *Kel*, but not *Xk* transcripts were detected in erythroid progenitors [256].

7.13.3 McLeod phenotype and McLeod syndrome (MLS)

Routine tests on medical students led Allen *et al.* [257] to recognise that one of the students, Mr McLeod, had an unusual Kell phenotype. In the McLeod red cell phenotype all high frequency Kell antigens are expressed weakly, the degree of depression of these antigens varying in different individuals. K is also weakly expressed when present [59]. McLeod phenotype red cells lack the Kx (XK1) and Km (KEL20) antigens (Section 7.13.5).

McLeod phenotype is very rare and no frequency estimate has been published. Two unrelated men with the McLeod phenotype were found as a result of testing, with anti-k, red cells from many thousands of donors from South East England [258].

McLeod phenotype is only one of a number of characteristics that collectively make up a wider phenomenon known as McLeod syndrome (MLS), a form of neuroacanthocytosis [259]. Originally described as a 'benign X-linked myopathy with acanthocytes' [258], MLS is now recognised as a multisystem degenerative disorder sharing many features with Huntington's disease [260]. McLeod cells are acanthocytic [258,259,261] with decreased whole cell deformability [262] and reduced *in vivo* survival [263]. A variety of muscular, neurological, and psychiatric defects have been associated with MLS, with an average age of onset of around 40 years [260, 264,265]. Symptoms include muscle wasting, diminished deep tendon reflex, choreiform movements, and cardiomyopathy, sometimes leading to chronic heart failure and death, personality disorders, and generalised seizures. Elevated serum creatine phosphokinase is almost a constant feature. The reason for the association between Xk deficiency and neuroacanthocytosis and muscular defects is unknown, but the relationship of Xk protein to mammalian neurotransmitter transporters could provide a clue.

MLS red cells appear biochemically and physiologically relatively normal, despite their grossly abnormal morphology and the virtual absence of Kell system antigens. The protein profile as determined by SDS PAGE is normal, suggesting no defect of the membrane skeleton [246,266,267]. Phospholipid content is essentially normal [268,269], but there is evidence for abnormalities in the composition of the membrane lipid bilayers [270] and enhanced transbilayer mobility of phosphatidylcholine [269]. Electrolyte transport in McLeod cells is normal, but osmotic water permeability is reduced [268].

7.13.4 Inheritance and molecular genetics of Kx and McLeod syndrome

Expression of Kx antigen, present on all red cells save those of the McLeod phenotype, is controlled by an X-borne gene. MLS is, therefore, inherited as an X-linked recessive disease. With only rare exceptions [271], McLeod has always been found in males and the rare gene is inherited from the mother.

MLS has resulted from hemizygosity for a large variety of inactivating mutations including deletions of the whole gene, exon deletions, deletion of one or a few nucleotides introducing frameshifts, nonsense mutations, and splice site mutations (listed in [206,207,260, 264,272,273]). Two missense mutations, encoding Arg-222Gly (*XK*N.27*) [272,273] and Glu327Lys (*XK*N.29*) [274], have been found in two pairs of brothers manifesting no or minimal symptoms of MLS, despite all being over 50 years of age. Xk with Arg222Gly did not travel to the surface of transfected COS-1 cells [272]. A deletion of exon 2 of *XK* (*XK*N.04*) was detected in a man with MLS and in his grandson, predicting that the boy will be afflicted with the disease in the future [275]. Studies of sets of brothers with identical *XK* mutations revealed significant variation in clinical presentation, including causes of morbidity and mortality [276]. A nonsense mutation in *XK* exon 3 (*XK*N.21*) of a man with MLS was probably a new mutation as it was not present in his mother or sister [277].

A mutation in the fifth nucleotide of the 5′ donor splice site of intron 2 of *XK* (*XK*N.25*) was found in a man with almost no Kell antigens on his red cells and in his two daughters [278]. He did not have neuroacanthocytosis or muscle defects, possibly because of some degree of normal *XK* RNA splicing. The extreme reduction in Kell antigen was attributed to the combined effects of homozygosity for a Kp^a allele and the Kx deficiency (Section 7.7.3).

Chronic granulomatous disease (CGD) is an inherited immunodeficiency that may be X-linked (about 60% of cases) or autosomal. X-linked CGD result from deletion of *CYBB*, an X-linked gene that encodes the NADPH oxidase NOX2, or from mutations within that gene [279]. A small minority of CGD patients, all of the X-linked type, have MLS. The locus for X-linked CGD and the *XK* locus are discrete and the association of MLS with CGD results from a deletion of part of the X-chromosome that

encompasses both genes. Some large deletions also include genes for Duchenne Muscular Dystrophy (*DMD*), retinitis pigmentosa (*RPGR*), and ornithene transcarbamylase deficiency (*OTC*), causing a condition known as contiguous gene deletion syndrome [280–284].

7.13.4.1 X-chromosome inactivation

XK is subject to the phenomenon of X-chromosome inactivation, in which all somatic cells in female mammals have one active X-chromosome and one inactive X-chromosome (described in Section 12.7.1). Mixed populations of Kx+ and Kx– red cells, or of red cells with strong and weak Kell antigen expression, have been recognised in many female carriers of genes responsible for MLS. The proportion of McLeod phenotype red cells in female McLeod carriers usually varies from 5 to 85% [211]. This dual population of red cells is often difficult to detect serologically, especially if Kell antibodies and not anti-Kx are used, but flow cytometry permits an accurate estimation of the two red cell populations [211,285].

The only female described with MLS was heterozygous for a single basepair deletion in exon 2 of *XK* (*XK*N.08*) [271]. Her severe neurological and muscular defects and weakening of her Kell antigens were attributed to extreme skewing of inactivation of her X-chromosome carrying the normal *XK* gene.

7.13.5 Anti-Kx and -Km (-KEL20)

A five-year-old boy afflicted with recurrent infections (later presumed to be CGD), the second example of the McLeod phenotype, had anti-Kx and -Km (initially called anti-KL) and suffered an HTR. The antibody reacted with all cells tested, except for his own and those of Mr McLeod [286,287]. Anti-Kx reacts strongly with K_o cells, weakly with red cells of common Kell phenotype, and not at all with McLeod phenotype cells [208] (Table 7.6).

Table 7.6 Expression of Kx, Km, and Ku on red cells of common, K_o, McLeod, and K_{mod} phenotypes.

Phenotype	Kx	Km	Ku*
Common	Weak	Strong	Strong
K_o	Strong	Negative	Negative
McLeod	Negative	Negative	Weak
K_{mod}	Strong	Not tested	Weak

* Ku represents all high frequency Kell antigens.

Adsorption of anti-Kx + -Km serum with K_o cells removes anti-Kx and isolates anti-Km. The anti-Kx can be recovered by elution. Unfortunately, the separation of anti-Kx from anti-Km is often difficult to achieve and sera containing these antibodies are in very short supply. Anti-Km reacts with red cells of common Kell phenotype, but not with K_o or McLeod phenotype red cells [208] (Table 7.6). The Kx determinant is located on the Xk protein whereas Km is probably a discontinuous antigen, the product of interaction between Kell glycoprotein and Kx protein. Although Km has been numbered KEL20, it could belong to the Kx system.

Anti-Kx + -Km is the typical immune response of McLeod phenotype patients with CGD following transfusion [208,286,288], and has been responsible for an HTR [263,286]. An untransfused McLeod phenotype CGD patient made anti-Km during septic shock [289]. Two transfused McLeod individuals without CGD made anti-Km, but no anti-Kx [290,291]. When one of these patients required further transfusion, a monocyte monolayer assay was strongly positive and no radiolabelled red cells of common Kell phenotype survived, *in vivo*, 24 hours after injection. Consequently, the patient received four units of K_o and one unit of K_{mod} blood. The transfusion was successful and, despite receiving strongly Kx-positive red cells, the patient did not make anti-Kx [291]. However, some patients with MLS, but no CGD, can, make anti-Kx [292,293] or anti-Kx + -Km [294] in response to transfusion. Whether the molecular background to MLS affects the immune response is not clear.

An IgG autoanti-Kx in a man with common Kell phenotype did not cause haemolysis of autologous or transfused Kx+ cells [295].

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8 Duffy Blood Group System

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8.1 Introduction

Fy^a and Fy^b, the products of *FY*A* and *FY*B*, give rise to three phenotypes in white people: Fy(a+b–), Fy(a+b+), and Fy(a–b+). Another allele, *FY*X*, produces a weak Fy^b antigen. In people of African origin the most common Duffy phenotype is Fy(a–b–), the result of homozygosity for an allele that is silent in erythroid cells (*FY*Null*) . Fy(a–b–) is extremely rare in other races.

Fy3, Fy5, and Fy6, are high frequency antigens in white and Asian people, polymorphic in African Americans, and private antigens in parts of West Africa. Fy3 and Fy6 are expressed on red cells of all Duffy phenotypes apart from Fy(a–b–). Fy3 is defined by alloantibodies occasionally made by Fy(a–b–) individuals, Fy6 is defined by murine monoclonal antibodies. Fy5 resembles Fy3, but is not present on Fy3-positive Rh_{null} cells. The antigens of the Duffy system are listed in Table 8.1.

Duffy antigens are located on a glycoprotein of apparent MW 35–50 kDa, a chemokine receptor of the G protein-coupled family (Sections 8.2 and 8.7). Fy^a and Fy^b result from a Gly42Asp substitution within the Duffy glycoprotein (Section 8.3.2). Duffy glycoprotein is present on endothelial cells of post-capillary venules and on other cells throughout the body (Section 8.6).

The Fy(a–b–) phenotype common in people of African origin is caused by homozygosity for a mutation within an erythroid-specific, GATA-1 transcription-factor binding site upstream of the coding region of the Duffy gene (Section 8.4.1). This mutation prevents expression of the Duffy glycoprotein on red cells, but not on other cells.

Individuals with the Fy(a–b–) phenotype are resistant to infection by the malarial parasite *Plasmodium vivax* and Fy(a–b–) red cells are refractory to invasion by *P. vivax in vitro*. Interaction between the Duffy glycoprotein and receptors on *P. vivax* merozoites are essential, but not sufficient, for red cell invasion (Section 8.8).

Duffy glycoprotein is a binding protein for chemokines and often referred to as Duffy antigen receptor for chemokines (DARC) (Section 8.7).

The Duffy (*FY* or *DARC*) locus is on chromosome 1q21-q22.

8.2 DARC, the Duffy glycoprotein, and the gene that encodes it

Moore *et al.* [1] found by immunoprecipitation that Fy^a was associated with components of apparent MW 39.5, 64, and 88 kDa. Immunoblotting with anti-Fy^a or -Fy6 revealed a broad band with an intense region of apparent MW 35–50 kDa [2–5], which inhibited anti-Fy^a after electroelution from the gel [2]. DARC aggregates readily *in vitro* [2,6], and may exist in the membrane in oligomeric form [6,7].

DARC is *N*-glycosylated, but has no, or very little, *O*-glycosylation. Treatment of red cells with the *N*-glycanase endo F prior to solubilisation and immunoblotting with anti-Fy^a resulted in a dramatic reduction in apparent

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Table 8.1 Antigens of the Duffy system.

No.	Name	Relative frequency		Comments
		White people	Black people	
FY1	Fy^a	Polymorphic	Polymorphic	Antithetical to Fy^b (FY2), Gly42
FY2	Fy^b	Polymorphic	Polymorphic	Antithetical to Fy^a (FY1), Asp42
FY3	Fy3	High	Polymorphic	Absent from Fy(a–b–) cells
FY4	Fy4	Low	Polymorphic	Possibly antithetical to Fy3. Obsolete
FY5	Fy5	High	Polymorphic	Absent from Fy(a–b–) and Rh_{null} cells
FY6	Fy6	High	Polymorphic	Very similar to Fy3. Defined by monoclonal antibodies

MW and sharpening of the band on the blot [3]. Similar results were obtained by treatment of purified DARC or of tryptic peptide derived from it [5,8]. Variation in the degree of *N*-glycosylation may account for the range of MW.

From an internal peptide sequence obtained from DARC purified with anti-Fy6, Chaudhuri *et al.* [6,9] constructed degenerate oligonucleotide primers and amplified, by PCR, a segment of cDNA derived from Fy(a–b+) individuals. This amplified product was then used to isolate cDNA clones from a human bone marrow library. An open reading frame of 1267 bp, predicting a 338 amino acid polypeptide of 35 733 Da, was identified. Hydropathy analysis supported by *in silico* modelling suggested that the mature protein has seven membrane-spanning α-helices, an extracellular N-terminus, and a cytoplasmic C-terminus [10–12] (Figure 8.1). This arrangement is characteristic of the G protein-coupled superfamily of receptors, which includes chemokine receptors [13–15]. The 65 amino acid extracellular domain contains three asparagine *N*-glycosylation sites, at residues 16 (Asn-Ser-Ser), 27 (Asn-Ser-Ser), and 33 (Asn-Asp-Ser). Site directed mutation experiments revealed that all three sites are used for sugar attachment [16].

DARC appears to be part of the junctional or 4.1R protein complex containing band 3, glycophorin C, Rh, Kell, and Xk, and attached to the spectrin–actin junction through protein 4.1R and p55 (see Section 10.7) [17].

The first *FY* cDNA to be cloned was encoded by a single exon [18–20]. Subsequently, Iwamoto *et al.* [21]

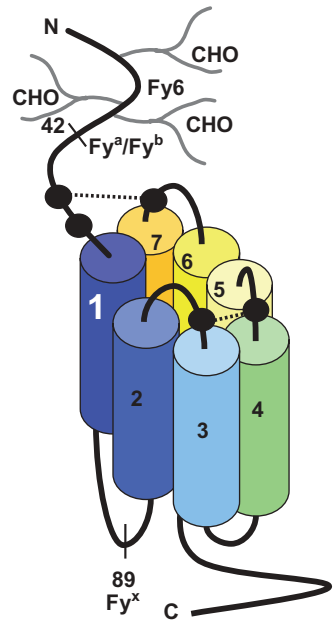


Figure 8.1 Three-dimensional model of DARC (adapted from [37], with permission from John Wiley & Sons). An extracellular N-terminal domain of 61 amino acids, containing three *N*-glycosylation sites (CHO) and the site of the Fy^a/Fy^b polymorphism at position 42, is followed by seven membrane-spanning domains and a cytoplasmic C-terminal domain. The first cytoplasmic loop contains the site of the amino acid substitution responsible for the Fy^x phenotype. There are five extracellular cysteine residues (•). Dotted lines represent the likely positions of disulphide bridges between cysteine residues in the N-terminal domain and the third loop and between the first and second loops.

showed that the predominant transcript in all tissues represented two exons separated by a 479 bp intron, the first exon encoding the seven N-terminal amino acids of DARC, including the translation-initiating methionine codon. Furthermore, the sequence of the minor transcript that encodes the nine N-terminal amino acid residues is part of the intron of the predominant, spliced transcript. The N-terminal sequence of the majority of DARC molecules is, therefore, MGNCLHRAEL [21] and not MASSGYVLQAEL [18], and the former protein is two amino acids shorter than the latter. The major erythroid transcription start point is 34 bp upstream of the first methionine codon in erythroid cells, but 82 bp upstream in lung and kidney [21]. In this chapter, nucleotides and amino acids will be numbered from the translation-initiating methionine residue of the major form of the glycoprotein.

The 5′ upstream region of *FY* contains no TATA or CAAT boxes, but it does contain several transcription-factor binding site motifs, including those for Sp1 and GATA [19,20].

In 1968, *FY* was the first human gene to be assigned to an autosome when Donahue *et al.* [22] showed *FY* to be linked to a visible deformity on the long arm of chromosome 1. More precise assignment was achieved by *in situ* hybridisation with *FY* cDNA [23].

Genes homologous to *FY* have been cloned from non-human primates, cow, pig, rabbit, and mouse [18,24].

8.3 Fy^a and Fy^b (FY1 and FY2)

Anti-Fy^a was discovered in the serum of a transfused haemophiliac, Mr Duffy, by Cutbush *et al.* in 1950 [25,26]. Only one year elapsed before Ikin *et al.* [27] identified the antithetical antibody, anti-Fy^b. Fy^x is the name given by Chown *et al.* [28] for a quantitative variant of Fy^b.

8.3.1 Frequency of Fy^a and Fy^b

Fy^a and Fy^b are useful anthropological markers. An extensive study of 2182 white Canadians, in which anti-Fy^b capable of detecting Fy^x was included, provided the following phenotype and deduced gene frequencies: Fy(a+b–) 0.1823; Fy(a+b+) 0.4735; Fy(a+b+^{w}) 0.0136; Fy(a–b+) 0.3302; Fy(a–b+^{w}) 0.0004; Fy(a–b–) 0; *FY*A* 0.425; *FY*B* 0.557; *FY*X* 0.016; *FY*Null* 0.002 [28,29]. The frequencies of Fy^a and Fy^b in African Americans and in Africans are variable, but low compared with Europeans because of the high frequency of the silent allele *FY*Null*. As in Northern Europeans, *FY*B* is more common than *FY*A*. In eastern Asia, *FY*B* has a far lower frequency than *FY*A* [30–35] (Tables 8.2 and 8.3).

8.3.2 The molecular basis of the Fy^a/Fy^b polymorphism

Sequencing of PCR products derived from reticulocyte cDNA and representing the entire coding sequence for the DARC revealed a SNP at position 125 encoding an amino acid substitution representing the Fy^a/Fy^b polymorphism: Fy^a, Gly42; Fy^b, Asp42 [18,19,36,37] (Figure 8.2). This was confirmed by expressing the appropriate cDNA clones in simian COS-7 cells and detecting Fy^a or Fy^b by flow cytometry [36]. The *FY*A* sequence creates a *Ban*I restriction site.

An Ala100Thr (G298A) polymorphism in the second membrane-spanning domain does not appear to affect antigenic expression [9,10,37,38]. In Swedish donors, the allele encoding Thr100 was detected in 22% of

Table 8.2 Genotype and allele frequencies in black South African, white Swedish, and Han Chinese populations, determined by molecular analysis of the Duffy genes of about 100 individuals from each population [32,33].

Phenotype	Genotype %				Allele %			
		Black	White	Chinese		Black	White	Chinese
Fy(a+b–)	*FY*A/A*	0	21	89	*FY*A*	3	41	94
	*FY*A/Null*	4	0	0	*FY*B*	17.5	59	6
Fy(a+b+)	*FY*A/B*	2	40	10	*FY*Null*	79.5	0	0
Fy(a–b+)	*FY*B/B*	2	39	1				
	*FY*B/Null*	29	0	0				
Fy(a–b–)	*FY*Null/Null*	63	0	0				

*FY*X* cannot be distinguished from *FY*B* by this method.

Table 8.3 Duffy genotype frequencies on four populations of American blood donors, obtained by testing on the BeadChip array [34].

		*FY*A/B*			*GATA1* mutation†			*FY*X*†		
Ethnic group	No. tested	*A/A*	*A/B*	*B/B*	*AB/AB*	*AB/Null*	*Null/Null*	*AB/AB*	*AB/X*	*X/X*
Caucasians	1243	0.18	0.43	0.39	0.97	0.02	0.01	0.98	0.02	0
African	690	0.04	0.19	0.77	0.12	0.33	0.55	0.99	0.01	0
Hispanic	119	0.15	0.45	0.40	0.68	0.26	0.06	0.99	0.01	0
Asian	51	0.69	0.25	0.06	0.98	0.02	0	1.00	0	0

†*AB* represents the alleles encoding Fy^a or Fy^b; *Null* is erythroid-silent.

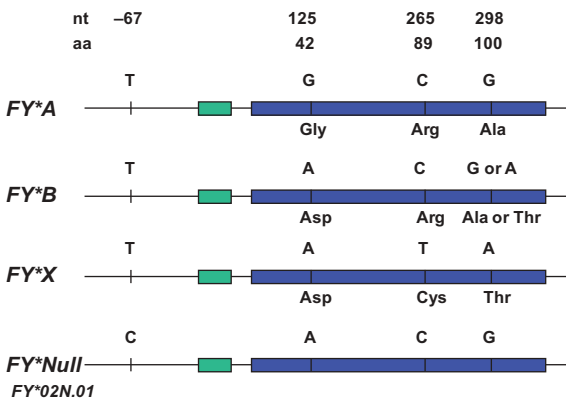


Figure 8.2 The four common Duffy alleles, showing nucleotide (nt) differences in the promoter regions and in exon 2 (blue) and encoded amino acid (aa) substitutions. Numbering from the major translation-initiating methionine residue [21].

Fy(a–b+), 9% of Fy(a+b+), but in 0% of Fy(a+b–); none of 100 black South Africans had Thr100 [39]. Other alleles in African Americans are listed in [40].

DARC homologues are present in non-human primates, with amino acid sequence homology of 99% between human and chimpanzee and 93–94% between human and squirrel, rhesus, and dourocouli monkeys [18]. All non-human primates have Duffy genes encoding Asp42, suggesting that *FY*B* is the ancestral allele [18,40,41].

8.3.3 Effects of enzymes on Fy^a and Fy^b

Fy^a and Fy^b are very sensitive to most proteolytic enzymes: they are completely destroyed by treatment of the red cells with papain, ficin, bromelin, pronase, or chymotrypsin, but trypsin does not abolish Fy^a and Fy^b activity. Use of impure preparations of trypsin containing chymotrypsin probably accounts for some early reports that Duffy antigens are trypsin-sensitive. Sialidase treatment of red cells does not affect the activity of Duffy antigens.

8.3.4 Fy^x

Fy^x behaves as a weak Fy^b antigen; there is no anti-Fy^x. Some anti-Fy^b sera react weakly with red cells of *FY*A/X* individuals, whereas others, particularly directly agglutinating anti-Fy^b, do not agglutinate those cells. The presence of Fy^x may be confirmed serologically by adsorption and elution of anti-Fy^b [28,42]. It is not possible to distinguish *FY*B/X* from *FY*B/B* serologically.

Fy^x has mostly been described in white populations, where it is not especially rare. Eleven Fy(a+b+^{w}) and one Fy(a–b+^{w}) were found among 1108 white people [29]. Fy^x has also been detected in a Canadian Cree kindred [43]. Several probable *FY*X* homozygotes have been reported, two of whom were originally thought to be Fy(a–b–) [29,42,44,45].

In addition to Fy^b, there is a very marked depression of Fy3, Fy5, and Fy6 in *FY*X* homozygotes [37–39, 43,44,46,47]. Flow cytometric analysis produced the following estimations of Fy6 site numbers per red cell: Fy(a–b+) (*FY*B/B*) 2200–2400; Fy(a–b+^{w}) (*FY*X/Null*) 150; Fy(a–b+^{w}) (*FY*X/X*) 250 [47]. Reduced levels of Fy6 binding associated with Fy^x were also detected by immunoblotting of DARC isolated from the red cell membrane [38,47]. This suggests that Fy^x represents reduced levels of DARC in the membrane rather than an altered Fy^b determinant or conformational changes affecting several

determinants and *FY*X* (also known as *FY*02M.01*) should be considered a mod allele.

*FY*X* and *FY*B* have the same coding sequence apart from 265C>T encoding Arg89Cys in the first cytoplasmic loop of DARC [39,41,47,48] (Figures 8.1 and 8.2). The replacement of a positively charged arginine residue by a neutral cysteine results in protein instability and might compromise the insertion of the molecule in the red cell membrane. Mammalian cells transfected with *FY* cDNA constructs in which the Cys89 codon had been introduced by site-directed mutagenesis had substantially reduced expression of Fy^b, Fy3, and Fy6, compared with cells transfected with normal *FY*B* cDNA [38,47] or with *FY*B* cDNA in which Arg89 had been replaced with positively charged lysine [49]. The *FY*X* mutation abolishes an *Aci*I restriction site. *FY*X* (*FY*02M.01*) encodes threonine at the site of the Ala100Thr polymorphism (Figure 8.2 and Section 8.3.2) [39,45]. Two Brazilians, one Caucasian, and one African, with extremely weak Fy^b, Fy3, and Fy6 expression, were heterozygous for *FY*Null* and an *FY*X* allele (*FY*02M.02*) containing an additional SNP, 145G>T encoding Ala49Ser [50].

*FY*X*, as determined by the presence of the Arg89Cys mutation, has an allele frequency of 0.025, 0.015, and 0.010 in Caucasian donors from Sweden, Austria, and the United States, respectively, and 0.005 in African Americans, but was not found in 100 black South Africans [34,39,48] (Tables 8.2 and 8.3). Fy^x phenotype, however, is associated with some degree of genetic heterogeneity. In some individuals with the Fy^x phenotype no change from the *FY*B* allele was detected in the coding region of the gene [37] and Fy^x has also been associated with a single base deletion in an Sp1 transcription-factor binding site upstream of the transcription start position [51].

8.3.5 Anti-Fy^a

The original anti-Fy^a of Mr Duffy was reported in 1950 [25,26]. Anti-Fy^a is estimated to be three times less frequent than anti-K [52]. Of 1778 alloimmunised Dutch patients, 9% of the antibodies were anti-Fy^a and 0.9% anti-Fy^b [53]. In Japan, however, where Fy(a–) phenotype is relatively uncommon, no anti-Fy^a was detected in 3554 patients with irregular blood group antibodies, but 4% of the antibodies were anti-Fy^b [54]. There is substantial evidence, from several different centres in the United States, that Fy^a is less immunogenic in black people than in white people [52,55–57], though one survey disputes this [58]. Anti-Fy^a often accompanies or precedes anti-Fy3 in Fy(a–b–) black people [59–62].

Some anti-Fy^a may have been stimulated by pregnancy, but most arise from blood transfusion. 'Naturally occurring' anti-Fy^a are very rare [63,64]. Anti-Fy^a may occur alone, but is often found in mixtures of antibodies. Duffy antibodies are not commonly detected in the first 6 months following transfusion, but, relative to other antibodies, are more common after 6 months and even more so after 5 years [53]. Anti-Fy^a are usually IgG, mostly IgG1 [65,66]. They generally react best by an antiglobulin test, but rarely anti-Fy^a may be directly agglutinating [67,68]. About 50% of anti-Fy^a activate complement up to the C3 stage [52].

Anti-Fy^a has been incriminated in immediate and delayed HTRs [69]. Though generally mild, a few immediate reactions have been fatal [70,71] and antigen-negative red cells should be selected for transfusion to patients with Duffy antibodies. The majority of radiolabelled Fy(a+) red cells injected into a patient with anti-Fy^a were eliminated within 10 minutes [72]. Anti-Fy^a in donor blood was responsible for a transfusion reaction in a Fy(a+b+) patient [73]. HDFN caused by anti-Fy^a is usually mild, but is occasionally severe. In a survey of 68 pregnancies in which the mother has anti-Fy^a, three resulted in a severely anaemic fetus, two requiring intrauterine transfusion [74]. A drop in the titre of anti-Fy^a in a D– Fy(a–b+) pregnant woman from 4096 to 256 following three injections of anti-D immunoglobulin could have resulted from an immunosuppressive effect of the prophylactic treatment [75].

There is a clear association between production of anti-Fy^a and *HLA-DRB1*04* [76,77], although an additional association with *HLA-DRB1*15* is also reported [77]. Duffy antigens could be minor histocompatibility antigens: Duffy mismatched renal grafts had significantly more chronic lesions compared with Duffy matched grafts [78].

8.3.6 Anti-Fy^b

Anti-Fy^b is a relatively rare antibody usually found only in mixtures of red cell antibodies. It has been stimulated by pregnancy and transfusion, and by intrauterine transfusion in the mother [79]; 'naturally occurring' anti-Fy^b has been found [80]. Often consisting entirely of IgG1 [65,66], Fy^b antibodies generally react best by an antiglobulin test, but directly agglutinating examples are known [28]. Some anti-Fy^b bind complement.

Anti-Fy^b has been responsible for immediate and delayed HTRs [69], two of which were reported to have been fatal [81,82]. One delayed reaction occurred in a man with no prior history of transfusion [83]. The single

reported case of HDFN caused by anti-Fy^b was treated by phototherapy and two transfusions [84].

Autoantibodies mimicking anti-Fy^b in Fy(a+b–) patients have been described, one responsible for AIHA [85,86]. The antibodies reacted more strongly with Fy(a–b+) and Fy(a+b+) cells than with Fy(a+b–) cells, and not at all with Fy(a–b–) cells. In one case, pure anti-Fy^b specificity was subsequently found [85].

8.3.7 Monoclonal anti-Fy^a and Fy^b

Murine monoclonal anti-Fy^a and -Fy^b have been produced [87], including anti-Fy^a from lymphocytes derived from transgenic mice expressing Fy^b and immunised with a transfected human embryonic kidney cell line expressing Fy^a [88]. One monoclonal IgM anti-Fy^b directly agglutinated Fy(b+) red cells and reacted weakly with Fy(a–b+^{w}) (*FY*X/X*) cells [87].

8.4 Fy(a–b–) phenotype; Fy3, Fy5, and Fy6 antigens

8.4.1 Fy(a–b–) in people of African origin

The Fy(a–b–) phenotype came to light when Sanger *et al.* [89] found that red cells of nearly 70% of African American blood donors failed to react with both anti-Fy^a and -Fy^b. A silent allele, recessive to *FY*A* and *FY*B*, was postulated in order to account for this null-phenotype. Fy(a–b–) has a frequency of about 63% in black New Yorkers, West Indians [90], and South Africans [32], but the frequency is higher in Africa, approaching fixation in parts of the continent [30,35] (Tables 8.2 and 8.3). All of 1168 donors from rural Gambia were Fy(a–b–) [91].

Although Fy(a–b–) red cells lack DARC [4,6], it is expressed in endothelial cells lining post-capillary venules of soft tissues and splenic sinusoids from black Fy(a–b–) individuals [92]. Duffy mRNA was not detected in the bone marrow of Fy(a–b–) individuals, but was present in their lung, spleen, and colon [18]. The coding sequence of the common erythroid-silent allele (*FY*Null* or, more precisely, *FY*02N.01*) is identical to that of an *FY*B* allele [9,18,19,36,37,92], but a T>C change is present in the promoter region of the gene, 33 bp upstream of the erythroid transcription start point and 67 bp upstream of the major translation start codon (position –67), introducing a *Sty*I restriction site [20,93]. This mutation is within a GATA consensus sequence (CTTATCT to CTTACCT), disrupting binding of the erythroid-specific GATA-1 transcription factor and preventing expression of the gene in erythroid cells, but not in other cells. A human erythroid cell line (HEL) and human microvascular endothelial cells transfected with a construct consisting of the promoter region of the *FY*B* allele and the reporter gene chloramphenicol acetyltransferase (CAT) had high levels of CAT activity. Transfection with a construct containing the –67T>C GATA mutation abolished CAT activity in the HEL cells, but not in the endothelial cells [20,93]. Transgenic mice with the human *FY*Null* allele expressed no DARC on their red cells, but did express it in non-erythroid tissues [94].

Twenty-three of 1062 individuals from the East Sepik of Papua New Guinea were heterozygous for *FY*A* and a Duffy allele with the *FY*A* coding sequence (encoding Gly42), but with the GATA mutation characteristic of *FY*Null* (–67C), an allele frequency of 0.022 [95]. This allele (*FY*01N.01*) is probably silent in erythroid cells and red cells of these individuals had lower levels of Fy6 than those homozygous for normal *FY*A*. An erythroid-null allele with –67C and an *FY*A* sequence has also been reported in five Brazilians from the malaria-endemic region of the Amazon [96] and in two tribes of the Sudan [191].

Resistance of people with the Fy(a–b–) red cell phenotype to the malarial parasite *Plasmodium vivax* is described in Section 8.8.

8.4.2 Fy(a–b–) in other ethnic groups

Fy(a–b–) is very rare in ethnic groups not originating from sub-Saharan Africa [30,35]. None of 6000 white Australian donors was Fy(a–b–), as determined by testing red cells with the original anti-Fy3 [97], but a remarkably high incidence of 1% *FY*Null/Null* homozygotes was found in New York Caucasians by genotyping [34].

Most examples of non-African Fy(a–b–) have been found through the presence of strong anti-Fy3. The molecular background to the Fy(a–b–) phenotype has been determined in four non-Africans with anti-Fy3, all of whom had been transfused and/or pregnant. The Duffy gene of a white Australian woman [97] contains a 14 bp deletion resulting in a reading frameshift and introduction of a translation stop codon (281–295del, *FY*01N.02*) [37]. Homozygosity for nonsense mutations that introduced translation stop codons was found in three examples:

1 a white British woman with 408G>A (Trp136Stop) in *FY*A* (*FY*01N.03*) [98];

2 a Lebanese Jewish woman with 407G>A (Trp136Stop) in *FY*B* (*FY*02N.02*) [98];

3 a native Canadian (Cree) with 287G>A (Trp96Stop) in *FY*A* (*FY*01N.04*) [43,98].

Table 8.4 Reactions of anti-Fy3 (from different ethnic groups), anti-Fy5, and monoclonal anti-Fy6 with red cells of various phenotypes.

Race and phenotype	Anti-Fy3		Anti-Fy5	Anti-Fy6
	Black	Other		
All races				
Fy(a+b–)	+	+	+	+
Fy(a+b+)	+	+	+	+
Fy(a–b+)	+	+	+	+
Cord cells†	– or +	+	+	+
Papain-treated cells†	+	+	+	–
Black				
Fy(a–b–)	–	–	–	–
White				
Fy(a–b–)	–	–	+¶	nt
Fy(a–b+^{w}) (*FY*X/X*)	w	w	w	w
Rh_{null}†	+	+	–	+
D––†	+	+	w	+

†Not Fy(a–b–) cells.
¶White Australian [97,101].
nt, not tested; w, weakly positive.

An Fy(a–b–) Japanese woman without anti-Fy3, but who had never been transfused or pregnant, was homozygous for an *FY*A* allele with a deletion of 327C, which introduces a stop signal 12 codons downstream at codon 120 (*FY*01N.05*) [99]. Each of these mutations would be expected to result in no expression of Duffy antigen on red cells or in any other part of the body.

8.4.3 Fy3 and anti-Fy3

8.4.3.1 Fy3 antigen

Fy3 is present on all red cells apart from those of the Fy(a–b–) phenotype. Fy3 is a public antigen in people of European and Asian origin, polymorphic in many black populations, and a private antigen in some parts of Africa. In contrast to Fy^a and Fy^b, Fy3 is resistant to the treatment of red cells with proteases [43,59,97,100]. Red cells of some primates have Fy3, but not Fy^a or Fy^b.

8.4.3.2 Anti-Fy3

The original anti-Fy3 was found by Albrey *et al.* [97] in the serum of an Fy(a–b–) transfused white Australian woman during her third pregnancy. The antibody reacted equally strongly with Fy(a+b–), Fy(a+b+), and Fy(a–b+) cells, and could not be separated into anti-Fy^a and -Fy^b components. It did not react with Fy(a–b–) cells (Table 8.4). Four other examples of anti-Fy3 have been produced by people who are not of African origin [43,98,100], one of which appeared to show a preference for Fy(a+) cells [100].

Anti-Fy3 is rare in Fy(a–b–) black people, though several examples have been reported [58–61,102–104], including two demonstrated to be homozygous for the *FY*Null* erythroid-silent allele [104]. Most anti-Fy3 in black people are found in mixtures of antibodies to red cell antigens, which often include anti-Fy^a. In some black patients with anti-Fy3, anti-Fy^a had preceded development of anti-Fy3 but was no longer detected when the anti-Fy3 was present [62]. Anti-Fy^a is a far more common antibody in the serum of multiply transfused black patients than anti-Fy3. Antibody screening tests on sera from 566 transfused Fy(a–b–) black patients in France revealed no Duffy antibodies [105].

Fy(a–b–) black people have Fy:–3 red cells, but they have DARC in other parts of the body [92]. The *FY*Null* allele has the *FY*B* sequence, so it is likely that *FY*Null* homozygotes express Fy3 and Fy^b in non-erythroid tissues, explaining the rarity of anti-Fy3, the absence of anti-Fy^b, and the relatively common occurrence of anti-Fy^a in transfused Fy(a–b–) black people. Anti-Fy3 made in black people probably recognise subtle differences between DARC expressed in different tissues, possibly a glycosylation or conformational difference. Anti-Fy3 made by black people react either very weakly or not at all with cord cell samples [60,61,102], whereas anti-Fy3 from three non-black women all reacted equally strongly with red cells of adults and newborn infants [43,100]. Perhaps Duffy antigen on fetal red cells resembles that on adult endothelial cells.

Anti-Fy3 is potentially haemolytic and has been responsible for immediate and delayed HTRs [62,100, 103,106,107], including intravascular haemolysis in an acute reaction [106] and hyperhaemolyis in a patient with sickle cell disease [107]. Fy(a–b–) red cells should be selected for transfusion to patients with anti-Fy3. The third child of the white Australian and the eighth child of the Cree woman with anti-Fy3 showed signs of HDFN, but in neither case was any treatment beyond phototherapy required [97,43].

Several mouse monoclonal antibodies have been produced that resemble anti-Fy3 in reactivity and detect protease-resistant epitopes on DARC [87,108]. Protein analyses have shown that these antibodies detect either a non-linear conformational epitope involving charged amino acids in all three extracellular loops [109] or epitopes located on the third extracellular loop [108,110] (see Figure 8.1).

8.4.4 Fy5 and anti-Fy5

Fy5 closely resembles Fy3 [101,111]; it differs by its absence from Fy3-positive Rh_{null} cells (amorph and regulator type), weak expression on red cells of D–– phenotype, and presence on Fy(a–b–) cells from people of non-African origin. Like Fy3, Fy5 is a protease-resistant antigen. Fy5 is expressed equally strongly on red cells of adults and newborns (Table 8.4). DARC appears to be part of a protein complex that contains the Rh proteins [17], so Fy5 expression could be dependent on an interaction between those proteins.

Several examples of anti-Fy5 have been reported, all in multiply transfused black Fy(a–b–) patients, mostly with sickle cell disease [62,101,111–113]. All were present in a mixture of other red cell antibodies; in two anti-Fy^a was present [111,113] and in another two anti-Fy^a had preceded anti-Fy5 but was no longer detectable [62]. Anti-Fy5 could not be separated into other antibody components by adsorption and elution. Some Rh e-variant red cells have weak Fy5 antigen [114]. Red cells from *FY*X* homozygotes have depressed Fy5 (Section 8.3.4).

Anti-Fy5 has been incriminated in delayed HTRs [62,112,113]. One patient had two separate reactions, one caused by anti-Fy^a, the other by anti-Fy5 [113].

8.4.5 Monoclonal anti-Fy6

Some monoclonal antibodies produced by immunising mice with red cells have a specificity very similar to that of anti-Fy3 [4,5], but were named anti-Fy6 because, unlike anti-Fy3, the determinant they detect is destroyed by papain (Table 8.4), ficin, and chymotrypsin; Fy6 is resistant to trypsin. Peptide scanning and site-directed mutagenesis have demonstrated that anti-Fy6 detect a linear epitope on the N-terminal extracellular domain of DARC between amino acid residues 19 and 26 (see Figure 8.1) [108,115,116]. In its distribution on red cells of non-human primates, Fy6 differs from Fy^a, Fy^b, and Fy3, but shows close accord with susceptibility to *Plasmodium vivax* invasion (see Section 8.8). Monoclonal anti-Fy6 proved invaluable in the isolation of DARC [5,6].

A recombinant dromedary antibody fragment, known as a VHH or nanobody, had anti-Fy6 specificity [117]. VHHs are the smallest intact antigen binding fragment derivative from the heavy chain-only antibodies present in camelids. The VHH recognises native DARC on red cells, inhibits *Plasmodium vivax* invasion, and displaces interleukin-8 bound to DARC.

8.4.6 Fy4 and anti-Fy4

Behzad *et al.* [118] described an antibody, produced by an Fy(a+b+) sickle cell patient, which gave most of the reactions expected of an antibody to the product of the *FY*Null* allele, though weak reactions confused some of the results. The antibody was numbered anti-Fy4, but as no second example was been found and the original is no longer available, the number Fy4 is obsolete. Although it is now known that DARC is not present on Fy(a–b–) cells, Fy4 might have recognised a changed conformation of an associated membrane component that occurs in the absence of DARC.

Table 8.5 Prediction of Duffy phenotypes from separate tests of two SNPs in the *FY* gene.

SNP 125	SNP −67	Predicted phenotype
G	T	Fy(a+b–)
A	T	Fy(a–b+)
G+A	T	Fy(a+b+)
A	C	Fy(a–b–)
A	T+C	Fy(a–b+)
G+A	T+C	Fy(a+b–)
G	C	Fy(a–b–) Very rare
G+A	C	Fy(a–b–) Very rare
G	T+C	Fy(a+b–) Very rare
G+A	T+C	Fy(a–b+) Very rare

8.5 Duffy genotype determination

To obtain accurate Duffy phenotype predictions by molecular genotyping, it is essential that the SNPs at positions 125 (*FY*A/FY*B*) and −67 (*FY*Null/FY*AB*) are interrogated. If separate tests are employed for the two SNPs, the results can be interpreted as shown in Table 8.5 and will give a high level of accuracy, though a false result will occur if the rare allele with −67C and 125G is present. A method employing a pair of sequence-specific primers at each SNP that give four potential combinations for allele-specific amplification of the 700 bp sequence between the SNPs avoids this potential error [32,119]. Further tests of the SNP at position 265 can be added to detect the presence of *FY*X* [34,39,47,48,120]. If this is not used, an *FY*X* allele would be interpreted as an *FY*B* allele, which would have no adverse affect on transfusion.

Duffy genotyping is valuable for the determination of Duffy phenotype of fetuses of mothers with anti-Fy^a, in order to assist in assessing the risk of HDFN [74]. It will also avoid Fy^x red cells being mistyped as Fy(b–).

Sickle cell disease (SCD) patients are regularly transfused and often make multiple red cell antibodies, making subsequent transfusion difficult. Phenotype-matched blood is often requested for these patients and Duffy genotyping might assist in providing matched blood. Fy(a+b–) patients who are *FY*A/A* would be capable of making anti-Fy^b, but those who are *FY*A/Null* would not. In a study of Brazilian SCD patients, 28% were Fy(a+b–), but of these 96% were *FY*A/Null* and only 4% *FY*A/A* and capable of making anti-Fy^b [121].

8.6 Site density, development, and distribution of Duffy antigens

Fy(a+b–) and Fy(a–b+) red cells were estimated, by quantitative immunoferritin microscopy, to have 13 000–14 000 Fy^a or Fy^b sites; Fy(a+b+) cells have about half that number of Fy^a sites [122]. With two radioiodinated monoclonal anti-Fy6, estimates of 12 200 and 6000 sites per red cell were obtained [4,5].

Fy^a and Fy^b are fully developed at birth and have been detected on red cells from embryos as early as 6–7 weeks gestation [123,124]. The expression of Fy^a and Fy^b is as strong on red cells of very young fetuses as on those of adults, and remains unmodified throughout fetal life.

There is almost 50% higher level of expression of Fy6 on reticulocytes than on mature erythrocytes [125].

In addition to its presence on red cells, DARC, detected with anti-Fy6, is abundant on endothelial cells lining post-capillary venules throughout the body, except for liver [126,127], and on Purkinje neurons of the cerebellum [128]. Sequence analyses of cDNA indicate that the renal and erythroid isoforms of the Duffy polypeptide are identical and any small differences in MW of the glycoproteins are probably accounted for by altered glycosylation [10,126,127]. DARC was also detected, with a rabbit polyclonal antibody specific for the glycosylation of DARC, on some other vascular endothelial cells and on epithelial cells of renal collecting ducts and pulmonary alveoli [127]. The effect of the GATA mutation in the *FY*Null* allele is erythroid-specific, so DARC is present on non-erythroid tissues in black people with the Fy(a–b–) red cell phenotype [92] (Section 8.4.1). Duffy mRNA has been detected in lung, muscle, spleen, colon, heart, pancreas, kidney and brain [10,18,126]. This transcript is present in tissues from Fy(a–b–) black individuals [18].

Fy^a and Fy^b are not present on lymphocytes, monocytes, granulocytes, or platelets [129,130].

8.7 The Duffy glycoprotein is a chemokine receptor

The Duffy glycoprotein binds a variety of pro-inflammatory chemokines and is often known as the Duffy Antigen Receptor for Chemokines (DARC). Chemokines are chemotactic cytokines that are involved in many cellular processes, especially the recruitment, activation, and directional movement of leucocytes [15,131]. There are two main classes of chemokines, called CXC

and CC based on the position of two highly conserved cysteine residues at their N-termini, plus two minor classes, C and CXXXC. Most chemokine receptors belong to a very large family of integral cell-membrane glycoproteins, G protein-coupled receptors, which traverse the membrane seven times and have an extracellular N-terminal domain (Figure 8.1) [13]. Most chemokine receptors are specific for one or more chemokines of a single class, but DARC, a promiscuous receptor, binds with high affinity to 60% of inflammatory chemokines from both CX and CC classes, but not with homeostatic chemokines [14,132]. Unlike almost all other G protein-coupled receptors, DARC lacks the Asp-Arg-Tyr (DRY) motif in the second cytoplasmic loop and does not appear to be coupled to a guanosine triphosphate-binding protein (G-protein) [24]. Consequently, Duffy is a chemokine-binding protein with no signalling function and has been referred to as a 'silent receptor' or interceptor (internalising receptor) [133].

Fy(a–b–) red cells do not bind chemokines [47,134]. Fy(a–b+^{w}) (*FY*X/X*) bind chemokines in substantially reduced quantities compared with Fy(a–b+) cells [38,47].

The chemokine-binding pocket of DARC appears to involve charged residues in the first four extracellular domains, which are brought into close proximity by two disulphide bridges [109,135] (Figure 8.1). Anti-Fy^{a}, -Fy^{b}, and -Fy6 (including VHH), which bind to the first extracellular domain of DARC, and a monoclonal anti-Fy3, which binds the fourth extracellular domain, all compete with chemokines for binding [110,117,134–137]. Interleukin-8 (IL-8, CXCL8) bound to red cells treated with trypsin, sialidase, or *N*-glycanase, but not to cells treated with papain or α-chymotrypsin [138], which cleave the N-terminal extracellular domain of DARC (see Section 8.3.3). Glycosylation of the protein is not required for chemokine binding. The Asp42Gly (Fy^{a}/Fy^{b}) Duffy polymorphism is associated with about 20% of variation in serum concentration of the chemokine monocyte chemoattractant protein-1 (MCP-1, CCL2), with Asp42 (Fy^{b}) associated with the greater red cell-bound MCP-1, and also with serum concentrations of IL-8 and RANTES (CCL5) [139].

The function of DARC on red cells and on endothelial cells remains controversial. DARC on red cells has long been considered to function as a sink, binding excess chemokines to prevent inappropriate activation of neutrophils and disruption of chemokine gradients [140]. The sink theory, however, is supported by responses of healthy volunteers to infusions of endotoxin (lipopolysaccharide, LPS). Following LPS infusion, plasma chemokine levels were 2–3 times higher and red cell-associated chemokine levels 20–50 times higher in Duffy-positive Caucasians compared with Duffy-negative Africans [141]. However, lower levels of plasma chemokines in Fy(a–b–) persons and in Duffy knockout mice suggests that DARC on red cells could provide a reservoir of chemokines, maintaining plasma chemokine concentrations [142,143]. One mechanism for releasing chemokines from DARC is clotting [139].

A degree of neutropenia, often referred to as 'benign ethnic neutropenia', is prevalent in Africans and in people of African origin. It is closely associated with homozygosity for the erythroid-silent Duffy allele [144–146]. This provides further support for the proposal that red cell DARC is involved in the regulation of chemokine concentration and, as a consequence, neutrophil production and migration. Associations between Duffy phenotype and disease are described in Section 8.9.

DARC is present on the endothelial cells of post-capillary venules throughout the body, in both Duffy-positive people and Fy(a–b–) Africans (Section 8.6). The kidney isoform binds chemokines with the same affinity as the red cell isoform [126]. It has been assumed that DARC on endothelial cells functions as a chemokine reservoir or decoy receptor, dampening chemokine effects in the local circulation [133,147]. However, it now appears that a primary role of endothelial DARC is the transport of tissue-derived chemokines across the endothelial barrier to the luminal surface, where they are presented to receptors on leucocytes [148]. DARC internalises chemokines, but does not scavenge them. Instead, the chemokines remain active and support optimal leucocyte migration across the endothelial layers, a fundamental component of inflammation [148].

DARC in endothelial cells forms dimers with the chemokine receptor CCR5, inhibiting CCR5 signalling [7]. Therefore, DARC, which is non-signalling, could be involved in the regulation of chemokine signalling though heterodimerisation with other chemokine receptors.

Several people have the Fy(a–b–) red cell phenotype owing to homozygosity for inactivating mutations within the coding region of their Duffy genes (Section 8.4.2). These individuals would have no DARC in any of their tissues, yet all were apparently healthy.

8.8 Duffy antigens and malaria

Plasmodium vivax is responsible for between 70 and 390 million cases of malaria annually, mostly in South and

Southeast Asia. Although often considered to be a less severe form of malaria than that caused by *P. falciparum*, vivax malaria is far from benign and can often cause severe, life-threatening syndromes that are similar to those associated with falciparum malaria [149]. For several decades it had been known that most black people are resistant to *P. vivax* infection. When Miller *et al*. [150] showed that Fy(a–b–) red cells are refractory to invasion by the simian parasite *P. knowlesi*, which can invade human red cells *in vitro*, it was not long before the association between the Fy(a–b–) phenotype and resistance to *P. vivax* infection became apparent [151]. Of 11 black and six white volunteers exposed to *P. vivax*, all became infected except for the five Fy(a–b–) black subjects [151].

Fy(a–b–) human red cells are refractory to invasion by *P. knowlesi* and *P. vivax* merozoites, *in vitro*, whereas Fy(a+b+) cells are invaded [150,152,153]. This is not a purely racial characteristic as Fy(a–b–) red cells from two Cree Indians and a white Australian (Section 8.4.2) were not invaded by *P. knowlesi* [154]. Merozoites are able to attach to Fy(a–b–) cells, but they cannot form a junction and eventually become detached [150,154,155]. Invasion of Duffy-positive red cells by *P. knowlesi* and *P. vivax* can be blocked by the presence of monoclonal anti-Fy6; invasion of Fy(a+) cells can be partially blocked by anti-Fy^a [150,152]. The chemokines IL-8 and MGSA (CXCL1) blocked invasion of Duffy-positive cells [134]. Treatment of Duffy-positive red cells with chymotrypsin renders them resistant to invasion by *P. knowlesi* and *P. vivax*, whereas trypsin treatment has no effect [150,152]. This parallels the effects of these proteases on Fy^a, Fy^b, and Fy6 antigens, but not on Fy3 and Fy5, which are resistant to chymotrypsin cleavage. *P. vivax* prefers to invade reticulocytes, which have about 50% more Fy6 sites than mature erythrocytes [125].

Despite being Fy(b+) and Fy:3, red cells of the Old World rhesus monkey are Fy:–6 and are not invaded by *P. vivax*, but are invaded by *P. knowlesi*. New World capuchin monkey cells are Fy(a–b–) Fy:3,–6 and are not invaded by *P. knowlesi* or *P. vivax* merozoites. These data suggest that the Fy6 epitope is important for the invasion of *P. vivax* [154,156–158].

Proteins that bind Duffy-positive human red cells, but not Fy(a–b–) cells, have been isolated from the supernatants of cultured *P. knowlesi* and *P. vivax* at the time of merozoite release and reinvasion [159,160]. These Duffy-binding proteins (DBLs), Pkα-DBL and Pv-DBL for *P. knowlesi* and *P. vivax*, respectively, belong to the Duffy-binding-like erythrocyte-binding protein family (DBL-EPB), which includes *P. falciparum* EPBs [161]. Binding could be inhibited by anti-Fy6 and by the chemokines IL-8 and MGSA [157,159,160]. The purified parasite proteins bound specifically to purified DARC. The extracellular domains of each protein have been classified into six regions of amino acid homology [161]. COS-7 cells expressing the cysteine-rich region II (PvDBP-RII) formed rosettes with Duffy-positive, but not Fy(a–b–), red cells [162]. This rosetting could be blocked by a synthetic peptide representing amino acid residues 8–42 of the N-terminal domain of DARC [163]. Fine mapping restricts the binding site to between Gln19 and Trp26 on the N-terminal domain of DARC [164], the location of the Fy6 epitope, although sulphation of Tyr41 is also essential for association [165]. PvDBP-RII and PkαDBP-RII share extensive sequence homology with the *P. falciparum* glycophorin A-binding protein, EBA-175 [161] (Section 3.21.1). Analysis of the Duffy-binding ligands has led to the identification of the *var* genes that encode the variant endothelial cytoadherence proteins of *P. falciparum* (for review see [24]). Naturally occurring antibodies to PvDBP or, more likely, inhibitory antibodies produced by immunisation with recombinant PvDBP-RII, could prove valuable in vaccine production [166].

The Duffy gene is subject to opposing selection pressures: the need to maintain effective chemokine binding versus the benefits of reducing *P. vivax* invasion. It is likely, therefore, that the high incidence of the *FY*Null* allele in Africa results from the selective advantage it confers in providing resistance to vivax malaria. In parts of West Africa the frequency of *FY*Null* is almost 100%, yet *P. vivax* is not present in these areas [91]. It is probable that the parasite has been eliminated by the disruption of its life cycle resulting from a shortage of susceptible hosts. Heterozygosity for the silent allele *FY*Null* may have a selective advantage in areas where *P. vivax* is endemic: individuals heterozygous for *FY*Null* are less susceptible than *FY*Null* homozygotes to *P. vivax* infection [167] and their red cells bind substantially less PvDBP than those of individuals with two active Duffy alleles [168]. Furthermore, Fy(a+b–) (*FY*A/A*) red cells had up to 50% lower PvDBP binding than Fy(a–b+) (*FY*B/B*) cells and *FY*A/A* individuals had substantially reduced risk of clinical vivax malaria than individuals of *FY*B/B* genotype [169]. Consequently, *FY*A* appears to have a survival advantage over the ancestral allele, *FY*B*, which could explain the high incidence of *FY*A* in regions where vivax is endemic but *FY*Null* is rare.

Occasional cases of Fy(a–b–) individuals infected by *P. vivax* have been observed in the Brazilian Amazon and various parts of Africa [170–173] and a substantial number of Fy(a–b–) people in Madagascar were susceptible to *P. vivax* blood-stage infection and clinical vivax malaria [174]. *P. vivax* therefore, appears to be evolving alternative pathways for red cell penetration.

8.9 Other disease associations

In contrast to resistance to vivax malaria, susceptibility to a number of other diseases appears to be associated with the presence or absence of Duffy on red cells, probably as an effect of differences in chemokine regulation and their influence on leucocyte levels and inflammation. The association between the erythroid Duffy-null phenotype and benign ethnic neutropenia [144–146] was mentioned in Section 8.7.

HIV-1 attaches to red cells via DARC, effecting infection of target lymphocytes [175]. Individuals homozygous for *FY*Null* are reported to have a 40% increase in HIV-1 infection, but slower HIV disease progression once infected [175] (although the evidence is disputed [176]). In a cohort of South African women, the risk of acquiring HIV infection was about three-fold greater in those with Fy(a–b–) phenotype-associated low neutrophil counts, compared with other study participants [177]. Among leukopenic HIV-positive African Americans, however, there was a survival advantage of being *FY*Null/Null*, but this did not apply to *FY*Null/Null* patients with high white cell counts [178].

Red cell Duffy expression is strongly associated with severity of sickle cell disease (SCD) and especially with organ damage. Twice as many Duffy-negative patients had evidence of organ damage compared with Duffy-positive patients; Duffy-negative patients were nearly four times more likely to have proteinuria [144]. Duffy-positive SCD patients have higher plasma levels of chemokines IL-8 and RANTES than Duffy-negative patients [179]. Activation through clustering of integrin $\alpha 4\beta 1$, which may play an important role in vaso-occlusive crises, was induced by IL-8 and RANTES on Duffy-positive, but not Duffy-negative, sickle reticulocytes [180]. The presence of DARC, therefore, may determine the extent of inflammation in SCD and Anstee [181] has suggested that the enhanced propensity for alloimmunisation in SCD could be related to inflammation and that Fy(a–b–) phenotype may play a part in this process.

Fy(a–b–) African Americans have lower renal allograft survival in the presence of delayed graft function [182].

The release of chemokines from Duffy-positive red cells during clotting suggests localised increased chemokine levels in thrombotic states, which could modulate the inflammatory effects associated with haemostasis and thrombosis [139].

The surface expression of Duffy antigens and red cell chemokine scavenging function is reduced with red cell storage [183] and Duffy antigen has been identified on exocytic vesicles from stored red cells [184]. Although the overall loss of Duffy from banked red cells is modest, this might contribute to the 'storage lesion' associated with some adverse effects of transfusion. In mice, transfusion of red cells stored for 10 days increased neutrophil counts and chemokine concentrations in the air spaces of the lung and transfusion of red cells from Duffy knockout mice into endotoxaemic Duffy-positive mice increased airspace neutrophils, inflammatory cytokine concentrations, and lung microvascular permeability, compared with transfusion of Duffy-positive red cells [184]. Consequently, loss or modification of Duffy antigen could be a contributing factor in transfusion-associated lung injury in the critically ill.

A genetic study in the Caribbean and South America suggests that absence of red cell Duffy antigen accounts, at least in part, for higher prevalence and severity of asthma, associated with high total IgE concentration in people of African descent compared with those of European origin [185].

African American men have substantially higher levels of prostate cancer and associated mortality rates than Caucasian men. Some chemokines have angiogenic properties, and it has been proposed that DARC on red cells may reduce angiogenesis and consequently the progression of prostate cancer, by clearing angiogenic chemokines from the tumour [186]. A case-control study among Jamaican men of African origin, however, did not support any effect or red cell DARC expression on the risk or progression of prostate cancer [187].

DARC on vascular endothelium also interacts directly with a tetraspanin CD82 on cancer cells. CD82 is a suppresser of metastasis: interaction between Duffy and CD82 inhibits the spread of the cancer to remote sites and also appears to induce cancer cell senescence [188]. Duffy antigen expressed on breast cancer cells inhibits tumour growth and metastatic potential, through the clearance of angiogenic chemokines and inhibition of neovascularity

[189]. Similar effects were apparent with lung cancer cells [190].

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9 Kidd Blood Group System

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9.1 Introduction

Jk[a] (JK1) and Jk[b] (JK2) of the Kidd system are the products of alleles and are polymorphic in all populations tested. The Jk[a]/Jk[b] polymorphism is associated with an Asp280Asn substitution in the Kidd glycoprotein. Kidd antibodies are often difficult to work with and are potentially dangerous, as they are a common cause of delayed HTRs.

A rare null phenotype, Jk(a–b–), is generally inherited recessively and is most commonly found in Polynesians. Jk(a–b–) cells lack the high incidence antigen Jk3. A variety of mutations are responsible for Jk(a–b–).

The Kidd glycoprotein functions as a urea transporter.

The *JK* (*SLC14A1*) locus is on chromosome 18 at 18q11-q12.

9.2 The Kidd glycoprotein and the gene that encodes it

Before the Kidd glycoprotein had been purified or the *JK* gene cloned, failure of Jk(a–b–) red cells to lyse in 2 *M* urea led to the supposition that the Kidd glycoprotein might be a red cell urea transporter (Section 9.4.2). A red cell membrane structure of apparent MW 45 kDa was isolated by affinity purified IgG anti-Jk[a], -Jk[b], and -Jk3 bound to nylon membranes [1]. Immunoprecipitation with anti-Jk3 isolated a glycoprotein of 46–60 kDa from red cells of all phenotypes except Jk(a–b–) [2]. The MW was reduced to 36 kDa by removal of *N*-glycosylation with *N*-glycanase [2]. Jk(a+b–) red cells were estimated to have around 14 000 Jk[a] antigen sites by immunoelectron microscopy with anti-Jk[a] and ferritin labelled anti-human IgG [3]. This is compatible with an estimate of less than 32 000 sites per cell obtained by determining the quantity of a mercurial required to inhibit facilitated urea transport [4].

Olivès *et al.* [5] produced a cDNA probe from human erythroblast mRNA by reverse transcriptase PCR with primers derived from the amino acid sequence of a rabbit urea transporter. They used this probe to isolate a cDNA clone (*HUT11*) by screening a human bone marrow library. A 36 kDa polypeptide produced by coupled *in vitro* transcription-translation of the cDNA was immunoprecipitated by anti-Jk3. Immunoblotting with a rabbit antibody raised to peptides predicted from the cDNA sequence revealed 46–60 kDa components from human red cell membranes, except those of the Jk(a–b–) phenotype [2]. *HUT11* has subsequently been shown to be an aberrant transcript or a cloning artefact [6]. Another transcript (*HUT11A*) encoding glutamic acid in place of lysine at position 44 and two Val-Gly dipeptides instead of three after position 227, produces the Kidd glycoprotein and red cell urea transporter [6,7]. The predicted gene product is a 43 kDa, 389 amino acid polypeptide with about 63% identity with the rabbit urea transporter. The protein contains 10 potential membrane-spanning domains, with intracellular N- and C-terminals, and is *N*-glycosylated at Asn211 on the third extracellular loop, the glycan expressing ABO activity [8] (Figure 9.1). In addition to erythroid cells and kidney, the Kidd transcript was also found in brain, heart, pancreas, prostate, bladder, tested, intestine, and colon tissues [9].

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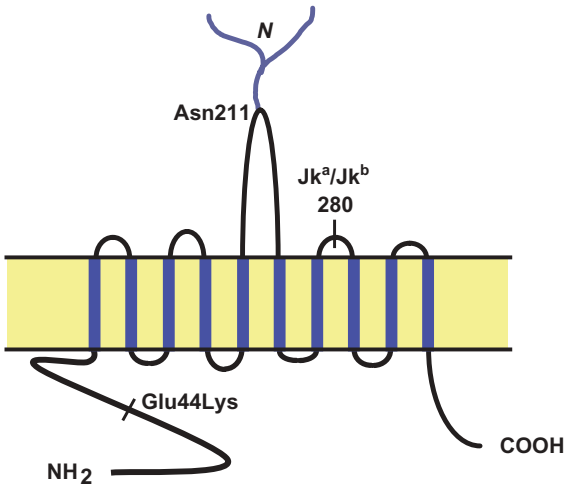


Figure 9.1 Diagrammatic representation of the Kidd glycoprotein in the red cell membrane, showing 10 membrane-spanning domains, cytoplasmic N- and C-termini, a single *N*-glycan on the third extracellular loop at Asn211, the position of the Jk[a]/Jk[b] polymorphism on the fourth extracellular loop, and the position of Glu44Lys associated with weak Jk[a] and Jk3 expression.

Table 9.1 Organisation of the *JK* (*SLC14A1*) gene.

Exon	Size (bp)	Amino acids	Intron size (kb)	
			[10]	[11]
1	93		0.7	
2	64		2.4	
3	157		3.1	
4	172	1–50	0.6	0.543
5	190	51–113	3.55	3.0
6	129	114–156	1.9	2.0
7	193	157–221	2.5	2.5
8	148	222–270	0.27	0.217
9	135	271–315	8.6	9.0
10	50	316–332	1.4	1.4
11	551	333–389		

The *JK* (*SLC14A1*) gene has 30 kb and contains 11 exons [10,11]. Exons 1–3 and part of 4 represent the 3′ untranslated region; exons 4–11 encode the mature protein (Table 9.1). The transcription initiation site is 335 bp upstream of the translation start codon in exon 4. The region between nucleotides −837 and −336 contains erythroid-specific GATA-1 and Sp1 transcription-factor binding sites, plus TATA and inverted CAAT boxes [10]. Two equally abundant erythroid transcripts, of 4.4 and 2.0 kb, have been identified, the smaller arising from skipping of exon 3 [8].

The Kidd gene, *SLC14A1*, and *SLC14A2*, a gene encoding another urea transporter (UT-A), were both localised to chromosome18q by *in situ* hybridisation [3,9].

9.3 Jk[a] and Jk[b] (JK1 and JK2)

An antibody in the serum of an American woman, Mrs Kidd, was named anti-Jk[a] in 1951 by Allen *et al.* [12], from the initials of Mrs Kidd's sixth child, who showed signs of HDFN. The antibody reacted with the red cells of 77% of Bostonians. The anticipated antithetical antibody, anti-Jk[b], was found in England two years later by Plaut *et al.* [13].

9.3.1 Frequency of Jk[a] and Jk[b]

Jk[a] and Jk[b] are inherited as the products of co-dominant alleles. The following phenotype frequencies were obtained from six series of tests with anti-Jk[a] on a total of 4275 Europeans [14]: Jk(a+), 76.4%; Jk(a–), 23.6%. The following gene and genotype frequencies are derived from these figures: *JK*A* 0.5142, *JK*B* 0.4858; *JK*A/A* 0.2644, *JK*A/B* 0.4996, *JK*B/B* 0.2360. Very similar gene frequencies were obtained from tests with anti-Jk[a] and -Jk[b] on red cells from 2102 Canadians: *JK*A* 0.5162, *JK*B* 0.4838 [14,15]. Numerous other population studies, many conducted with anti-Jk[a] only, have been summarised [16,17]. The gene frequency of *JK*A*, usually about 50% in Europeans, rises to 75% in some parts of Africa, but this frequency is by no means representative of all African populations. The frequency of *JK*A* is around 30% in Chinese and falls to as low as 20% in some Japanese studies, but this low frequency does not occur throughout Asia.

Table 9.2 shows genotype frequencies on four populations of American blood donors, obtained by molecular testing [18].

9.3.2 The molecular basis of the Kidd polymorphism

The Jk[a]/Jk[b] polymorphism results from 838G>A in exon 9, encoding Asp280Asn on the fourth extracellular loop of the Kidd glycoprotein [19] (Figure 9.1). Jk[a]/Jk[b] is also associated with a silent 588G>A SNP in exon 7 and

Table 9.2 Kidd genotype frequencies on four populations of American blood donors, obtained by testing on the BeadChip array [18].

		Genotypes		
Ethnic group	No. tested	*JK*A/A*	*JK*A/B*	*JK*B/B*
Caucasians	1243	0.30	0.44	0.26
African Americans	690	0.54	0.37	0.09
Hispanic	119	0.36	0.42	0.22
Asian	51	0.22	0.53	0.25

another SNP at position −46 from the 3′ end of intron 9 [11]. G838 of *JK*A* introduces an *Mnl*I restriction site [6]. The 838G>A polymorphism can be interrogated in a variety of ways to provide reasonably reliable phenotype predictions [20]. In populations with a relatively high level of the most common silent allele (*JK*02N.01*), however, it may also be prudent to test for the IVS5−1g>a inactivating mutation to avoid false prediction of Jk(b+) (see Section 9.4.1).

Aberrant expression of Jk^a and Jk^b in two *JK*A/B* African Americans was associated with mutations in *JK* exon 7: 511T>C, Trp171Arg in *JK*01W.02* and 548C>T, Ala183Val in *JK*02W.01*, respectively [21]. Both amino acid changes are within the third extracellular loop. Another allele responsible for weak Jk^a is described in Section 9.4.6.

The Jk^a/Jk^b polymorphism was used as a model for the production of 'designer' red cells by genetic modification of erythroid cells cultured from $CD34^+$ progenitors. [22]. Transfection of the cultured cells with lentiviral vectors containing *JK* cDNA representing *JK*A* or *JK*B* alleles, converted Jk(a−b+) or Jk(a+b−) cells, respectively, to Jk(a+b+) red cells. On the other hand, lentiviral transfer of shRNA designed to interfere with *JK* transcription, reduced expression of Jk^a and Jk^b antigens, so that they were not detectable by routine serological testing. This technology clearly has great potential for the production of reagent red cells that express antigens of choice.

9.3.3 Effect of enzymes and reducing agents

Jk^a, Jk^b, and Jk3 are papain-, ficin-, trypsin-, chymotrypsin-, and pronase-resistant; treatment of red cells with these enzymes generally enhances reactivity with Kidd antibodies. Kidd antigens are not affected by sialidase or by AET.

9.3.4 Development and distribution of Kidd antigens

Jk^a and Jk^b are well developed on the red cells of neonates. Fetal cells have the same distribution of Kidd phenotypes as that found in the adult population. Jk^a and Jk^b antigens have been detected on red cells of 11- and 7-week-old fetuses, respectively [23].

Kidd antigens were not detected on lymphocytes, monocytes, granulocytes, or platelets [24–27]. Jk3 first appears on erythroblasts at a late stage of erythropoiesis [28].

9.3.5 Anti-Jk^a and -Jk^b

9.3.5.1 Alloantibodies

Anti-Jk^a and -Jk^b are often encountered in transfusion practice, with anti-Jk^a being more common than anti-Jk^b. They are most commonly detected within a month of transfusion, but then decline rapidly, often becoming undetectable after 3 months [29]. Jk^a and Jk^b are traditionally considered of relatively low immunogenicity, but when antibody evanescence is taken into account their calculated immunogenicity is substantially increased [30]. The only reports of 'naturally occurring' Kidd antibodies are anti-Jk^a in two Jk(a−b+) 9-month-old non-identical twins, detectable only by a solid-phase method [31], and an IgG anti-Jk^a in a 7-month-old boy with an *Escherichia coli* urinary tract infection [32]. Anti-Jk^a has been found during the first pregnancy of an untransfused woman [33] and a case of anti-Jk^a sensitisation following amniocentesis and intrauterine transfusion is reported [34]. Anti-Jk^a production appears to be associated with an *HLA-DRB1*01* genotype [35,36].

Kidd antibodies are often difficult to detect. Some directly agglutinate antigen-positive cells, but the reactions are usually weak by this method. Generally an antiglobulin test is required to detect Kidd antibodies. Use of enzyme-treated cells may be necessary to detect weaker antibodies. Anti-Jk^a detectable only by the manual Polybrene test was responsible for an HTR, emphasising the importance of detecting weak Kidd antibodies. Many anti-Jk^a react more strongly with Jk(a+b−) than with Jk(a+b+) cells, and some anti-Jk^a can only be detected with Jk(a+b−) cells [37]; some anti-Jk^b also demonstrate dosage. Panels for screening patient sera for antibodies should, therefore, contain Jk(a+b−) and Jk(a−b+) red cells.

Kidd antibodies are usually IgG or a mixture of IgG and IgM; they are rarely pure IgM [38,39]. Most IgG anti-Jk^a are IgG3 or a mixture of IgG3 and IgG1, and occasionally IgG1 alone. IgG2 may also be present. Four anti-Jk^b contained IgG1; one also contained IgG3 and one IgG4 [39,40]. Around 40–50% of sera containing Kidd antibodies bind complement; some Kidd antibodies can only be detected in the antiglobulin test when polyspecific antiglobulin or anti-complement is used [41,42]. Some Kidd antibodies may not be detectable by techniques incorporating a diluent that binds calcium [43]. Only those Kidd sera with an IgM component are capable of complement-binding as IgG Kidd antibodies are unable to fix complement [42].

9.3.5.2 Clinical significance

Kidd antibodies, which are often difficult to detect, are a hazard in blood transfusion. Anti-Jk^a has been responsible for severe and fatal immediate HTRs [44–48] and is regularly associated with delayed HTRs, which may be severe, leading to oliguria, renal failure, and even death [49,50]. Anti-Jk^b has also been incriminated in severe delayed HTRs [51–53]. A major reason why Kidd antibodies are such a common cause of delayed HTRs is their tendency to fall rapidly to low or undetectable levels in the plasma [29,30]. Pineda *et al.* [49,54] estimated that over one-third of delayed HTRs were caused by anti-Jk^a. Antigen-negative red cells should be selected for transfusion to patients with Kidd antibodies.

In an unusual case, a Jk(a–b+) patient with chronic lymphocytic leukaemia was found to have alloanti-Jk^b 14 days after transfusion of two units of Jk(b+) red cells [55]. The transfused red cells clearly survived in the patient and the DAT was negative, but Jk^b could not be detected in the patient's blood. It has been suggested that this represents a case of alloantibody induced antigen loss [56].

In contrast to the haemolytic activity of Kidd antibodies in incompatible blood transfusion, Kidd antibodies are only very rarely responsible for severe HDFN, though there are a few reports of severe and fatal HDFN caused by anti-Jk^a [57] and -Jk^b [58–60].The reason why Kidd antibodies so rarely cause HDFN, even when present in relatively high titre, is unclear.

There are two reported cases of passenger lymphocyte syndrome caused by anti-Jk^a, following peripheral blood progenitor cell transplantation, one causing severe haemolysis [61] and the other non-haemolytic [62].

Kidd may be a minor histocompatibility antigen. Anti-Jk^a has been implicated in a severe vascular rejection of a kidney transplant [63]. An analysis of 370 renal transplants in a single centre suggested that Jk^a/Jk^b mismatched grafts had more interstitial inflammation than matched grafts [64].

9.3.5.3 Autoantibodies

Several cases of autoanti-Jk^a associated with AIHA have been described [65–70]. Red cells of one patient, who also developed idiopathic thrombocytopenic purpura (Evans syndrome), were initially typed as Jk(a–b+), but later became Jk(a+b+), their true phenotype as demonstrated by family study [70]. AIHA in a Jk(a+) patient taking Aldomet (methyldopa), with anti-Jk^a in her serum and in an eluate from her red cells, declined on cessation of the drug and the autoantibody gradually disappeared [66]. A Jk(a+b+) patient taking chlorpropamide, a hypoglycaemic agent, had an apparent anti-Jk^a in her serum and acute AIHA [68]. In a post-transfusion specimen the anti-Jk^a only reacted with Jk(a+b+) cells in the presence of chlorpropamide or related structures and the AIHA declined when chlorpropamide administration was stopped.

Autoanti-Jk^a has also been found in healthy individuals [71]. Four examples of benign autoanti-Jk^a reacted preferentially with red cells in the presence of parabens or certain other neutral aromatic compounds, the antibodies being detected because of the presence of parabens as preservatives in commercial low ionic-strength solutions [72,73].

A Jk(a–b+) nephrectomy patient who had suffered from chronic proteus infections showed signs of an HTR, although no transfusion had taken place [74]. The patient's serum contained autoanti-Jk^b. Jk(b–) red cells incubated with *Proteus mirabilis* reacted with anti-Jk^b reagents.

9.3.5.4 Monoclonal antibodies

IgM human monoclonal anti-Jk^a and -Jk^b have been produced by Epstein-Barr virus-transformation of lymphocytes from immunised donors and fusion with mouse myeloma cells to form heterohybridomas [75,76]. Some of these antibodies make excellent blood grouping reagents.

9.4 Jk(a–b–) phenotype and Jk3 antigen

The Kidd-null phenotype, Jk(a–b–), was first described by Pinkerton *et al.* in 1959 [77]. A Filipino woman of Chinese and Spanish ancestry, with two children, became

Table 9.3 Frequency of Jk(a–b–) phenotype in several populations, ascertained through screening by the urea lysis test.

Population	No. tested	No. Jk(a–b–)	Frequency (%)	References
Polynesian	17 300	47	0.272	[75]
Thai	25 340	5	0.020	[79]
Japanese	648 460	12	0.002	[80]
Taiwanese	95 451	22	0.023	[81]
Chinese	201 194	16	0.008	[82]
Chinese Han	100 000	19	0.019	[83]
Finnish	79 349	24	0.030	[84]
New Zealand Caucasian	120 000	0		[85]
English	52 908	0		[86]

jaundiced after blood transfusion. Her serum reacted with all cells save her own, which had the novel phenotype Jk(a–b–). Adsorption of her serum with Jk(a+b–) cells left some activity for Jk(a–b+) cells, but adsorption with Jk(a–b+) cells removed all antibody. Eluates from the adsorbing cells reacted with Jk(a+b–) and Jk(a–b+) cells. Thus, her serum contained a mixture of anti-Jk3 and anti-Jk^b.

9.4.1 Jk(a–b–) and *JK* silent alleles

Although rare in much of the world, many examples of Jk(a–b–) have been found in many different ethnic groups. Often they have been ascertained through the production of anti-Jk3. Jk(a–b–) is most abundant amongst Polynesians. Of 17 300 random Polynesian blood donors screened by the urea lysis method (Section 9.4.2) and confirmed serologically, 47 (0.27%) were Jk(a–b–) Jk:–3 [78]. The highest frequency was found in Niueans and Tongans, with 1.4% and 1.2% Jk(a–b–), respectively [78].The urea lysis method has been used to search for Jk(a–b–) in other ethnic groups (Table 9.3).

Homozygosity or compound heterozygosity for a variety of inactivating mutations in the *JK* gene have been responsible for Jk(a–b–) phenotype (Table 9.4). Others have been found in individuals of common Kidd phenotype through apparent discrepancy between genotype and serological result. The most common, often called the Polynesian mutation, is g>a in the invariant 3′ acceptor splice site of intron 5 of a *JK*B* allele (*JK*02N.01*), causing loss of exon 6 from mRNA transcripts [10,11]. The predicted, truncated Kidd glycoprotein could not be detected in *Xenopus* oocytes transfected with the abnormal transcript. Of 46 Polynesians, eight were heterozygous for the intron 5 mutation, a gene frequency of 8.7%, which predicts an incidence of 0.76% for Jk(a–b–) [11]. The same mutation was also found in Asians with the following allele frequencies: indigenous Taiwanese, 1–8%; Fujians (China), 2.5%; Filipinos, 9%; Indonesians, 1% [94].

Screening of 674 Black Brazilians for a mutation encoding Tyr187Stop (*JK*01N.05*), initially found in a Jk(a–b–) African American, disclosed one homozygote and five heterozygotes (gene frequency 0.52%) [90].

9.4.2 The urea lysis test for Jk(a–b–) phenotype

The urea lysis test for detecting Jk(a–b–) phenotype was discovered serendipitously when a Samoan man with aplastic anaemia appeared to have excessively high platelet counts in an automated system dependent on lysing red cells with 2 *M* urea [85]. These false platelet counts were shown to be due to failure of his Jk(a–b–) red cells to lyse. Red cells of common Kidd phenotypes lysed within one minute in 2 *M* urea; Jk(a–b–) red cells required at least 30 minutes for lysis. Red cells of individuals heterozygous for a null allele demonstrated intermediate lysis time in a modified urea lysis test [95].

9.4.3 Anti-Jk3

Anti-Jk3, the typical antibody of immunised Jk(a–b–) individuals, may be accompanied by separable anti-Jk^a or anti-Jk^b [77,96,97]. Only a minority of immunised Jk(a–b–) people produce anti-Jk3 [80,98]. An apparently 'naturally occurring' IgM anti-Jk3 was found in an untransfused Jk(a–b–) male, the only reported example;

Table 9.4 Some *JK* alleles responsible for Jk(a–b–) phenotype.

Allele symbol	Nucleotide change	Exon / intron	Amino acid change	Population	Refs
*JK*01N.01*	del exons 4 & 5	4, 5	Initiation Met absent	English, Tunisian	[87,88]
*JK*01N.02*	202C>T	5	Gln68Stop	American Caucasian	[89]
*JK*01N.03*	582C>G	7	Tyr194Stop	Swiss	[87]
*JK*01N.04*	956C>T	10	Thr319Met	African American	[89]
*JK*01N.05*	561C>A	7	Tyr187Stop	African American, Black Brazilian	[90]
*JK*02N.01*	IVS5–1g>a	Intron 5	Exon 6 skipped	Polynesians, Asians, others	[10,11]
*JK*02N.02*	IVS5–1g>c	Intron 5	Exon 6 skipped	Chinese	[91]
*JK*02N.03*	222C>A	5	Asn74Lys	Chinese, Taiwanese	[82,92]
*JK*02N.04*	IVS7+1g>t	Intron 7	Exon 7 skipped; fs, Leu223Stop	French Caucasian	[10]
*JK*02N.05*	723delA	8	fs, Ile262Stop	Hispanic American	[89]
*JK*02N.06*	871T>C	9	Ser291Pro	Finnish	[11,93]
*JK*02N.07*	896G>A	9	Gly299Glu	Chinese, Taiwanese, Thais	[79,82,83,92]
*JK*02N.08*	956C>T	10	Thr319Met	Indian	[89]

fs, frameshift.

his Jk(a–b–) sister had been pregnant seven times without making anti-Jk3 [99].

Jk3 antibodies react optimally by an antiglobulin test, the reaction being enhanced by enzyme treatment of the cells. Enzyme-treated cells may be haemolysed by anti-Jk3 in the presence of fresh serum [100]. Anti-Jk3 are usually IgG. Like other Kidd antibodies, anti-Jk3 may decline rapidly *in vivo* [96].

Anti-Jk3 has been responsible for severe immediate [101] and delayed [96,98,102] HTRs. Most babies of mothers with anti-Jk3 are clinically unaffected, although the baby's red cells may give a positive DAT, and in a few cases phototherapy was administered [97,98,103].

Two examples of autoanti-Jk3, or mimicking autoanti-Jk3, have occurred during pregnancy [104,105]. In one, which was associated with AIHA, mimicking autoanti-Jk^b was also present [104]. Autoanti-Jk3 may block Jk^a and Jk^b antigen sites, resulting in false serological results [106]. Anti-Jk3 in a patient with transient Jk(a–b–) phenotype is described in Section 9.4.5.

9.4.4 Jk(a–b–) of the dominant type

Red cells from two of 14 Jk(a–b–) Japanese blood donors found by the urea lysis test (Section 9.4.2) proved to be different from those of the other 12 and from Jk(a–b–) cells previously reported [80]. A family study suggested a dominant mode of inheritance: the presence of a Jk(a+b+) mother of two Jk(a–b–) daughters, both with Jk(a–b–) children, excludes homozygosity of a silent gene. The dominant inhibitor gene proposed to account for these observations was named *In(Jk)*, as it was considered analogous to *In(Lu)* of the Lutheran system (Section 6.8). Two further examples have been found in Japan. The molecular basis for In(Jk) remains unknown.

Jk(a–b–) red cells of the dominant type can bind anti-Jk3 and anti-Jk^a and/or anti-Jk^b, as detected in adsorption and elution tests. Kidd genotypes deduced in this way demonstrated that *In(Jk)* is not inherited at the *JK* locus. In(Jk) cells are less readily lysed in the urea lysis test than cells of common Kidd type and less resistant to lysis than Jk(a–b–) cells of the recessive type [80].

9.4.5 Transient Jk(a–b–)

An 85-year-old Russian woman with myelofibrosis and bleeding secondary to colon carcinoma was found to be Jk(a–b–) and to have anti-Jk3, which was responsible for a severe HTR [101]. Neither anti-Jk^a nor -Jk^b could be adsorbed and eluted from her red cells. Two years later her cells appeared to have a normal Jk(a+b–) phenotype and no anti-Jk3 or -Jk^b was present.

9.4.6 A *JK*A* allele responsible for a Jk_{mod} phenotype

A *JK*A* allele containing 130G>A in exon 4 (*JK*01W.01*), encoding Glu44Lys in the cytoplasmic N-terminal domain of the Kidd glycoprotein, was responsible for

reduced expression of Jk^a and Jk3 [21,107]. *JK*01W.01* also contained the 588G and intron 9 –46g, silent SNPs usually associated with *JK*B*. *JK*01W.01* homozygotes display substantial weakening of Jk^a and Jk3 and could be mistaken for Jk(a–b–), but weakened Jk^a and Jk3 were also apparent in *JK*A/01W.01* and *JK*B/01W.01* heterozygotes [107]. Such a dramatic effect of a glutamic acid to lysine substitution in the cytoplasmic domain might be unexpected, but analogies are seen in other blood groups, for example Fy^x (Section 8.3.4) and some D variants (Section 5.6). Furthermore, deletion of the cytoplasmic N-terminal domain, or just mutation of both Cys25 and Cys30 to serines, abolished membrane expression in oocytes, demonstrating the vital role of the N-terminal to successful localisation in the plasma membrane [8]. Immunoblotting indicated that the *JK*01W.01*-encoded protein hinders expression of the normal protein in heterozygotes, suggesting interaction between the proteins either in the membrane or during transport to the membrane, although the Kidd glycoprotein is generally considered monomeric red cell [107].

*JK*01W.01* is not rare in Caucasians: screening of 300 Swedish donors for 130A revealed an allele frequency of 4.2% [107].

9.5 The Kidd glycoprotein is the red cell urea transporter UT-B

Failure of Jk(a–b–) red cells to lyse in aqueous solutions of urea provided the first clue that the Kidd glycoprotein might function as a urea transporter (Section 9.4.2). Red cell lysis in the presence of urea results from an osmotic imbalance. In cells of common Kidd phenotype urea is transported very rapidly across red cell membranes, so in 2 *M* urea these cells rapidly take up urea, become hypertonic, and lyse because of the rapid diffusion of water into the cell. Jk(a–b–) red cells lack the Kidd glycoprotein. They take up urea slowly and, therefore, lyse very slowly in 2 *M* urea. Treatment of normal red cells with a mercurial inhibitor of urea and water transport resulted in a substantial retardation of lysis in 2 *M* urea (aqueous solution) [108]. Measurements of unidirectional urea and thiourea fluxes revealed that urea crosses the membrane of Jk(a–b–) red cells about 1000 times slower than normal cells [109].

The Kidd glycoprotein, UT-B, has substantial sequence homology with another human urea transporter (UT-A), present only on renal cells [9]. Physiological levels of expression of UT-B cDNA in *Xenopus* oocytes strongly facilitated urea transport, but not water permeability [6]. This activity is blocked by the urea transport inhibitors [5].

The Kidd glycoprotein is present on endothelial cells of the vasa recta, the vascular supply of the renal medulla, but is not present in renal tubules [110]. Urea transporters in the kidney play an important role in concentrating urea in the renal medulla, whilst conserving water, in order to produce concentrated urine [111]. UT-B in human colon epithelium could participate in the transport of urea across the colon mucosa and assist in the maintenance of a normal colonic bacterial population [112,113]. In red cells, UT-B has two main functions:

1 transporting urea rapidly in and out of the cells to prevent shrinkage as they pass through the high urea concentration of the renal medulla, and to prevent swelling as they leave;

2 to prevent the red cells from carrying urea away from the renal medulla, which would decrease the urea concentrating efficacy of the kidney [114].

The Kidd-null phenotype is not associated with any clinical defect, although, like UT-B knockout mice, Jk(a–b–) individuals have urine concentrating ability reduced by about one third [115,116]. This may be because other urea transporters, especially UT-A, compensate for the absence of UT-B in the kidney, and because maximal urea concentrating ability is rarely required under normal conditions. One-year old UT-B knockout mice, however, have severe renal dysfunction and structural damage, probably because of long-term hydronephrosis and polyuria [117].

Genome-wide association studies reveal an association between SNPs in *SLC14A1* and bladder cancer, identifying *SLC14A1* as a potential susceptibility gene [118,119].

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10.1 Introduction

The Diego system consists of 22 antigens, which includes three pairs of antithetical antigens – Di^a and Di^b, Wr^a and Wr^b, Wu and DISK – plus 16 antigens of very low frequency (Table 10.1). Di^a is a useful anthropological marker because it is polymorphic in most Mongoloid populations, but virtually absent from other ethnic groups. Di^a represents Leu854 and Di^b Pro854 in the red cell anion exchanger, band 3 or AE1 (CD233). Low frequency Wr^a (DI3) and high frequency Wr^b (DI4) represent Lys658 and Glu658 in band 3, respectively. Glycophorin A (GPA)-deficient red cells are Wr(a–b–), as Wr^b requires the presence of both band 3 and GPA for expression. The other low frequency Diego system antigens are all associated with amino acid substitutions in band 3. No true Diego-null phenotype has been reported, reflecting the functional importance of band 3. Band 3 is associated with the Rh proteins and RhAG, GPA, and many other proteins, as part of at least two red cell membrane protein macrocomplexes.

SLC4A1, the gene encoding band 3, is located on chromosome 17q12-q21.

10.2 Band 3, the red cell anion exchanger (AE1), and the gene that encodes it

Band 3, anion exchanger 1 (AE1), or CD233, is a major intrinsic red cell membrane glycoprotein, with approximately 1.2 million copies per red cell. After SDS PAGE of red cell membranes, band 3 is easily detected by Coomassie blue staining. It migrates as a diffuse band of about 100 kDa. Band 3 exists in the red cell membrane mostly as tetramers and dimers. For reviews on band 3 see [34–37].

The band 3 gene (*SLC4A1*) covers 18 kb of DNA and contains 20 exons [38] (Table 10.2). Cloning and sequencing of band 3 cDNA confirmed that the protein consists of three domains: a cytoplasmic N-terminal domain of 403 amino acids; a hydrophobic transmembrane domain of 479 amino acids; and a C-terminal cytoplasmic tail of 29 amino acids [39,40] (Figure 10.1). In the original model the transmembrane domain has 14 α-helical membrane-spanning domains and cytoplasmic N- and C-termini [34,40], but this model has been challenged [41] (Figure 10.1). The single *N*-linked oligosaccharide on Asn642, on the fourth extracellular loop, carries H, A, B, I, and i activity. Variation in the number of repeating *N*-acetyllactosamine units accounts for the broadness of the band on SDS PAGE. Associations of band 3 with other red cell membrane proteins and with components of the cytoskeleton are discussed in Section 10.7.

10.3 Di^a and Di^b (DI1 and DI2)

Di^a was first described in a Venezuelan family by Layrisse *et al.* [1] in 1955. It soon became apparent that Di^a is relatively common in South American Indians, but rare in people of European origin [1,42]. Two examples of

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Table 10.1 Antigens of the Diego blood group system.

Antigen				Molecular basis			
No.	Name	Frequency	Antithetical antigen	Nucleotides	Exon	Amino acids	References
DI1	Di^a	Low*	Di^b (DI2)	2561C>T	19	Pro854Leu	[1,2]
DI2	Di^b	High	Di^a (DI1)	2561C	19	Pro854	[2,3]
DI3	Wr^a	Low	Wr^b (DI4)	1972G>A	16	Glu658Lys	[4,5]
DI4	Wr^b	High	Wr^a (DI3)	1972G	16	Glu658	[5,6]
DI5	Wd^a	Low		1669G>A	14	Val557Met	[7,8]
DI6	Rb^a	Low		1643C>T	14	Pro548Leu	[9,10]
DI7	WARR	Low		1654C>T	14	Thr552Ile	[11,12]
DI8	ELO	Low		1249C>T	12	Arg432Trp	[13–15]
DI9	Wu	Low	DISK (DI22)	1694G>C	14	Gly565Ala	[15–17]
DI10	Bp^a	Low		1707G>A	14	Asn569Lys	[15,18]
DI11	Mo^a	Low		1967G>A	16	Arg656His	[15,19]
DI12	Hg^a	Low		1966G>T	16	Arg656Cys	[15,20]
DI13	Vg^a	Low		1663T>C	14	Tyr555His	[15,21]
DI14	Sw^a	Low		1. 1937G>A	16	Arg646Gln	[22,23]
				2. 1936C>T	16	Arg646Trp	
DI15	BOW	Low		1681C>T	14	Pro561Ser	[15,24,25]
DI16	NFLD	Low		1287A>T, 1681C>G	14	Glu429Asp, Pro561Ala	[26,27]
DI17	Jn^a	Low		1696C>T	14	Pro566Ser	[28,29]
DI18	KREP	Low		1696C>G	14	Pro566Ala	[25]
DI19†	Tr^a	Low		1653C>G	14	Lys551Asn	[10,18]
DI20	Fr^a	Low		1438G>A	13	Glu480Lys	[30,31]
DI21	SW1	Low		1936C>T	16	Arg646Trp	[32,33]
DI22	DISK	High	Wu (DI9)	1694G	14	Gly565	[33]

*Polymorphic in people of Mongoloid origin.
†Provisional assignment.

antibodies detecting an antithetical antigen, Di^b, were described by Thompson *et al.* [3] in 1967.

10.3.1 Frequencies

Frequency studies on Di^a are prodigious because of its usefulness as an anthropological marker. Di^a occurs almost exclusively among Mongoloid people. Extensive reviews of the frequency data are provided elsewhere [43–45]; studies on selected populations are shown in Table 10.3.

Di^a is most common in native South Americans, where the frequency is variable, but reaches over 70% in some populations. Di^a is also found in most Central and North American native populations, although the incidence in the northern continent is generally not as high as in the southern continent. Surprisingly, Di^a is rare among the Inuit of Alaska and Canada, but relatively common in Siberian Inuits. Di^a also occurs in eastern Asian populations where the frequency varies between about 2 and 12% [59].

Very few Di(a+) white people with no apparent Mongoloid admixture are reported [1,60,61]. Invasion of parts of Poland by Tartars in the thirteenth to seventeenth centuries was used to explain Di^a in 0.46% of Poles [56]. Di^a appears to be absent from Australian Aborigines and many Pacific Oceanic populations, and absent, or extremely rare, among Africans (Table 10.3).

A few large surveys have been carried out with anti-Di^b [43,44,49,62]. All Di(b–) individuals were Di(a+); Di(a–b–) phenotype has not been described (but see Section 10.6).

Table 10.2 Organisation of the band 3 gene, *SLC4A1* [38].

Exon	Size (bp)	Amino acids	Intron size (kb)
1	582		>3
2	83	1–5	0.125
3	91	6–36	0.998
4	62	36–56	0.757
5	181	57–117	0.095
6	136	117–162	0.472
7	124	162–203	0.227
8	85	204–232	0.152
9	182	232–292	0.539
10	211	293–363	0.232
11	195	363–428	0.178
12	149	428–477	0.114
13	195	478–542	1.503
14	174	543–600	0.377
15	90	601–630	0.543
16	167	631–686	1.126
17	254	686–771	1.527
18	170	771–827	0.086
19	174	828–885	0.620
20	2146	886–911	

10.3.2 The molecular basis of the Diego polymorphism

Di^a expression is associated with 2561C>T in exon 19: *DI*A* encodes Leu854; *DI*B* encodes Pro854 [2]. *DI*A* is associated with loss of *Msp*I and *Nae*I restriction sites. According to the 14 transmembrane-domain model, this amino acid substitution occurs in the seventh extracellular loop and amino acid 854 is also exposed at the cell surface in alternative models (Figure 10.1).

When red cells are treated with pronase or chymotrypsin prior to SDS PAGE, a band 3 polymorphism can be detected [63]. In most cases a 60 kDa band is stained representing the N-terminal region of band 3, but in a minority of subjects a second band of reduced mobility (63 kDa) is also apparent. The variant band, band 3 Memphis, results from 166A>G in exon 4 of *SLC4A1*, encoding Lys56Glu within the cytoplasmic N-terminal domain of band 3 [64,65]. Band 3 Memphis was initially detected in 6–7% of random blood samples [64]; a higher incidence has been found in African Americans (16%), Native Americans (17–25%), Chinese (13%), Filipinos (17%), and Japanese (29%) [66,67]. Red cell anion exchange inhibitors diisothiocyanatostilbene (DIDS) and its dihydro derivative H_2DIDS bind covalently to Lys539 of band 3 [39]. In some individuals with band 3 Memphis, the variant band 3 has markedly increased binding of H_2DIDS [68]. This is called band 3 Memphis variant II in order to distinguish it from band 3 Memphis variant I, which has normal H_2DIDS binding. Di(a+) red cells were found to have the band 3 Memphis variant II [69]. Band 3 Memphis from Di(a+) red cells bound about three times the normal quantity of radiolabelled H_2DIDS, whereas the band 3 Memphis from Di(a–) red cells bound normal quantities of H_2DIDS. Di^a is generally associated with band 3 Memphis variant II; Di^b is associated with either normal band 3 and with band 3 Memphis variant I. The enhanced H_2DIDS binding occurring with Leu854 (Di^a) could result from a localised conformational change that affects the rate of covalent cross-linking between Lys851 in the seventh extracellular loop and Lys539 in the fifth transmembrane domain [70].

*Msp*I cleavage of PCR products to determine *DI*A/B* genotype of 70 Parakanã (Amazonian) Indians revealed 27 *DI*A/A*, 26 *DI*A/B*, and 17 *DI*B/B*, giving gene frequencies of *DI*A* 0.57 and *DI*B* 0.43 [46]. Four of the *DI*A* homozygotes were heterozygous for 166G/A, revealing a new allele encoding Di^a, but not band 3 Memphis.

Studies on non-human primates suggest that 2561C (Di^b) *in cis* with 166G (band 3 Memphis) is the ancestral band 3 gene [71].

One Di(a+b–) Japanese donor genotyped as *DI*A/B* had a *DI*B* allele containing a CAC trinucleotide insertion, encoding histidine, at nucleotide 2359 in exon 18 [72].

10.3.3 Effect of enzymes and reducing agents

Di^a and Di^b are resistant to treatment of red cells with papain, trypsin, α-chymotrypsin, pronase, sialidase, and AET.

10.3.4 Weak Di^b

A healthy Mexican woman with many weak red cell antigens initially appeared to have Di(a–b–) red cells, but, on further testing, was shown to have weak Di^b [73]. Weaker than expected Di^b reactions were detected with red cells from several Di(a+) Mexican Americans [49]. All of 784 Hispanic Americans were Di(b+), but 11 had weaker than average Di^b antigens, yet only one of them was Di(a+) [62].

Di^b is depressed in South-East Asian ovalocytosis [74] (Section 10.9).

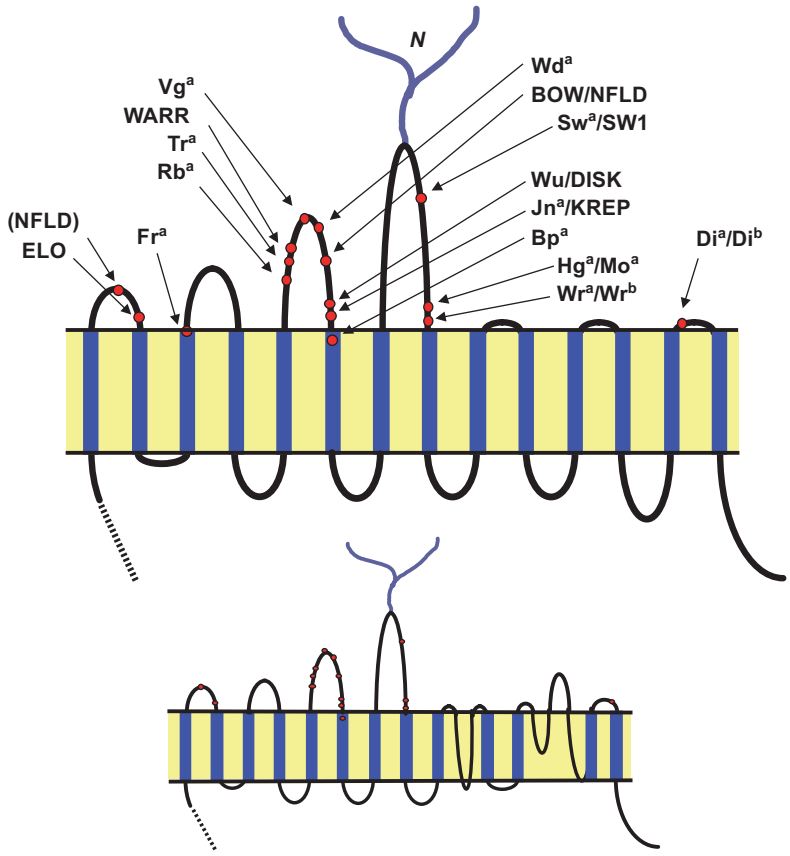


Figure 10.1 Two models for the topography of the membrane domain of band 3 glycoprotein. In the conventional model (above) the protein spans the membrane 14 times and has cytoplasmic N- and C-terminal domains [34,40]. Positions of the amino acid substitutions associated with the Diego system antigens are shown. *N* represents the single *N*-glycan at Asn642. The large cytoplasmic N-terminal domain is not shown. Below is an alternative model [41].

10.3.5 Anti-Di^a and -Di^b

The original anti-Di^a was responsible for fatal HDFN [42] and other cases of severe HDFN caused by anti-Di^a have been reported since [55,56,61,75]. Most anti-Di^a have been stimulated by pregnancy. One example of anti-Di^a, in an Australian Caucasian, was apparently 'naturally occurring' [60]. Anti-Di^a is implicated in a delayed HTR [76] and may have caused an immediate HTR, but the presence of anti-c and incomplete information confused the picture [77]. Red cell panels used for antibody screening often do not contain Di(a+) cells and examples of anti-Di^a remain undetected, even in regions where anti-Di^a is relatively common. In Brazil, four of 112 (3.6%) multitransfused patients had anti-Di^a [78], and in Singapore introduction of Di(a+) cells to an antibody screening panel revealed 19 anti-Di^a in 1383 samples referred for antibody investigation [79].

One of the original two anti-Di^b may have been responsible for a delayed HTR [3]. An HTR caused by anti-Di^b, in which the haemolysis appeared to have been largely intravascular, was considered to be a contributory cause of the patient's death [80]. Mochizuki *et al.* [81] list 27 published cases of HDFN caused by anti-Di^b, of which 10 required exchange transfusion, six received phototherapy (with or without top-up transfusion), one was treated with high dose intravenous immunoglobulin, and 10 required no therapy. In monocyte monolayer assays with anti-Di^b, significantly higher scores of adherence and phagocytosis were obtained with Di(a–b+) red cells than with Di(a+b+) cells [82]. No examples of 'naturally

Table 10.3 Results of some selected studies on the frequency of Di^a.

Population	No. tested	No. Di(a+)	Di^a frequency	References
Carib Indians (Venezuela)	121	43	0.3554	[42]
Arawaco Indians (Venezuela)	152	8	0.0526	[42]
Kainganges Indians (Brazil)	48	26	0.5416	[42]
Parakanã Indians (Brazil)	70	53*	0.7571	[46]
Chippewa Indians (USA)	148	16	0.1081	[47]
Penobscot Indians (USA)	249	20	0.0803	[48]
Inuits (Alaska, Canada)	1477	2	0.0014	[43]
Inuits (Siberia)	86	18	0.2093	[43,44]
Mexicans (USA)	1685	172	0.1021	[49]
Japanese	2427	244	0.1005	[50]
Chinese	617	32	0.0519	[43]
Chinese	1766	125*	0.0708	[51]
Chinese (Taiwan)	1000	32	0.0320	[52]
Koreans	277	17	0.0614	[53]
Indian (North India)	377	15	0.0400	[43]
Europeans	4462	1	0.0002	[42,49,54,55]
Poles	9661	45	0.0047	[56]
White Americans	1000	0	0	[42]
African Americans	827	1	0.0012	[43]
Ghanaians	107	0	0	[57]
Australian Aborigines	1374	0	0	[58]
Papua New Guineans	1741	0	0	[58]

*Predicted from molecular genotyping.

occurring' anti-Di^b are reported. Two of 74 sera containing red cell autoantibodies contained autoanti-Di^b together with other autoantibodies [83]. One of the autoanti-Di^b may have been responsible for AIHA.

Although anti-Di^a has been detected in sera containing multiple antibodies to low incidence antigens, anti-Di^a and -Di^b are most often found alone. They usually require antiglobulin to agglutinate cells, but directly agglutinating anti-Di^a [60] and -Di^b [84] have been described. Anti-Di^a and -Di^b are often IgG1 and IgG3 [85] and occasionally anti-Di^a binds complement and may haemolyse untreated cells [56].

Monoclonal anti-Di^a and -Di^b have been produced by human-mouse heterohybridomas incorporating lymphocytes derived from immunised individuals [50,86].

10.4 Wright antigens

10.4.1 Wr^a and Wr^b (DI3 and DI4)

Wr^a, first described by Holman in 1953 [4], has an incidence of around one in 1000 in white populations (Table 10.4). Very few large surveys have been carried out on other ethnic groups. No Wr(a+) was found among 2000 Australian Aborigines or 2000 Papua New Guineans [58]. One family gave a suggestion of close linkage between the genes controlling Wr^a and Sd(a++) [97]. Wr^a shows individual variation in strength of expression and is fully developed at birth.

The name anti-Wr^b was provisionally used in 1971 for an antibody, in the serum of a Wr(a+) woman, that detected a high frequency antigen [6]. Her red cells appeared to have a double dose of Wr^a antigen and the antibody reacted more strongly with Wr(a–) cells than with Wr(a+) cells. Wr^a and Wr^b dosage on Wr(a+b+), Wr(a–b+), and Wr(a+b–) cells was confirmed by an enzyme-linked antiglobulin test [98]. Two other examples of Wr(a+b–) phenotype have been described briefly [99,100].

Sequencing of PCR-amplified band 3 cDNA from the original Wr(a+b–) propositus revealed 1972G>A in exon 16, predicting Glu658Lys within the fourth extracellular loop of band 3 (*DI*02.03*) [5] (Figure 10.1). Three Wr(a–b+) individuals had the normal sequence (Glu658); seven

Table 10.4 Frequency of Wr^a in predominantly white populations.

Population	No. tested	No. Wr(a+)	Wr^a frequency	References
English	45 631	36	0.0008	[87,88]
English	5253		0.0004	[89]
Norwegians	5138	0	0	[90]
Norwegians	3140	2	0.0006	[90]
Swiss	3753	2	0.0005	[91]
Spanish	110 000	140	0.0012	[92]
Italians	6350	7	0.0011	[93]
Czechs	1500	1	0.0007	[94]
Americans (Boston)	2784	3	0.0011	[95]
Americans (New York)	5000	5	0.0010	[96]

Wr(a+b+) individuals were heterozygous for Glu658 and Lys658 codons. Another Wr(a+b–) individual was also homozygous for the Lys658 codon [100].

Wr^a and Wr^b are resistant to treatment of red cells with trypsin, chymotrypsin, pronase, papain, and sialidase, and with the reducing agent AET. Neither Wr^a nor Wr^b was detected on peripheral blood lymphocytes, granulocytes, or monocytes.

10.4.2 Association of band 3 with glycophorin A (GPA) and its importance in Wr^b expression

Band 3 and GPA are closely associated in the membrane. A monoclonal antibody to the cytoplasmic C-terminal domain of band 3 precipitated both band 3 and GPA [101], although most antibodies to either band 3 or GPA do not co-precipitate [35]. Binding of GPA antibodies to red cells significantly reduced rotational mobility of band 3 [102–104]. GPA may facilitate the translocation of newly synthesised band 3 to the membrane [105]. GPA is not essential for band 3 expression at the cell surface, but in red cells deficient in GPA, band 3 is imperfectly folded and moves slowly to the surface. In GPA-deficient cells the *N*-glycan on band 3 is of increased molecular weight and, despite normal quantities of band 3, anion transport is impaired (Sections 3.5.1 and 3.23), as a result of altered band 3 structure [106]. Whereas the cytoplasmic C-terminal tail of GPA is responsible for enhancing trafficking of band 3 to the cell surface, extracellular residues 68–70 of GPA increase band 3 anion transport activity [107]. Red cells of band 3 knockout mice are deficient in both band 3 and GPA, suggesting that band 3 plays a chaperone-like role and is essential for expression of GPA at the red cell surface [108]. This is not applicable to humans: GPA appears before band 3 on erythroid progenitor cells during human erythropoiesis [109,110]; a human erythroleukaemia cell line (K562) expresses GPA, but no band 3 [111,112]; and an infant with almost total band 3 deficiency has markedly reduced, but clearly present, GPA [113,114]. Red cells of transgenic mice expressing human GPA had reduced levels of mouse GPA, implying a competition between human and murine GPA for band 3 [115].

GPB, which lacks the cytoplasmic tail of GPA, does not appear to have any affect on band 3 [105]. GP(B-A-B) Mur, however, which resembles GPB but has an additional amino acid sequence in the external domain (Section 3.11.1.2), increases levels of band 3 in the membrane by 25–67% in the GP.Mur phenotype, resulting in superior HCO_3^- transporting capacities [116]. This affect could be attributed to GPA and GP(B-A-B)Mur interaction through the formation of heterodimers.

Despite a single amino acid substitution being the primary cause of the Wr^a/Wr^b dimorphism, Wr^b is not expressed in the absence of GPA. GPA-deficient, En(a–), red cells are Wr(a–b–) [117,118]. The only other Wr(a–b–) cells are those with some rare phenotypes involving hybrid glycophorins, which lack the part of GPA that reacts with antibodies called anti-En^aFR (Sections 3.5 and 3.10). Red cells with GP(B-A-B)Mur had enhanced expression of Wr^b [119]. Red cells of the three Wr(a+b–) patients demonstrated no abnormality in expression of the MNSsU or En^a antigens, of GPA, or of red cell sialic acid levels [100,120]. There was no abnormality in the sequence of *GYPA* cDNA from a Wr(a+b–) person [5].

Most monoclonal antibodies to GPA and band 3 do not co-precipitate both proteins, but monoclonal

anti-Wr^b either precipitated GPA [121,122] or GPA together with band 3 [123,124]. Six sera containing red cell autoantibodies co-precipitated band 3 and GPA [125]. Three of the sera contained autoanti-Wr^b, but the other three sera contained no anti-Wr^b, suggesting that other epitopes may exist that depend on band 3 and GPA interaction. Haemagglutination with alloanti-Wr^b could be inhibited by purified fragments of GPA, but, like anti-En^{a}FR, inhibitory activity was low and only detectable in the presence of lipid [120]. One monoclonal anti-Wr^b was inhibited by a synthetic peptide representing amino acid residues 65–70 of GPA; alloanti-Wr^b, autoanti-Wr^b, and two other monoclonal anti-Wr^b were not inhibited [126]. Monoclonal anti-Wr^b do not bind to human cell lines unless they express both GPA and band 3 [123]. The erythroleukaemia cell line K562 expresses GPA, but no band 3 or Wr^b, but transfection with band 3 cDNA induces Wr^b expression [112]. During *ex vivo* erythropoiesis, Wr^b appears at the cell surface at the same time as band 3, slightly after GPA [110]. Binding of antibodies to the extracellular domain of GPA causes immobilization of band 3, an effect that is significantly reduced in Wr(a+b–) cells [104].

Amino acid sequences of GPA and the hybrid glycophorin GP(B–A)Sch, which are associated with Wr^b expression, were compared with those of GP(A–B)Hil and GP(B–A)Dantu, which are associated with the Wr(a–b–) phenotype. The results suggested that amino acid residues 55–68, an α-helical region close to the insertion of GPA into the membrane, might be important in Wr^b expression [5,127]. Gln63Lys and Ala65Pro substitutions within GPA are both associated with abnormal Wr^b expression (Section 3.15). Hybrid glycophorins expressing SAT, however, do not express Wr^b, despite having amino acids 1–70 or 1–71 derived from GPA, with a transmembrane domain derived from GPB (Section 3.10.3) [127]. It is likely, therefore, that the association between band 3 and GPA occurs between the single membrane-spanning domain of GPA and the eighth membrane-spanning domain of band 3, and the extracellular regions adjacent to these domains.

Whether GPA is required for Wr^a expression is not known. Attempts to identify any membrane component by immunoprecipitation with monoclonal anti-Wr^a were unsuccessful [124].

10.4.3 Anti-Wr^a

Anti-Wr^a is a relatively common antibody. The reported incidence of anti-Wr^a in the sera of normal donors varies in different studies: the highest frequency was one in 13 sera [128], but other studies have provided figures of between one in 37 and one in 100 [89,92,96,129]. The incidence increases dramatically in patients, post-partum women, and in people with other alloantibodies [89,96]. About one in three patients with AIHA has anti-Wr^a [88,92,96]. Some anti-Wr^a are directly agglutinating, but most require an antiglobulin test for detection. Most anti-Wr^a in healthy donors are IgM or IgM plus IgG; IgG1 and IgG3 were the subclasses most commonly detected in pregnant women and transfused patients [92,129].

Anti-Wr^a is clinically significant. It has been implicated in acute HTRs [91,130–132] and has caused severe HDFN [4,133–135]. Red cells compatible by IAT should be selected for transfusion to patients with anti-Wr^a, although anti-Wr^a in patients are not usually detected because Wr(a+) cells are not generally used for antibody screening. Consequently, in the absence of a serological crossmatch, Wr^a incompatible transfusions will occur at a rate of between one in 40 000 and one in 100 000 and the risk of a resultant HTR has been estimated to be about two in a million [136,137]. Anti-Wr^a is common, so use of Wr(a+) screening cells means that many examples are detected and require identification. Consequently, the consensus opinion is that the expense of using Wr(a+) screening cells cannot be justified.

IgG1 mouse monoclonal anti-Wr^a (BGU1-WR) was produced as a result of immunising mice with Wr(a+) red cells [138].

10.4.4 Anti-Wr^b

Alloanti-Wr^b has been found in the sera of the three Wr(a+b–) patients [6,99,100], plus some Wr(a–b–) individuals with certain rare MNS phenotypes (Sections 3.5 and 3.10). Wr^b is generally considered resistant to treatment of red cells with proteases, but one anti-Wr^b did not react with ficin- or papain-treated red cells [100]. There is little information regarding the clinical importance of alloanti-Wr^b. An En(a–) patient with anti-Wr^b and -En^a suffered a mild delayed HTR after receiving six units of En(a+) blood [139] and red cells of a baby born to a mother with alloanti-Wr^b gave a positive DAT, but transfusion was not required [140].

Anti-Wr^b is a relatively common autoantibody [141,142]. Analysis of eluates from the DAT-positive red cells of 150 individuals revealed 110 antibodies unrelated to Rh, 46 of which contained anti-Wr^b, and 34 of those came from patients with AIHA [142]. Two anti-Wr^b autoantibodies in patients with DAT-positive red cells have been responsible for fatal intravascular haemolysis [143,144].

Many examples of murine and one rhesus monoclonal anti-Wr^b have been reported [86,145–147].

10.5 Other Diego antigens, DI5 to DI22

Diego was a simple system consisting of a pair of allelic antigens, Di^a and Di^b, from 1967 until Wr^a and Wr^b joined the system in 1995. Since 1996, a further 18 antigens have joined the Diego system. All except DISK (DI22), which is antithetical to Wu (DI9), are of very low frequency (Table 10.5) and are associated with amino acid substitutions in or close to extracellular loops of band 3 (Figure 10.1).

The low frequency antigens DI5 to DI21 (Table 10.1) were assigned to the Diego system following recognition of an association between antigen expression and a missense mutation in the band 3 gene [8,10,12,14,15,17,23,25,27,29,31,157]. In most cases at least two unrelated individuals with the low frequency antigen were shown to have the associated mutation. Only one Tr(a+) individual was available [10], so the assignment of Tr^a to the Diego system remains provisional. The WARR mutation was found in a Native North American kindred, the only family containing WARR+ members [12], and the mutations associated with Vg^a [15] and with KREP [25] were only identified in single families.

All the antigens are associated with a single amino acid substitution, apart from NFLD, in which two substitutions were found, and Sw^a, which represents either of two amino acid substitutions at the same position (Table 10.1). All substitutions are in the putative extracellular

Table 10.5 Frequencies of other antigens of the Diego system.

Antigen			Population	No. tested	No. positive	Antigen frequency	References
DI5	Wd^a	Waldner	North American	4000	0		[7]
			Norwegian	7151	0		[148]
			African Hei//om	114	2*	0.0175	[149]
DI6	Rb^a	Redelberger	English	10 200	1	0.0001	[9]
DI7	WARR	Warrior	North American	8275	1†		[11]
DI8	ELO		English	16 223	1	<0.0001	[13]
DI9	Wu	Wulfsberg	Norwegian	7000	1	0.0001	[16]
			Danish	2021	4‡	0.0020	[150]
			Australian	16 472	4	0.0002	[151]
DI10	Bp^a	Bishop	English	75 000	1	<0.0001	[18]
DI11	Mo^a	Moen	Norwegian	9000	0		[19]
			Belgian	9793	2	0.0002	[19]
DI12	Hg^a	Hughes	Welsh	5434	2	0.0004	[20]
DI13	Vg^a	Van Vugt	Australian	17 209	1	<0.0001	[21]
DI14	Sw^a	Swann	English	55 410	9	0.0002	[18,22,87,88]
			English	17 661	1	<0.0001	[11]
			Swiss	~7000	3	~0.0004	[152]
			Canadian	5000	3	0.0006	[153]
DI15	BOW	Bowyer	English	>55 000	0	<0.0001	[24]
DI16	NFLD	Newfoundland	North American	1125	0		[26]
			Japanese	45 825	2	<0.0001	[154]
DI17	Jn^a	Nunhart	Norwegian	13 824	0	<0.0001	[28,148,150]
DI19	Tr^a	Traversu	English	38 069	2	<0.0001	[18]
DI20	Fr^a	Froese	Canadian	1400	1§	0.0007	[30,155]

*Sisters. Only other Wd(a+) individuals have been Canadian Hutterites with the surname Waldner [7] and a black South African [149].
†Sister of WARR+ propositus.
‡Hov+. Later shown to be the same as Wu [156].
§Mennonite. Most Fr(a+) propositi have been found among Canadian Mennonites [32,155].

loops of band 3 (Figure 10.1), with the exception of that associated with Bp^a, which is in a membrane-spanning domain, but very close to loop 3, so Bp^a may represent a conformational change in the third loop [15]. Most of the amino acid changes are clustered on the third and fourth extracellular domains, but ELO and one of the NFLD substitutions are on the first loop, Fr^a is close to the second loop, and the Di^a/Di^b polymorphism is on loop 7. None of the positions of these amino acid changes challenges any of the models of protein topology (Figure 10.1). There is no evidence that any of the substitutions has an effect on the function of the band 3 molecule or on binding of the red cell anion exchange inhibitor H_2DIDS (see Section 10.7). It is likely, therefore, that the third and fourth extracellular loops are not involved in anion exchange [15]. Most of the substituted amino acids are not well conserved in evolution, but Asn569, which is changed to lysine in the Bp^a-associated substitution, is conserved in 12 anion exchanger homologues [15].

Adsorption of some sera containing anti-Wu, -NFLD, and -BOW with Wu+, NFLD+, or BOW+ cells will often remove all three antibodies [158]. BOW and NFLD represent different substitutions of Pro561, whereas Wu represents a substitution of Gly565 [15,17,25,27]. NFLD is associated with amino acid changes in the first and third extracellular loops [27]. The serological associations of NFLD with BOW and Wu suggest that Ala561 is most important in determining NFLD expression. Similar serological cross-reactivity occurs between Jn^a and KREP, which represent different substitutions of Pro566 [25,29]. Some Sw(a+) red cells are SW1+, whilst others are SW1– [23,32]. Anti-Sw^a appears to recognise changes associated with conversion of Arg646 to Gln or Trp, whereas anti-SW1 is specific for the Arg646Trp substitution [23]. Hg^a and Mo^a represent different substitutions of Arg656 [15], but no serological association is reported.

The high frequency antigen DISK (DI22) is antithetical to Wu. The only example of anti-DISK was produced by a woman who was homozygous for the Wu mutation (1694C) and whose red cells were DISK– and expressed higher levels of Wu than other Wu+ cells [33]. Her heterozygous brother was Wu+ DISK+. An IgM murine monoclonal antibody directed at the third external loop of band 3 did not react with red cells of another probable Wu homozygote and, therefore, behaves like anti-DISK [159].

Band 3 has two chymotrypsin cleavage sites in the third extracellular loop, at Tyr553 and Tyr555. Consequently, the antigens in the third loop are mostly sensitive to α-chymotrypsin, whereas those in the fourth and seventh loop are resistant [160]. This provides further evidence that Pro561Ala is the most important substitution in determining NFLD expression. There are two unexpected effects. One example of anti-ELO did not react with chymotrypsin-treated cells, though ELO is on the first loop. It is possible that the Arg432Trp substitution associated with ELO creates a new chymotrypsin site, giving rise to partial band 3 cleavage and loss of activity with one example of anti-ELO. Diego-system antigens are papain- and trypsin-resistant.

With the exception of anti-ELO, antibodies to the low frequency antigens from DI5 to DI22 have not been found to be clinically significant. A second ELO+ child of a woman with anti-ELO had mild HDFN and her third ELO+ child had severe HDFN, necessitating exchange transfusion [161,162]. The anti-ELO was IgG (mostly IgG3) and haemolytic IgM. Anti-Fr^a caused a positive DAT on cord red cells, but no other signs of HDFN [163].

Some antisera contain antibodies to numerous red cell antigens of low frequency. These antibodies are produced with no obvious immunising stimulus (see Chapter 29). Antibodies to all low frequency Diego antigens (with the possible exception of anti-Di^a) have been found in multispecific sera. Anti-ELO [161], -Sw^a, and -Fr^a [163] have also been produced as a result of immunisation by antigen-positive red cells. No anti-Rb^a was found in five Rb(a–) mothers of Rb(a+) children [9], no anti-Wd^a was found in six Wd(a–) Hutterite mothers of a total of 30 Wd(a+) children [7], and no anti-NFLD was found in an NFLD– mother of three NFLD+ children [154]. The only anti-DISK was a very strong antibody, reactive by direct agglutination [33].

Two cases of severe AIHA were caused by autoantibodies directed at chymotrypsin-sensitive determinants on band 3. One was IgA optimally active at 37°C [164]; the other cold reactive IgM plus IgG [165].

10.6 Band 3 deficiency

No Diego-null phenotype has been reported and it is likely that total band 3 deficiency would be lethal because of the vital importance of band 3 as an anion exchanger (Section 10.7). There is one description of a baby with only trace quantities (2%) of band 3, resulting from homozygosity for a band 3 mutation (band 3 Coimbra, Val488Met) [113,114]. The severely hydropic, anaemic baby, with dramatic erythroblastosis and poikilocytosis, was delivered by emergency caesarean section, resuscitated, and kept alive by blood transfusion. At 3 months

the baby developed distal renal tube acidosis (dRTA) (Section 10.8). At 3.5 years the patient was splenectomised and became largely transfusion independent with no major complications. Two other patients, with less severe symptoms, are homozygous mutations in the band 3 gene: band 3 Neapolis (deletion of 11 N-terminal amino acids) and band 3 Courcouronnes (Ser667Phe), with 88% and 65% band 3 deficiency, respectively [166,167].

Band 3 knockout mice and cattle with a band 3 nonsense mutation were able to survive, despite spherocytosis, haemolytic anaemia, and growth retardation [168–170].

10.7 Functional aspects and band 3 membrane complexes

Band 3 plays an important role in the efficient transport of respiratory gases in the blood. In the presence of H_2O, carbonic anhydrase II (CAII) in red cells hydrates CO_2 to HCO_3^- and H^+. Band 3, an antiporter, transfers HCO_3^- ions out of the cell in exchange for Cl^-. The proton then interacts with haemoglobin promoting the release of O_2. As HCO_3^- is more soluble than CO_2, this process supports the transport of CO_2 and the release of O_2 in the tissues, with the reverse process occurring in the lungs. The short C-terminal domain of band 3 associates with CAII and the N-terminal domain associates with deoxyhaemoglobin, glycolytic enzymes, and hemichromes [34–37]. This has led to the concept that band 3 is part of a metabolon, a functional complex of proteins that catalyse sequential reactions in a metabolic pathway [36,114].

In addition to being a membrane transporter and other metabolic functions, band 3 has a structural role, linking the membrane to the red cell cytoskeleton, a lattice of glycoproteins beneath the red cell membrane that is vital for maintaining the shape and integrity of the red cells (see Section 18.7 for reviews). The N-terminal domain of band 3 tetramers interacts with the red cell cytoskeleton (Figure 10.2). Reduced levels of red cell band 3 resulting from heterozygosity for a variety of mutations within the band 3 gene often causes abnormally shaped red cells, especially spherocytosis, a common, familial haemolytic anaemia characterised by small spheroid red cells [171]. There is no evidence that any of the mutations responsible for the low frequency Diego system antigens affects red cell morphology.

Band 3 tetramers form the core of a macrocomplex of membrane proteins, which includes trimers of the Rh proteins and the Rh-associated glycoprotein (RhAG), ICAM-4 (LW), CD47, GPA and GPB, and is linked to the cytoskeleton through ankyrin-1 and protein 4.2 [114,172] (Figure 10.2 and see Section 5.7). All of these proteins are either dramatically reduced or absent from red cells of the patient with only trace quantities of band 3 (Coimbra) and a similar effect was found in red cells of band 3 knockout mice [114]. Cotransfection experiments in K562 erythroleukaemia cells show that band 3 enhances expression of Rh proteins and RhAG at the cell surface [93]. Bruce *et al.* [114] speculate that RhAG could be a relatively non-specific channel for neutral small molecules and function as a gas channel for CO_2, O_2, and/or NO. As part of the band 3 metabolon, RhAG is ideally located to channel CO_2 to and from CAII, and O_2 to and from haemoglobin. In addition, the adhesion molecules ICAM-4 and CD47 might assist in facilitating transient adhesive interactions between the red cell and the vascular endothelium to maximise gas transfer, and may even have signalling functions, switching on the Rh/RhAG complex at the appropriate sites. The band 3/Rh macrocomplex may also be associated with AQP1 in the membrane, which provides the H_2O necessary for the hydration of CO_2.

Studies on mice deficient in the cytoskeleton protein 4.1R revealed another red cell membrane macrocomplex involving band 3 [174]. Dimers of band 3 are linked to the spectrin-actin junction through adducin and protein 4.1R, together with glycophorin C (Gerbich blood group antigen), which is linked to actin through p55 and protein 4.1R (see Section 18.7) [173,174]. In addition, reduced levels of Rh proteins, Kell, Xk, and Duffy glycoproteins in 4.1R-deficient mice suggest that these structures are also part of this 'junctional' macrocomplex, whereas RhAG does not appear to be involved. Phosphorylation of 4.1R at two sites in human red cells, which weakens the affinity of 4.1R for β-spectrin, renders GPC, Duffy, Kell, and Xk readily extractable by non-ionic detergent [175]. The glucose transporter, GLUT1, which is linked to the spectrin-actin junction through dematin and adducin, may also be part of the junctional complex [176] (Figure 10.2).

In addition to the two complexes, band 3 can also exist in the membrane as freely diffusing dimers [174].

Band 3 also plays a role in removal of senescent and oxidatively damaged red cells from the circulation, through the action of 'naturally occurring' autoantibodies [177,178]. Haemoglobin is denatured to form hemichromes. Hemichromes have strong affinity for the N-terminal domain of band 3 and could cause

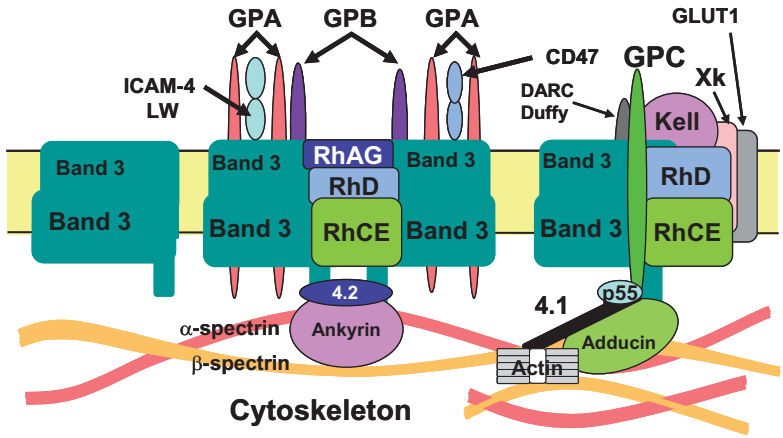


Figure 10.2 Diagrammatic representation of band 3 in the red cell membrane, showing the band 3 macrocomplex containing tetramers of band 3 linked to the spectrin matrix through protein 4.2 and ankyrin-1, the junctional complex linked to spectrin through adducin, and freely diffusing dimers of band 3.

cross-linking and disruption of band 3 attachment to the cytoskeleton, resulting in the formation of band 3 clusters. Binding of autoantibodies to the band 3 clusters may then lead to complement activation and phagocytosis of the aging red cells by splenic macrophages [178]. Similar mechanisms could be involved in the band 3 clustering and exposure of neoantigens in *Plasmodium falciparum*-infected red cells [179].

The sialic acid-rich glycoproteins, GPA, GPB, and GPC, are red cell receptors for the malarial parasite *P. falciparum* (Sections 3.21.1 and 18.8). In addition, interaction between a chymotrypsin-sensitive region of band 3 and *P. falciparum* merozoite surface protein 1 (MSP1) plays a role in a sialic acid-independent invasion pathway [180].

10.8 Tissue distribution

In addition to erythroid cells, band 3 has only been detected in acid-secreting intercalated cells of the kidney collecting duct. The renal isoform of band 3 lacks the N-terminal 65 amino acid residues of erythroid band 3, as a result of transcription initiation from an alternative promoter region in intron 3. It plays an important role in acid secretion by removing H^+ in the form of HCO_3^- ions [181]. Recessive and dominant forms of distal renal tubule acidosis (dRTA), a condition associated with impaired acid secretion in the distal nephron and inability to acidify urine resulting in symptoms including metabolic acidosis, hypokalaemia, nephrocalcinosis, kidney stones, and metabolic bone disease, result from mutations in the band 3 gene (reviewed in [36,171,182]). Mutations responsible for dRTA do not usually affect red cell band 3 function because of the presence of GPA in red cells, which is not present in kidney. The Gly701Asp mutant, for example, which causes recessive dRTA but no red cell defects, is absolutely dependent on GPA for movement to the red cell surface. The reduction in anion transport associated with these mutations in transfected *Xenopus* oocytes is rescued, at least partially, by co-transfection with GPA cDNA [183,184].

Despite the limited band 3 tissue distribution, band 3 was unexpectedly expressed on gastric cancer cells. Expression of band 3 is modulated by the microRNA miR-24, which decreases expression of band 3 through binding to the 3′-untranslated region of band 3 mRNA. Transfection of miR-24 into gastric cancer cells reduced band 3 expression, whereas miR-24 inhibitor co-operated with hemin to induce band 3 expression on K562 erythroleukaemia cells [185].

10.9 South-East Asian ovalocytosis (SAO)

A form of hereditary ovalocytosis, known as South-East Asian Ovalocytosis (SAO), is relatively common in the southern Pacific region and confers protection against cerebral malaria in children (reviewed in [35,36,171]).

SAO results from heterozygosity for a band 3 gene with a 27 bp deletion, which encodes a variant protein (band 3 SAO) with a deletion of amino acids 400–408, the region of the protein at the boundary of the cytoplasmic N-terminal domain and the first membrane-spanning domain (Figure 10.1). Band 3 SAO also has the Memphis I variant and is non-functional as an anion transporter. Despite its common occurrence, no person homozygous for this mutation has been found, supporting the assertion that homozygosity for any mutation that inactivates band 3 would normally be lethal.

Many antigens are depressed on red cells of Melanesians with SAO, including Di^b and Wr^b, but also S, s, U, En^a, D, C, e, Kp^b, Jk^a, Jk^b, Xg^a, Sc1, LW, Ge2, Ge3, Ge4, I^T, and I^F [74,186]. Reduced binding to SAO cells was also detected with rodent monoclonal antibodies to extracellular epitopes on band 3 and with monoclonal anti-Wr^b [187]. Depression of antigens on band 3 in SAO may result from the disturbance of co-operative interactions between the membrane-spanning domains, important in maintaining structural integrity of band 3. Depression of antigens of the Rh membrane complex (RhD, RhCcEe, LW, SsU) could be due to reduced translocation of the complex to the cell surface [188]. In addition there may be wider effects on other membrane proteins, caused by disruption of protein complexes involving band 3 and to an abnormal interaction between band 3 SAO (and dimers of band 3 and band 3 SAO) and the cytoskeleton [187].

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11 Yt Blood Group System

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11.1 Introduction

Anti-Yt^a, an antibody detecting an antigen of high frequency, was first found through crossmatching in 1956 [1]. The antithetical antibody, anti-Yt^b, detects an antigen on red cells of about 8% of white people and was found eight years later [2]. Yt, also known as the Cartwright system, remains a two-allele system (Table 11.1); an inherited Yt(a–b–) phenotype has not been found.

Yt^a and Yt^b represent His353Asn in acetylcholinesterase (AChE) on red cells. *YT* (*ACHE*) is located on chromosome 7q22.

11.2 Yt antigens and red cell acetylcholinesterase

Yt^a and Yt^b are on the GPI-linked red cell glycoprotein, acetylcholinesterase (AChE). Immunoprecipitation with anti-Yt^a and -Yt^b isolated structures of apparent MW 72 kDa under reducing conditions and 160 kDa under non-reducing conditions; structures of identical electrophoretic mobility were obtained by immunoprecipitation with monoclonal antibodies to AChE [3]. The structures precipitated by anti-Yt^a and -Yt^b had AChE enzyme activity. Further proof that Yt antigens are on AChE was provided by a MAIEA assay with alloanti-Yt^a and -Yt^b, and with monoclonal anti-AChE [4].

Estimates of 3000–5000 sites per red cell were obtained in quantitative analyses with monoclonal IgG anti-AChE and estimates of 7000–10 000 sites were obtained with Fab fragments, suggesting that the enzyme exists in dimeric form in the red cell membrane [3].

The complement-sensitive population of red cells (PNHIII) from patients with paroxysmal nocturnal haemoglobinuria (PNH) are deficient in several glycoproteins, including AChE, that are anchored to the membrane by means of glycosylphosphatidylinositol (GPI) (see Chapter 19). Most anti-Yt^a were non-reactive with PNHIII cells, but reacted with the relatively normal, complement-insensitive population of cells (PNHI) from the same patient [5]. Furthermore, in two PNH patients, the PNHI cells were Yt(b+), yet their PNHIII cells failed to react with anti-Yt^b.

AChE plays an essential part in neurotransmission. Acetylcholine is a neurotransmitter that permits the transmission of an electrical signal when released from a nerve terminal at a neuromuscular junction. AChE rapidly hydrolyses acetylcholine to terminate the signal. AChE exists in different tissues in a variety of forms, a result of post-translational modification and alternative splicing [6,7]. The function of red cell membrane-bound AChE is not known.

Partial human *ACHE* cDNA clones were obtained by screening a cDNA library constructed from fetal muscle and adult brain RNA with an oligonucleotide complementary to the amino acid sequence of a peptide derived from *Torpedo* AChE [8]. The complete gene encoding human AChE was cloned by screening a human genomic cosmid library with an oligonucleotide probe prepared by PCR amplification of human DNA with primers corresponding to a conserved region of mouse AChE gene [7]. Three exons encode the signal peptide and N-terminal 535 amino acids; alternative splicing of the next exon results in structural divergence of the C-terminal domain so that a GPI-anchor may be attached in erythroid cells, but not in nervous tissue [7].

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ACHE was assigned to chromosome 7q22 by *in situ* hybridisation and by PCR-based somatic cell hybridisation techniques [9,10]. An analysis of 31 families informative for segregation of *YT* and *KEL* revealed loose linkage between these loci with maximum likelihood of a recombination fraction of 0.26 [11].

11.3 Yt^a and Yt^b

11.3.1 Frequency, inheritance, and molecular basis of the Yt antigens

Some population studies are shown in Table 11.2. Around 1 in 500 Caucasians are Yt(a–) [1,12,15]. The phenotype is less common in African Americans, with about 1 in 2000 Yt(a–) [15]. About 8–10% of Caucasians are Yt(b+) [12,14]. Genotype frequencies of *YT*A/A* 0.8966, *YT*A/B* 0.1006, and *YT*B/B* 0.0028 calculated from the results of tests with anti-Yt^a and -Yt^b on 659 white Canadians correlated closely enough with the observed figures to suggest that no third allele is present [14]. Tests with anti-Yt^a and -Yt^b on Israeli Arabs and Druse and on a variety of populations of Israeli Jews revealed a relatively high frequency of Yt^b [17]. None of 5000 Japanese was Yt(a–) and Yt^b has not been detected in relatively small samples of Japanese, Inuits, Thais, and Native Americans [18,19]. The results of two large series of family studies are consistent with simple Mendelian inheritance of *YT*A* and *YT*B* in a co-dominant fashion, and absence of a silent allele [12,14].

Two single nucleotide changes in *ACHE* are associated with Yt^a/Yt^b polymorphism. One, 1057C>A in exon 2, encodes His353Asn; the other, 1432C>T, is a silent mutation in exon 3 in the codon for Pro477 [20]. The His353Asn substitution has no effect on the catalytic activity of the enzyme [21].

Table 11.1 Antigens of the Yt blood group system.

No.	Name	Relative frequency	Molecular basis
YT1	Yt^a	High	1057C His353
YT2	Yt^b	Low	1057A Asn353

11.3.2 Effects of enzymes and reducing agents

Yt^a is not affected by trypsin, but is destroyed by α-chymotrypsin treatment of the red cells; papain and ficin may also destroy the antigen, but this appears to depend on the anti-Yt^a used. Yt^a is not sialidase-sensitive.

Yt^a and Yt^b are sensitive to disulphide bond reducing agents. Eight anti-Yt^a were non-reactive with red cells treated with 200 m*M* dithiothreitol (DTT) [22]. Nine of

Table 11.2 Population studies with anti-Yt^a and -Yt^b.

		Phenotype frequencies		Gene frequencies		
Population	No. tested	Yt(a+)	Yt(b+)	*YT*A*	*YT*B*	References
English	2568	0.998	nt	0.9559	0.0441	[1,12]
South Welsh	29 802	0.999	nt	0.9761	0.0239	[13]
European	1399	nt	0.081	0.9587	0.0413	[12]
White Canadians	659	1.000	0.106	0.9469	0.0531	[14]
Hispanic Americans	9933	0.999	nt	0.9638	0.0362	[15]
African Americans	10 622	0.999	nt	0.9783	0.0217	[15]
African Americans	714	nt	0.084	0.9571	0.0429	[16]
Israeli Jews	2549	0.986	0.213	0.8845	0.1154	[17]
Israeli Arabs	85	0.976	0.235	0.8706	0.1294	[17]
Israeli Druse	77	0.974	0.260	0.8571	0.1429	[17]
Japanese	5000	1.000	nt			
	70*		0	1.0000	0.0000	[18]

*These 70 Japanese donors are also included in the 5000 tested with anti-Yt^b.
nt, not tested.

15 anti-Yt^a did not react with cells treated with 6% 2-aminoethylisothiouronium bromide (AET) and the other six sera showed reduced reactivity [23]. Yt^b, determined by two anti-Yt^b, was destroyed by 200 m*M* DTT and 500 m*M* 2-mercaptoethanol [24].

11.3.3 Development and distribution of Yt^a and Yt^b

Yt antigens are present on red cells from cord blood samples. Yt^b appears to be fully developed at birth [25], but the strength of Yt^a on cord cells is weaker than that on red cells of adults [1,25,26]. Of 10 fetal red cell samples, taken at less than 32 weeks' gestation, eight did not react with anti-Yt^a and the other two reacted only very weakly [26].

AChE is present in nervous tissue and on erythroid cells [6], but little is known about the tissue distribution of Yt antigens. Yt^a was not detected by flow cytometry on lymphocytes, granulocytes, or monocytes [27].

11.4 Anti-Yt^a and -Yt^b

Despite the relatively low incidence of Yt(a–b+) phenotype, numerous examples of anti-Yt^a have been identified. Of 79 sera containing anti-Yt^a, 57 were monospecific and 22 contained a mixture of antibodies [28]. Yt^b appears to be a poor antigenic stimulus as anti-Yt^b is rare and generally found in antibody mixtures; only a few examples are reported [2,12,25,29–31]. Anti-Yt^a and -Yt^b have been stimulated by pregnancy or transfusion; neither has been 'naturally occurring'.

Yt antibodies are mostly IgG and require an antiglobulin test to agglutinate red cells. Of those anti-Yt^a sera that could be subclassed, most contained IgG1, some IgG1 plus IgG4, and a few IgG4 alone; none contained IgG3 [32–35]. Some anti-Yt^a bind complement [36], others do not [26,37].

Yt antibodies have not caused HDFN, despite several cases of women with anti-Yt^a having Yt(a+) children, and one case of a woman with anti-Yt^b having a Yt(b+) child [25]. Anti-Yt^a is alleged to have been responsible for a fatal delayed HTR in a patient with sickle cell disease [38] and has been implicated in an immediate HTR [39]. Many patients with anti-Yt^a, however, have received multiple transfusions of Yt(a+) red cells with no ill effects [28,33,35,40]. Of 18 patients with anti-Yt^a who received Yt(a+) red cells, only three showed evidence of decreased red cell survival [28]. Survival studies with radiolabelled antigen-positive red cells have given widely variable results with Yt antibodies [26,33,35–37,40–42]; in a few cases it was predicted that incompatible red cells would be removed rapidly from the circulation [36,36,37]. Cellular assays have also given variable results [26,28, 31,32,35,39,42,43]. Of 73 anti-Yt^a tested by a monocyte monolayer assay, 47 gave a positive result (>5% reactivity), 19 of those giving reactivity scores over 20%. Both anti-Yt^b tested gave scores >5%, and one of them was >20% [35]. One example of anti-Yt^a appeared benign before transfusion of incompatible blood, but subsequently *in vivo* and *in vitro* assays gave indications of haemolytic potential [43].

For transfusion purposes, each example of anti-Yt^a must be assessed independently. For patients with anti-Yt^a least incompatible blood should usually be selected for transfusion, but Yt(a–) red cells should be selected for patients with strong examples of the antibody.

An apparent alloanti-Yt^a in a Yt(a+) patient led to speculation of an inherited Yt^a variant [44]. The antibody did not react with the patient's own cells or with those of his Yt(a+) father, but did react with his mother's red cells. The antibody appeared 9 days after transfusion of 5 units of blood and disappeared after a few months.

11.5 Transient Yt(a–b–) phenotype, anti-Yt^{ab}, and red cell AChE deficiency

In view of the vital role of AChE in neurotransmission, it is not surprising that no inherited null phenotype resulting from deletion or inactivating mutation of *ACHE* has been found. A cardiac transplant candidate appeared to have the Yt(a–b–) phenotype, but some anti-Yt^a could be adsorbed and eluted from his cells [45]. His serum contained an antibody, anti-Yt^{ab}, that did not react with his own cells or with PNHIII cells, which lack GPI-linked proteins. Reduced 24-hour *in vivo* survivals of radiolabelled Yt(a–b+) and Yt(a+b–) red cells suggested that only autologous red cells would be suitable for transfusion. Red cells of the patient contained about 10% of normal AChE and about 15% of normal AChE enzyme activity was detected. Four months after the initial investigation, weak Yt^a activity was apparent by an antiglobulin test and red cell AChE content was 54–60% of normal.

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12 Xg Blood Group System

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12.1 Introduction

The human sex chromosomes began to arouse the interest of blood groupers with the discovery in 1962 of an X-linked blood group locus, *XG*. Mann *et al.* [1] found an antibody in the serum of a much transfused white man (Mr And) that detected an antigen with a frequency that, unlike all previous blood groups, differed in males and females. Family studies confirmed the suspicion that expression of the antigen, named Xg^a (XG1), was controlled by an X-linked gene. No antigen antithetical to Xg^a has been found, so the allele of *XG*A* is denoted *XG*0* here.

CD99, an antigen produced by a gene on both X and Y chromosomes, is closely related to the Xg blood group [2]. Because Xg^a and CD99 are encoded by closely linked homologous genes, CD99 has become XG2 of the Xg system. Xg^a and CD99 have been invaluable tools in the study of X- and Y-linkage, X–Y pairing and recombination, X inactivation, sex determination, X and Y aneuploidy, and various sex upsets, especially XX maleness.

12.2 Xg^a frequencies

The normal human chromosomal complement or karyotype is 46,XX in females and 46,XY in males. (Karyotypes are written with the total number of chromosomes first followed by the complement of sex chromosomes.) The *XG* gene resides on the X chromosome, but not on the Y chromosome. If a man has an *XG*A* allele on his X chromosome his red cells are Xg(a+) and if he has an *XG*0* allele they are Xg(a–). Homozygous *XG*A/A* and heterozygous *XG*A/0* women are Xg(a+); only homozygous *XG*0/0* women are Xg(a–). Consequently, more women than men express Xg^a on their red cells.

Results of testing 6784 unrelated Northern Europeans with anti-Xg^a were as follows: males, 66% Xg(a+) and 34% Xg(a–); females, 89% Xg(a+) and 11% Xg(a–) [3]. Phenotype, gene, and genotype frequencies are shown in Table 12.1. Results of other studies on a variety of ethnic groups are listed in Table 12.2. The incidence of *XG*A* differs remarkably little between most of the populations studied.

12.3 Xg^a inheritance

Analysis of the Xg groups of 2540 Northern European families with a total of 5824 children [3], together with many other families [20], proved that the *XG* locus is X-linked. With very few exceptions, Xg^a is inherited in a way expected of a character produced by an X-linked dominant gene. This expected pattern of inheritance is shown in Table 12.3.

Although the overwhelming majority of families with normal karyotypes fit the simple model presented in

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Table 12.1 Phenotype, gene, and genotype frequencies calculated from results of tests with anti-Xg^a on 6784 unrelated Northern Europeans [3].

		All	Males	Females
Phenotype	Xg(a+)	0.767	0.656	0.887
	Xg(a–)	0.233	0.344	0.113
Gene	*XG*A*	0.659		
	*XG*0*	0.341		
Genotype	*XG*A*		0.659	
	*XG*0*		0.341	
	*XG*A/A*			0.434
	*XG*A/0*			0.450
	*XG*0/0*			0.116

Table 12.3, there have been a few families that appear to break the rules of X-linked inheritance. According to these rules an Xg(a+) male must have received his *XG*A* gene from his mother, yet 16 Xg(a+) sons of Xg(a–) mothers are recorded [3,6,20–22]. Race and Sanger [21] suggested that a small portion of the father's X chromosome, including the *XG* locus, may have been translocated onto his Y chromosome, this Y chromosome being transmitted to his sons. A more probable explanation also involves X–Y recombination, with Xg^a expression in the sons being regulated by a gene (*XGR*) derived from the paternal Y chromosome (Section 12.9). In one of the families, one of two Xg(a+) men with an Xg(a–) mother has an Xg(a–) son [21]. Xg(a+) men must have Xg(a+) daughters, who inherit their father's single X chromosome. Cases of Xg(a–) daughters with Xg(a+) fathers have been considered less significant violations of X-inheritance because of the difficulties involved in ruling out non-paternity [3,20].

The first firm evidence that *XG* is located near the tip of the short arm of X was provided by an Xg(a–) woman who had failed to inherit her father's *XG*A* allele [23]: she had an X-Y translocation, 46,X,t(X;Y)(p22.3;q11), in which the distal segment of the short arm of one of her X-chromosomes was missing and replaced by part of the long arm of a Y-chromosome. The woman's son, who had inherited the abnormal chromosome and was nullisomic for Xpter–p22.3, was also Xg(a–). He suffered from a generalised ichthyosis as a result of zero steroid sulphatase activity, locating the steroid sulphatase (*STS*) gene, known to be closely linked to *XG*, on Xpter–p22.3.

Table 12.2 *XG* gene frequencies in various populations.

Population	No.	*XG*A*	*XG*0*	References
Northern European*	15716	0.66	0.34	[3–9]
Spaniards	636	0.59	0.41	[10]
Greeks	638	0.55	0.45	[11]
Sardinians	322	0.76	0.24	[12]
Israelis, non-Ashkenazi	201	0.68	0.32	[13]
Black New Yorkers and Jamaicans	219	0.55	0.45	[14]
Indians, Bombay	100	0.65	0.35	[15]
Thais	181	0.57	0.43	[16]
Japanese	529	0.68	0.32	[17]
Chinese	171	0.60	0.40	[5]
Chinese, Taiwan	178	0.53	0.47	[5]
Chinese, Hong Kong	1300	0.49	0.51	[18]
Taiwanese (Aboriginal)	164	0.38	0.62	[5]
Native Americans, Navajo	308	0.77	0.23	[5]
Australian Aborigines	352	0.79	0.21	[19]
New Guineans	263	0.85	0.15	[19]

*All the studies with people of Northern European extraction, which included people from North America, gave very similar gene frequencies.

12.4 Xg^a antigen

12.4.1 Biochemistry of Xg^a and the effects of enzyme treatments

Xg^a is destroyed by treatment of the red cells with the proteases bromelin, ficin, papain, pronase, trypsin, and chymotrypsin [24,25]. Xg^a is not destroyed by sialidase.

Immunoblotting with alloanti-Xg^a revealed that Xg^a resides on a red cell membrane component, probably a sialoglycoprotein, of apparent MW 22.5–28 kDa [25]. Sialidase treatment of red cells reduced the apparent MW of this structure by about 1.5 kDa. Whether the Xg glycoprotein is associated with CD99 in the red cell membrane is controversial [26,27] (see Section 12.6).

The sequence of *XG* (Section 12.8) predicts a 180 amino acid peptide with an extracellular N-terminal domain of about 142 residues, containing 16 potential *O*-glycosylation sites and no sites for *N*-glycosylation, a 20 residue transmembrane domain, and a 24 residue

Table 12.3 Phenotypes and genotypes of possible mating types and expected progeny.

Father		Mother		Sons		Daughters	
Xg(a+)	*XG*A*	Xg(a+)	*XG*A/A*	Xg(a+)	*XG*A*	Xg(a+)	*XG*A/A*
Xg(a+)	*XG*A*	Xg(a+)	*XG*A/0*	Xg(a+)	*XG*A*	Xg(a+)	*XG*A/A*
				Xg(a–)	*XG*0*	Xg(a+)	*XG*A/0*
Xg(a+)	*XG*A*	Xg(a–)	*XG*0/0*	Xg(a–)	*XG*0*	Xg(a+)	*XG*A/0*
Xg(a–)	*XG*0*	Xg(a+)	*XG*A/A*	Xg(a+)	*XG*A*	Xg(a+)	*XG*0/A*
Xg(a–)	*XG*0*	Xg(a+)	*XG*A/0*	Xg(a+)	*XG*A*	Xg(a+)	*XG*0/A*
				Xg(a–)	*XG*0*	Xg(a–)	*XG*0/0*
Xg(a–)	*XG*0*	Xg(a–)	*XG*0/0*	Xg(a–)	*XG*0*	Xg(a–)	*XG*0/0*

C-terminal cytoplasmic domain [28]. A putative 21 amino acid leader peptide is cleaved after membrane insertion.

12.4.2 Development and loss of Xg^a

Xg^a is developed at birth, but cord red cells may give weaker reactions than red cells of adults [8,29]. Xg^a appears to develop quite late in fetal life: of 54 samples from fetuses between 6 and 20 weeks' gestation, only 19 were Xg(a+), a significantly lower Xg^a frequency than that found in adults [30]. The youngest Xg(a+) fetus was 12 weeks old.

Expression of Xg^a declines exponentially with red cell age, with an *in vivo* half-life of 47 days [31]. Between 5 and 10% of red cells from an Xg(a+) male lack Xg^a antigen [32]. During *in vitro* erythropoiesis, Xg glycoprotein appears on the erythroblasts after glycophorin A and band 3, but before the Rh proteins [33].

12.4.3 Xg^a dosage and site density

Xg^a is expressed equally strongly on the red cells of hemizygous males as on those of homozygous females, but Xg^a expression on red cells of heterozygous females may be weaker. About 5–10% of Xg(a+) females, all heterozygotes, have very weak expression of Xg^a. Weak Xg^a in males is very rare.

The number of Xg^a binding sites per Xg(a+) red cell was estimated to be about 9000 in one study [32], but only 159 in another [34].

12.4.4 Xg^a on other cells

In 1974, Fellous *et al.* [35] utilised a microcomplement fixation test to detect Xg^a on cultured fibroblasts from Xg(a+) donors and on human–rodent somatic cell hybrids. Xg^a co-segregated with a number of known X-linked characters and the results also suggested that Xg^a on fibroblasts, like red cells, is not subject to X inactivation (see Section 12.7.1). Alas, Fellous and his colleagues were unable to repeat their feat of detecting Xg^a on cells other than red cells and neither, subsequently, could Hsu *et al.* [36].

XG transcripts were detected in fibroblasts [20], as well as in erythroid tissues and in heart, placenta, skeletal muscle, prostate, thyroid, spinal cord, and trachea [27]. Low levels of *XG* mRNA were also detected in some fetal tissues and in adult heart, lung, kidney, testis and some lymphoid cell lines [27,37].

12.5 Anti-Xg^a

Many examples of anti-Xg^a have been described [1,4,7,17,18,25,38–40]. Xg^a is apparently not very immunogenic yet, unlike other rare blood group antibodies, most sera containing anti-Xg^a are not 'contaminated' with other blood group antibodies. Twelve of the first 14 anti-Xg^a [21] and 11 of 13 anti-Xg^a found in Japanese volunteer donors [40] have been in men, somewhat surprising even considering the different frequencies of the Xg(a–) phenotype in men and women. In Hong Kong, four anti-Xg^a were identified in 325 serum samples referred for antibody investigation, and one anti-Xg^a was found in sera from 60 108 blood donors; all five antibodies were in men [18]. Anti-Xg^a often appear to be 'naturally occurring'. Although Xg^a antibodies occasionally

agglutinate red cells directly, they are generally IgG, react by the antiglobulin method, and are often capable of fixing complement.

Anti-Xg^a has never been held responsible for HDFN or for an HTR. One patient with anti-Xg^a received six units of Xg(a+) blood with no signs of a haemolytic reaction [38]. Repeated injections of small volumes of radiolabelled Xg(a+) red cells into a patient with anti-Xg^a survived normally [39]. Autoanti-Xg^a has been identified in a pregnant woman [41,42].

Murine monoclonal anti-Xg^a have been produced by immunising mice with human red cells [43] or with a peptide corresponding to a segment from the N-terminal of the Xg glycoprotein [37]. Two human monoclonal anti-Xg^a have also been described [44].

12.6 CD99, a quantitative polymorphism related to Xg

In 1981 Goodfellow and Tippett [2] started a new chapter in the Xg story when they observed that a monoclonal antibody to a determinant controlled by an X-borne gene (*MIC2*, now *CD99*), was defining a red cell polymorphism related to the Xg blood group. The monoclonal antibody, called 12E7, was produced as a result of immunising mice with human leukaemic T cells [45]. CD99, the antigen defined by the 12E7 antibody, is expressed on all human tissues tested [46], but on red cells, unlike other cells, the level of expression shows individual variation. By antiglobulin tests, radioimmunoassay, or flow cytometry, a quantitative polymorphism is observed and individuals can be subdivided into CD99 high expressors and CD99 low expressors [2,34].

Testing of red cells from over 300 Europeans demonstrated the following association between Xg and CD99 [2]:

all Xg(a+) individuals are CD99 high expressors;
all Xg(a–) females are CD99 low expressors;
about 68% of Xg(a–) males are CD99 high expressors and 32% low expressors.

Goodfellow and Tippett [2] postulated that a locus, *YG*, on the Y chromosome, controls the level of expression of the *CD99* gene. *YG* would have two alleles, *YG*A* (*Yg^a*) and *YG*0* (*Yg*). Individuals are CD99 high expressors if they have an *XG*A* or *YG*A* allele and CD99 low expressors if they have neither. Although family studies partially substantiated this theory [47], in light of information provided by some exceptional families the model was modified to involve a locus on X and Y chromosomes, which regulates both Xg^a and CD99 red cell expression [48] (described in Section 12.9). This putative regulator locus is called *XGR*.

CD99 became XG2 when two examples of alloanti-CD99 were found in healthy Japanese blood donors [49]. The antibodies gave the same pattern of reactions as monoclonal anti-CD99 with CD99 high- and low-expressor red cells.

Like Xg^a, CD99 is located on a sialoglycoprotein. CD99 is destroyed by the proteases papain, pronase, trypsin, and chymotrypsin [46,50,51]. It is generally sialidase-resistant [46,50], but one exceptional CD99-like antibody did not react with sialidase-treated cells [51]. Immunoblotting of red cells, lymphocytes, various human cell lines, and human–rodent hybrid cells containing a human X or Y chromosome showed that CD99 is associated with a glycoprotein of approximate MW 32 kDa [26,27,50–53]. Sialidase treatment of the cells resulted in a reduction in apparent MW of the CD99 structure [26,27,50,53]. Immunoblotting of immunoprecipitates confirmed that CD99 and Xg^a are located on separate structures [26,27]. Partially purified CD99 glycoprotein inhibited anti-CD99 and anti-Xg^a [54]. Petty and Tippett [26] found that alloanti-Xg^a co-precipitated Xg^a and CD99 glycoproteins, suggesting that these two homologous structures are associated in the membrane, but Fouchet *et al.* [27] were unable to repeat this result with a monoclonal antibody to the Xg glycoprotein.

CD99 is located on a protein of about 186 amino acids comprising an N-terminal signal peptide of 20 or 21 amino acids (cleaved after membrane insertion), about 100 extracellular residues, a hydrophobic transmembrane region, and a 36 amino acid cytoplasmic tail [54,55]. No difference in size or charge was detected between the X- and Y-encoded forms of the molecule [52].

There are an estimated 27 000 binding sites for anti-CD99 on lymphocytes, 4000 on platelets, and only 1000 on high-expressor and 100 on low-expressor red cells [50]. Reticulocytes from CD99 low expressors have lower levels of *CD99* transcripts than those from CD99 high expressors [34].

12.7 X-chromosome inactivation and the pseudoautosomal region

12.7.1 X-chromosome inactivation

Normal mammalian somatic cells have two X chromosomes in the female, but only one in the male. As most X-borne genes do not have a homologue on the Y

chromosome, the potential difference in dosage of X-linked genes between male and female cells is compensated by a process called X-chromosome inactivation [56,57]. In each somatic cell of an XX female only one of the X chromosomes is active, the other becoming permanently inactivated at an early stage in embryological development when a few million cells are formed. Whether the maternal or paternal X chromosome in any cell becomes inactivated is generally a matter of chance but, once inactivation has taken place, all descendants of that cell will have the same inactivated X chromosome. Female mammals are, therefore, mosaics of roughly equal numbers of cells with either the paternal or maternal X chromosome active. X inactivation is a *cis* phenomenon, which spreads along the chromosome from an X-inactivation centre, where the gene *XIST* produces a non-coding mRNA that appears to coat the inactive X.

Not all X-borne genes are subject to inactivation, with about 15% of the genes on the human X chromosome escaping inactivation [58]. The first locus known to deviate from the rule of X inactivation was *XG*. If *XG* were subject to inactivation, and assuming that Xg^a is a direct product of the *XG*A* gene and not manufactured outside the red cell, then heterozygous *XG*A/0* women would be expected to have a mixed population of Xg(a+) and Xg(a–) red cells. No such mosaicism occurs. Natural chimeras have proven that mixtures of Xg(a+) and Xg(a–) red cells can be produced by the same marrow [59,60] and post-bone marrow transplant conversion from Xg(a–) to Xg(a+) and *vice versa* provides further evidence that Xg^a production is restricted to the bone marrow [61].

CD99 also escapes inactivation [62]. Hybrid cell lines containing only an inactivated human X chromosome expressed CD99 and the antigen was expressed at increasing levels in hybrid cells with multiple inactive X chromosomes.

12.7.2 The pseudoautosomal region

During male meiosis it is important that the X and Y chromosomes segregate to separate spermatocytes. Consequently, like the autosomes, the X and Y chromosomes undergo pairing during the first meiotic division. This pairing, however, only involves the telomeric regions of the short and long arms of each chromosome. Within the pairing regions genes are shared and recombination occurs. The pairing regions are called pseudoautosomal regions (PAR): PAR1, with about 24 genes, is on the short arm and PAR2, with about five genes, is on the long arm [57,63].

PAR1 has a recombination rate about 20 times the average rate for the genome. Some genes within PAR1 and very close to the telomere undergo 50% recombination and cannot be distinguished from autosomal genes on family evidence. *CD99* is pseudoautosomal, but situated very close to the pseudoautosomal boundary and only recombines with X- and Y-linked genes on the other side of the boundary in about 2% of male meioses [64]. Pseudoautosomal genes are on both X and Y chromosomes, so no dosage compensation is required and they do not participate in X inactivation.

XG is not pseudoautosomal, but straddles to the pseudoautosomal boundary on the X chromosome [28]. It is likely that on very rare occasions *XG* is involved in recombination with the Y, providing an explanation for the rare families in which Xg(a–) mothers have Xg(a+) sons [3,6,20–22] (Section 12.3).

12.8 *CD99* and *XG* genes

Human–rodent somatic cell genetics has demonstrated that *CD99* is located on both X and Y chromosomes [65–67]. Hybrid cells express CD99 when either X or Y is the only human chromosome retained [66]. *CD99* cDNA was cloned by screening a cDNA expression library with a mixture of two monoclonal antibodies to CD99 epitopes [54,68]. A cDNA probe was used to show that *CD99* genes on the X and Y chromosomes are identical [68]. Somatic cell genetics [66] and *in situ* hybridisation [69] have mapped *CD99* to the tips of the short arms of the X and Y chromosomes, at Xp22.32 and Yp11.3.

Isolation of 95 kb of genomic DNA encompassing the entire *CD99* gene revealed a 52 kb gene orientated toward the centromere and with its 5′ end 95 kb from the pseudoautosomal boundary (*PAB1X*) [70] (Figure 12.1). The gene comprises 10 small exons: exon 1 encodes the leader peptide; exons 2–9 and 23 base pairs of exon 10 encode the CD99 protein (Table 12.4). No difference was detected between the organisation of the X- and Y-borne alleles.

Genomic sequences between the 3′ end of *CD99* and a CpG-rich region proximal to the pseudoautosomal boundary were used to isolate a 600 bp clone from a bone marrow cDNA library. This cDNA was, in turn, used to isolate an 820 bp transcript containing a 540 bp open reading frame capable of encoding a 180 amino acid polypeptide with a high level of homology with the CD99 protein, but not containing the sequence representing the CD99 epitope [28]. Mouse monoclonal and rabbit polyclonal antibodies raised to a 14 amino acid peptide

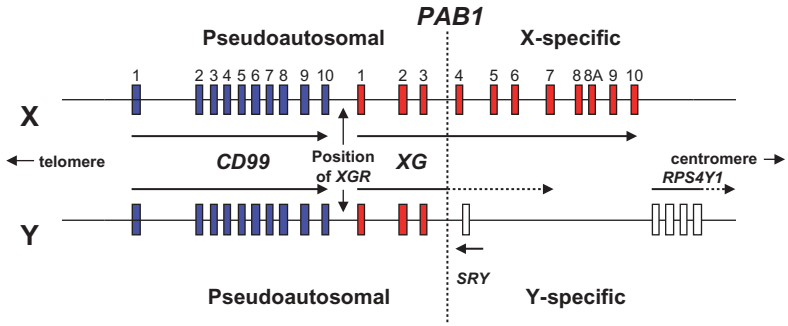


Figure 12.1 A region of about 250 kb the X and Y chromosomes spanning the pseudoautosomal boundary (*PAB1*), showing the organisation of *CD99* and *XG* [28,71]. Both genes contain 10 exons on X, but only 3 exons of *XG* are on Y. *CD99* and exons 1–3 of *XG* are pseudoautosomal; exons 4–10 of *XG* are X-specific. Exon 8A in *XG* was present in fibroblast RNA, but not erythroid RNA [20]. *SRY* and *RPS4Y1* are Y-specific genes. The position of *XGR*, a proposed regulator of *XG* and *CD99* expression on red cells [48], is shown between *CD99* and *XG*.

Table 12.4 Organisation of the *CD99* and *XG* genes [20,28,70].

	CD99			*XG*		
Exon	Exon size (bp)	Amino acids*	Intron size (kb)	Exon size (bp)	Amino acids*	Intron size (kb)
1	~244	1–23	23	246	1–21	12.5
2	33	23–34	3.2	42	21–35	3.3
3	48	34–50	2.4	24	35–43	8.3
4	45	50–65	0.6	63	43–64	7.0
5	69	65–88	1.0	63 (66)†	64–85	7.0
6	48	88–104	1.2	69	85–108	2.0
7	51	104–121	4.1	51	108–125	10
8A‡				45	(125–140)‡	
8	114	121–159	6.8	36	125–137 (140–152)	5.0
9	57	159–178	7.7	117	137–176 (152–191)	4.0
10	533	178–186		67 (244)	176–180 (191-195)	

*Amino acids encoded in CD99 by *CD99* and in the major erythroid form of Xg protein (fibroblast form in parentheses) by *XG*.
†In a minority of *XG* cDNA clones an additional 3 bp, inserting a serine at position 86, appeared between exons 5 and 6 [20].
‡Exon 8A is present in fibroblast RNA, but not in erythroid RNA [20].

synthesised to represent an N-terminal sequence encoded by this cDNA, behaved as anti-Xg^a in haemagglutination tests and were shown to bind the same membrane structure as anti-Xg^a by an antibody-specific immobilisation of antigens assay [37]. The antibodies gave an identical banding pattern to human anti-Xg^a on immunoblots of red cell membranes. The *CD99* homologue is, therefore, *XG*. Like *CD99*, erythroid *XG* transcripts consist of 10 exons, with exon 1 encoding the leader peptide and exons 2–10 the native protein (Table 12.4). An additional exon, exon 8A, is present in transcripts from fibroblasts [20,28].

XG spans the pseudoautosomal boundary, *PAB1X*. Exons 1–3 are situated within PAR1, whereas exons 4–10 are X-specific (Figure 12.1). Exons 1–3 of *XG* are also present on the Y chromosome. Transcription from the *XG* promoter on Y results in a low abundance of transcripts that contain exons 1–3 of *XG* plus sequences from two downstream Y-linked genes, the gene for the

testis-determining factor (*SRY*) and *RPS4Y1*. The *SRY* sequence is in antisense configuration and most of these transcripts do not maintain an open reading frame [71]. *XGPY*, an expressed pseudogene of *XG* with a frameshift mutation in exon 5, is on the long arm of Y at Yq11.21 [71].

XG has two modes of inheritance: the 5′ end is subject to a recombination rate 20 times that of the genome average and is also involved in X/Y recombination; the 3′ end is only subject to X/X recombination, with an average recombination rate.

There is substantial amino acid sequence homology between CD99 and the Xg protein, with corresponding blocks of regions rich in acidic amino acids, basic amino acids, proline residues, and glycine residues [20]. The Asp61-Gly-Glu-Asn sequence of CD99 recognised by 12E7 antibody (anti-CD99) is in a region rich in acidic amino acids not present in the Xg protein.

Transfection of mouse fibroblastic cells with *XG* and/or *CD99* cDNA molecules showed that Xg and CD99 proteins were expressed independently and at a similar level in single and double transfectants [27]. This suggests that the phenotypic association between Xg^a and CD99 polymorphisms is regulated primarily at the transcriptional level and not through association of the glycoproteins in the membrane.

12.8.1 Xg polymorphism

The molecular basis for the Xg polymorphism is not known. Monoclonal antibodies to at least two separate epitopes on the Xg glycoprotein, human anti-Xg^a, and rabbit antibodies raised to a 14 amino acid peptide corresponding to an N-terminal sequence of the Xg polypeptide, all reacted with Xg(a+), but not Xg(a–), red cells [37]. This suggests that the Xg(a–) phenotype results from absence of the Xg glycoprotein from the red cell. Reverse transcriptase PCR amplified *XG* fragments from Xg(a+) cord blood, but not Xg(a–) cord blood [20], indicating that substantially less RNA is produced by the *XG*0* allele of *XG*, than by the *XG*A* allele.

12.9 A model for explaining the association between the Xg and CD99 polymorphisms

When Goodfellow and Tippett [2] first recognised the unusual association between expression of Xg^a and CD99 on red cells, they proposed a hypothesis involving a gene (*YG*) on the Y chromosome. Both *XG* and *YG* regulate red cell expression of CD99 encoded by the structural gene *CD99*. However, families demonstrating recombination between *CD99* and *XG* and between *YG* and the X and Y chromosomes induced Goodfellow *et al.* [48] to modify their model.

In one family a normal 46,XX female had apparently received her father's *XG*A* on one of her X chromosomes and yet a *CD99* DNA probe showed that she had also received her father's Y chromosome PAR1 including his Y-borne *CD99* gene. Recombination must have separated *CD99* from the locus controlling expression of Xg^a.

Brothers receive the same Y chromosome from their father, yet seven of 172 Xg(a–) males did not have the same *YG* allele as their brother; that is, despite being Xg(a–), they differed in the level of their CD99 red cell expression [47]. In one family, part of which is shown in Figure 12.2, all the males had the same Y chromosome, but not all the Xg(a–) males were CD99 high expressors; one (II-1) was a low expressor [48]. A *CD99* RFLP showed that II-1 had inherited the same *CD99* gene from his father as that received on an X chromosome by his two sisters (II-2 and II-4) and that he had inherited a different *CD99* gene on a Y chromosome from his father from that received by his brother (II-3). Therefore, both *CD99* and

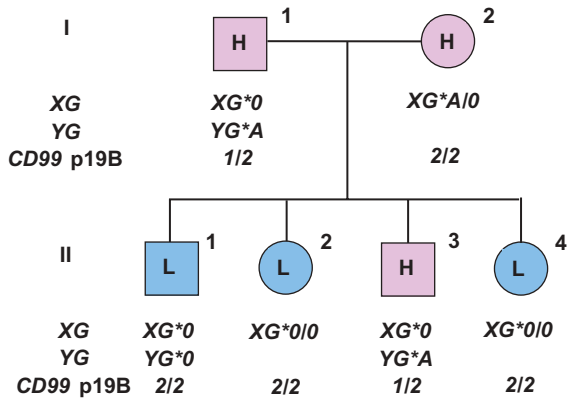


Figure 12.2 Part of family showing *XG* and *YG* genotypes and *CD99 Taq*I RFLP (p19B) genotypes, demonstrating recombination of *CD99* and the gene determining CD99 red cell expression (*YG*) with the remainder of the sex chromosomes [48]. The father (I-1) has passed an X chromosome with a *CD99 2* allele to his two daughters (II-2 and II-4) and a Y chromosome with *CD99 1* and *YG*A* (CD99 high-expressor) alleles to one of his sons (II-3), but a Y chromosome with *CD99 2* and *YG*0* (CD99 low-expressor) alleles to his other son (II-1). H, CD99 high; L, CD99 low.

YG have been involved in an X–Y exchange, are pseudoautosomal on the Y chromosome, and are distal to the male sex-determining gene *SRY*.

In order to explain these phenomena Goodfellow *et al.* [48] proposed the existence of a regulator locus, *XGR*, which controls *cis* expression of the structural loci *XG* and *CD99* on the X chromosome and of *CD99* on the Y chromosome. This gene is polymorphic. One allele (XGR^{high}) induces Xg^a antigen expression from the *XG* locus and high CD99 expression from the *CD99* locus; the other allele (XGR^{low}) prevents Xg^a expression and results in low level CD99 expression, probably by regulating transcription. All Xg(a+) individuals must have XGR^{high} on at least one X chromosome and so they have high expression of CD99. Xg(a–) females must have XGR^{low} on both X chromosomes and, therefore, must have low expression of CD99. Xg(a–) males must have XGR^{low} on their X, but can have either XGR^{high} or XGR^{low} on their Y, and so can have high or low expression of CD99 (Table 12.5). Although *XGR* is pseudoautosomal, it must be very close to the pseudoautosomal boundary (Figure 12.1) as recombination resulting in Xg^a inheritance contravening the rules of X-linkage is extremely rare (Section 12.3).

This model in which both Xg^a and CD99 expression on the red cells are controlled by the same gene (*XGR*) explains why the frequencies of Xg^a and CD99 high expression are so similar. It also provides an explanation for the families in which an Xg(a–) mother has an Xg(a+) son [3,10,20–22]. The son receives his mother's *XG* gene on his X chromosome, but his father's Y-derived XGR^{high} allele on the same chromosome as a result of X–Y recombination between *XGR* and *XG*. It should be emphasised that this theory is conjectural, but it does provide a useful model for explaining the data.

Table 12.5 Effects of *high*- and *low*-expression alleles of *XGR* on Xg^a and CD99 red cell phenotypes.

	XGR allele		Phenotype	
	X	X		
Females	*High*	*High*	Xg(a+)	CD99 high
	High	*Low*	Xg(a+)	CD99 high
	Low	*Low*	Xg(a–)	CD99 low
	X	Y		
Males	*High*	*High*	Xg(a+)	CD99 high
	High	*Low*	Xg(a+)	CD99 high
	Low	*High*	Xg(a–)	CD99 high
	Low	*Low*	Xg(a–)	CD99 low

12.10 XX males and sex chromosome aneuploidy

12.10.1 XX males (testicular disorder of sex development)

In order to maintain the chromosomal basis of sex determination, it is imperative that Y-borne genes outside the pseudoautosomal region are not normally involved in X–Y recombination, especially *SRY*, the gene controlling the testes determining factor. One occasion where this axiom appears to break down is in the rare case of XX males, sterile males with an apparently normal female karyotype. Approximately one in 20 000 men is 46,XX, yet is phenotypically and psychosexually male [72]. Xg and CD99 have played an important part in determining the aetiology of this phenomenon. In six families with informative Xg groups the XX male propositus had not received his father's *XG*A* allele, suggesting superficially that both X chromosomes were maternal in origin [21], probably caused by a small exchange of genetic material at the tips of the short arms of the X and Y chromosomes resulting in a paternally derived X chromosome that has lost its *XG* locus and gained the Y-derived testis determining genes [73]. This is consistent with the observed Xg distribution in XX males being much closer to that of XY males than to that of XX females [74,75]. A very informative family analysed for red cell expression of Xg^a and CD99 proved X–Y interchange as a cause of XX maleness [76]. Occasionally XX males express a paternally derived *XG*A* allele, suggesting that the X–Y recombination does not always involve the *XG* locus [21,72,77]. The X–Y interchange may involve an exchange of different amounts of genetic material, explaining how the recombinant chromosome could have both *XG* and *SRY* loci [78]. Although XX maleness can be explained by an exchange of genetic material between the X and Y chromosomes in about 80% of cases, other mechanisms, often involving autosomal genes, appear to be involved in the minority of examples of this form of sex reversal.

12.10.2 Sex chromosome aneuploidy

Aneuploidy is the term given to karyotypes in which the number of chromosomes is not a true multiple of the haploid number (23 in humans). Aneuploidies in which

one to five X chromosomes are involved can be viable, presumably because in somatic cells only one X chromosome is active (Section 12.7.1). One or more Y chromosomes may also be involved. The Xg blood group provided a great deal of information about the non-disjunctions that have caused these chromosomal upsets, in some cases pinpointing the meiotic division at which non-disjunction occurred [75,76]. More recent evidence on the origin of the X chromosomes in sex chromosome aneuploidy has been elicited from studies with DNA probes [79].

12.11 Functional aspects and association with disease

Although the natural ligands are not known, CD99 appears to function as a receptor and adhesion molecule. CD99 enhances α4β1 integrin-dependent T-cell adhesion to inflamed vascular endothelium under shear stress conditions [80] and modulates peripheral T-cell rosette formation and homotypic aggregation of thymocytes and B cells [81,82]. Yet CD99 can also induce apoptosis of thymocytes and mature T cells by a caspase-independent pathway [83,84]. A truncated isoform of CD99, which lacks most of the cytoplasmic domain as the result of alternative splicing of *CD99* transcripts, may function in an opposite way to full-length CD99 by inhibiting CD99-induced homotypic aggregation of B cells [82]. Expression of the CD99 long form in a T-cell line is sufficient to promote CD99-induced adhesion, whereas co-expression of both isoforms is required to trigger T-cell death [85].

Homophilic interaction between CD99 on endothelial cells and on monocytes and neutrophils is important in the migration of the leucocytes through endothelial vessel walls during inflammation [86,87]. CD99 may also play a role in migration of haemopoietic progenitors across the endothelium. CD99 is expressed on the majority of $CD34^+$ cells from bone marrow and mobilised peripheral blood, but only on 50% of $CD34^+$ cells from cord blood [88].

CD99 and HLA class I molecules co-localise both in the Golgi apparatus and at the cell surface, through a physical association via their transmembrane domains [89]. In B-cell and T-cell lines, decreased CD99 expression resulted in HLA class I retention in the Golgi [89,90]. In addition to HLA class I, both isoforms of CD99 are associated with HLA class II and the tetraspanin CD81 in T cells [91]. This tetraspanin web (see Section 23.2) may be involved in the regulation of T-cell proliferation. CD99 is expressed at around 8-fold higher levels on acute lymphoblastic leukaemia lymphoblasts than on normal T cells and could be used as a marker for minimal residual disease [92].

CD99 is expressed at the cell surface in most cases of Ewing sarcoma (EWS), a malignant, aggressive bone tumour, and is the best diagnostic marker for this disease [93]. CD99 contributes to oncogenesis by preventing neural differentiation through modulation of a mitogen-active protein kinase pathway [94]. Whereas 100% of EWS tumours express CD99, tumours in 24% of EWS patients also express Xg^a, which defines a subgroup of patients with worse prognosis than those not expressing Xg^a [95]. Forced expression of Xg^a in an EWS cell line enhanced cell migration and invasion *in vitro*, whereas knockdown had the opposite effect. Knocking down of CD99 expression in human EWS cell lines reduced their ability to form tumours and bone metastases when grafted into immunodeficient mice [94]; Xg^a expression increased the number of metastases [95].

CD99 is present in a variety of other tumours, including some carcinomas, leukaemias, and lymphomas. In osteocarcinoma, prostate cancer, and breast cancer cells the long isoform of CD99 (and in particular the presence of Ser168) inhibited metastasis, through increased motility, whereas the shorter variant appears to favour the phenomenon [96,97].

In contrast to CD99, almost nothing is known regarding the function of Xg^a, although structural homology between Xg^a and CD99 might suggest similar functions.

12.12 Xg^a and CD99 in animals

Gibbons (*Hylobates lar lar*) have Xg^a on their red cells: of 52 tested, 30% of males and 53% of females were Xg(a+), very suggestive of X-linkage. Other great apes (67 chimpanzees, two gorillas, 20 orangutans, and five gibbons of the species *Hylobates pileatus*) as well as various monkeys (including 60 baboons), and a few non-primates, were all Xg(a–). CD99 was not detected on the red cells or peripheral blood lymphocytes of 10 gibbons, regardless of their Xg phenotype. CD99 was detected on red cells and fibroblasts of chimpanzees and gorillas, but not of orangutans or any of the other mammals tested [21,98,99].

Genomic and cDNA analyses revealed *XG* and *CD99* in non-human primates. *XG* was also present in some other mammals and the position of *XG* at the pseudoautosomal boundary appears to be conserved in higher

primates [20]. The chromosomal organisation of the two homologous genes in tandem (Figure 12.1) probably arose from a duplication event occurring over 150 million years ago, before the mammalian radiation [20].

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13 Scianna Blood Group System

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13.3 Scianna antigens, 372

13.1 Introduction

The Scianna system consists of seven antigens: a pair of antithetical antigens, the high and low frequency antigens SC1 and SC2, an antigen of low frequency (SC4, previously Rd), and four antigens of very high frequency (Table 13.1). SC3 is absent only from cells of the Scianna null phenotype. Scianna antigens are located on the immunoglobulin-superfamily glycoprotein, ERMAP. The Scianna gene is linked to the Rh genes on chromosome 1p.

13.2 ERMAP, the Scianna glycoprotein, and the gene that encodes it

Spring *et al.* [1,2] demonstrated by immunoblotting of red cell membranes with alloantibodies that SC1, SC2, and Rd (SC4) are located on a glycoprotein of apparent MW between 60 and 68 kDa. No bands were present if the membranes were prepared in the presence of a thiol reducing agent, showing that one or more disulphide bonds are required for antigen integrity. Variation in sialic acid content of the *N*-glycans probably accounts for the diffuse nature of the bands representing the glycoprotein. The Scianna glycoprotein was also immunostained on protein blots by autoanti-SC1 [3].

Analysis of families with SC:2 members demonstrated linkage between *SC* and the Rh genes, located at chromosome 1p36, with a male recombination fraction of about 0.10 [4,5]. Other studies demonstrated linkage, with a similar recombination fraction, between the genes controlling SC4 and Rh [6,7].

Human cDNA encoding a protein with a high level of homology to mouse ERMAP (erythroid membrane-associated protein) [8] was cloned from human fetal liver cDNA libraries [9,10]. The encoded 475 amino acid protein belongs to the immunoglobulin superfamily (IgSF) of adhesion and receptor proteins (see Section 6.2.2). The N-terminal extracellular domain of about 156 amino acids contains a single IgSF V domain. There is a single membrane-spanning domain, and a C-terminal cytoplasmic domain of about 298 amino acids that contains a B30.2 domain, a conserved motif of 157 amino acids found in many proteins with diverse functions. Human ERMAP is highly expressed on erythroid cells and may also be expressed at low levels on other haemopoietic cells [10]. *ERMAP* mRNA was not detected in many other adult tissues. Almost nothing is known about the function of ERMAP, a member of the butyrophilin-like subset of the IgSF (reviewed in [11]).

The human *ERMAP* gene was then identified from the GenBank database with a sequence at chromosome 1p34 [10]. It consists of 11 exons spanning about 19 kb. Exon 1 is untranslated; exon 2 encodes the translation initiation methionine codon, the leader sequence, which is not part of the mature protein, and the N-terminal domain; exon 3 the IgSF domain; exon 4 the membrane-spanning domain; exons 5–10 are very small; and exon 11 encodes the B30.2 domain and the 3′ untranslated region [10].

In 2003 Wagner *et al.* [12] recognised from its chromosomal location that *ERMAP* was a good candidate for the Scianna gene. Furthermore, the MW of the Scianna glycoprotein was compatible with the predicted MW of ERMAP. By identifying nucleotide changes in *ERMAP* associated with SC:2, SC:4, and SC_{null} phenotypes, Wagner

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Table 13.1 Antigens of the Scianna blood group system.

Antigen				Molecular basis*		
No.	Name	Frequency	Antithetical antigen	Nucleotides	Exon	Amino acids
SC1	Sc1	High	SC2	169G (A)	3	Gly57 (Arg)
SC2	Sc2	Low	SC1	169G>A	3	Gly57Arg
SC3	Sc3	High		Various		Various
SC4	Rd	Low		178C>G	3	Pro60Ala
SC5	STAR	High		139G (A)	3	Glu47 (Lys)
SC6	SCER	High		242G (A)	3	Arg81 (Gln)
SC7	SCAN	High		103G (A)	2	Gly35 (Ser)

*Molecular basis of antigen-negative phenotype in parentheses.

Table 13.2 Frequency of SC2 in various populations.

Population	No. tested	No. SC:2	SC2 frequency	References
Canadian donors	1000	1	0.0010	[14]
White Canadians	348	5*	0.0144	[15]
White Canadians	1000	17	0.0170	[18]
Londoners	1039	7	0.0067	[21]
Oxford donors	5306	41	0.0077	[5]
Warsaw donors	1025	9	0.0088	[21]
Berlin donors	2015	15	0.0074	[22]
Czech donors	2100	7	0.0033	[23]
Black Canadians	212	0		[24]
Japanese	4900	5	0.0005	[25]

*Includes three Mennonites.

et al. [12] confirmed that the Scianna antigens are located on ERMAP. Soluble recombinant ERMAP, produced in human embryonic kidney cells, inhibited haemagglutination reactions with anti-SC1 and -SC5 [13].

13.3 Scianna antigens

13.3.1 SC1 and SC2

In 1962, Schmidt *et al.* [14] identified an antibody in a Caucasian woman (Mrs N.S.) defining a new, inherited antigen of very high frequency. They named the antigen Sm. The following year, Anderson *et al.* [15] described a new low incidence antigen, Bu^a. Lewis *et al.* [16] were quick to notice that the original Sm– cells were Bu(a+). Sm and Bu^a were renamed SC1 and SC2, respectively, following confirmation through family studies that they were the products of alleles [17,18].

Expression of SC2 and concomitant loss of SC1 results from a Gly57Arg substitution in the IgSF domain of ERMAP (Table 13.1) [12]. The mutation destroys an *Sma*I site.

Testing of 269 000 South London blood donors with anti-SC1 revealed one SC:–1 individual [19], suggesting the following gene frequencies: *SC*01* 0.9981; *SC*02* 0.0019. No SC:–1 person was found as a result of testing red cells from 1600 North Americans [14,18] and 29 737 Welsh donors [20]. Frequency studies with anti-SC2 are shown in Table 13.2. In Canada the frequency of SC2 is high amongst the Mennonite community, which may be

responsible for the high SC2 frequency in some Canadian studies.

Treatment of SC:1 red cells with the proteases papain, trypsin, and chymotrypsin does not affect their reactions with anti-SC1 in haemagglutination tests; pronase and a mixture of trypsin and chymotrypsin reduces their reactivity. As expected for antigens located within an IgSF domain, SC1 and SC2 are substantially weakened by the disulphide bond reducing agent 6% 2-aminoethylisothiouronium bromide (AET) [1]. In contrast, SC2 is sensitive or markedly weakened by trypsin or chymotrypsin, possibly as a result of Arg57, not present on the SC1 protein [26]. SC1 is fully developed at birth [15]. Fluorescent flow cytometry demonstrated that SC1 is not present on lymphocytes, granulocytes, or monocytes [27].

13.3.1.1 Anti-SC1 and -SC2

Scianna system antibodies are very uncommon. They react best by an antiglobulin test and do not fix complement. Directly agglutinating anti-SC1 is known. No 'naturally occurring' Scianna alloantibody has been described.

The original anti-SC2 was found in a transfused man; three of the donors were traced and red cells from one reacted with the antibody [15]. Four examples of anti-SC2 were found among 14 anti-D sera produced by immunising D– volunteers with D+ red cell samples, one of which was also SC:2 [21].

Neither anti-SC1 nor -SC2 been incriminated in an HTR. One anti-SC1 gave a high score in a monocyte monolayer assay, suggesting potential clinical significance [28]. IgG3 anti-SC1 was responsible for a positive DAT on a baby's red cells, but no treatment was required [19]. There are two reports of anti-SC2 causing HDFN: one case was very mild [29]; the other required neonatal transfusion [30].

Several examples of autoanti-SC1 have been described, some of which have been responsible for AIHA [3,31–34]; two of them were in individuals whose red cells had weakened expression of SC1 [32,34]. Two of the antibodies were only detectable in serum, not in plasma [31,33]. Some autoantibodies in patients with depressed SC1 and SC3 reacted strongly with SC:1,–2 cells, weakly with SC:–1,2 cells, and did not react with SC_{null} cells [35,36].

13.3.2 SC3 and the SC_{null} phenotype

The first three propositi with the SC:–1,–2 phenotype, now called SC_{null}, all had an antibody reactive with all red cells save those of the SC:–1,–2 phenotype [37–39]. Adsorption studies with SC:1,–2 and SC:–1,2 cells demonstrated that the antibody was not a mixture of anti-SC1 and -SC2, so the antibody was numbered anti-SC3 [38].The first propositus, who had been transfused seven months previously, was from the Marshall Islands in the South Pacific (Micronesia) [37]. Her cousin was also SC:–1,–2, but did not have anti-SC3 despite four pregnancies. The second propositus was a previously transfused white man [38]. The third SC:–1,–2 propositus with anti-SC3, a previously transfused four-year-old girl, was found in Papua New Guinea (Melanesia) [39]. Her mother and six other family members and villagers in her home area were also SC:–1,–2.

Two *ERMAP* mutations responsible for the Sc_{null} phenotype have been reported.

1 An SC_{null} individual from Saudi Arabia was apparently homozygous for a 2-bp deletion in exon 3 (307del2), causing a frameshift that would result in a truncated protein of 113 amino acids (*SC*01N.01*) [12].

2 Two SC_{null} individuals from California and three, plus two SC_{null} relatives, from Pacific Islands, were homozygous for 994C>T in exon 11 introducing a stop codon at the codon for Arg332 within the B30.2 domain (*SC*01N.02*) [40,41].

Survival studies with radiolabelled SC:1,–2 cells suggested that the original anti-SC3 may be clinically significant [37]. This antibody disappeared soon after its identification and was not restimulated by injection of SC:1,–2 red cells. Anti-SC3 in a Melanesian child could not be detected after splenectomy, even following transfusion of SC:1,–2 blood [39].

13.3.3 SC4, the Radin antigen (Rd)

Before Rd was assigned to the Scianna system and became SC4, it was 700015. The first five anti-SC4 were apparently stimulated through pregnancy. In the five families the gene for SC4 could be traced to Russian Jews, black people, Northern Europeans, and a Native American [42]. Rd frequencies are shown in Table 13.3. SC4 is inherited as an autosomal dominant character [42,43]. A person with the very rare SC:1,2,4 phenotype has been found [7].

Two SC:4 individuals had a SNP encoding Pro60Ala in the IgSF domain of ERMAP (*SC*01.04*) (Table 13.1) [12].

SC4 is resistant to papain, ficin, and sialidase treatment, and substantially weakened by AET. Variable results were obtained with anti-SC4 and red cells treated with trypsin or chymotrypsin [26].

Anti-SC4 has been stimulated by pregnancy and transfusion [42,43]. One example of apparently 'naturally occurring' anti-SC4 was found in an untransfused man

Table 13.3 Frequency of SC4 in various populations.

Population	No. tested	No. SC:4	SC4 frequency	References
Various ethnic groups	6773	0		[42]
New York Jews	562	3	0.0053	[42]
Danes	4933	24	0.0049	[43]
Canadians	770	3	0.0039	[6]
Winnipeg donors	2864	9	0.0031	[6]

[43]. Of 30 000 sera tested in Denmark, none contained anti-SC4 [43]. The first five anti-SC4 were all reported to have caused mild to moderate HDFN, but only one baby required exchange transfusion [42].

13.3.4 SC5 (STAR), SC6 (SCER), and SC7 (SCAN)

Three antibodies to high frequency antigens found in previously transfused Caucasian men with SC:1,–2 red cells failed to react with SC_{null} cells [44]. None of the antibodies reacted with autologous cells, but all three antibodies reacted with the red cells of the other two antibody makers and, therefore, have different specificities. The antigens they define joined the Scianna system when the antibody makers were found to be homozygous for mutations in *ERMAP* (Table 13.1) [40,45].

The only SC:–5 propositus was a previously transfused white man of Irish and English descent. He and his SC:–5 bother were homozygous for 139G>A encoding Glu46Lys in the IgSF domain of ERMAP (*SC*01.–05*); their seven children were all heterozygous 139G/A [45]. Anti-SC5 reacted with all of 8000 random donor samples [44].

The only SC:–6 propositus was a previously transfused white man of German descent homozygous for a mutation encoding Arg81Gln in the IgSF domain of ERMAP (*SC*01.–06*) [44].

The only SC:–7 propositus was a previously transfused white man of German, English, and native American descent homozygous for a mutation encoding Gly35Ser in the N-terminal domain of ERMAP (*SC*01.–07*) [45]. Anti-SC7 was reported to have caused a delayed HTR, but then disappeared over the next few months [44].

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14 Dombrock Blood Group System

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14.1 Introduction

Prior to 1992, Dombrock remained a simple, polymorphic blood group system with two antigens, Do^a (DO1) and Do^b (DO2), the products of alleles. The discovery by Banks *et al.* [1] that red cells of the rare phenotype Gy(a–) Hy– Jo(a–) were also Do(a–b–) led to Gy^a, Hy, and Jo^a joining the Dombrock system; three antigens of high frequency, DOYA, DOMR, and DOLG joined later (Table 14.1). Table 14.2 shows Dombrock phenotypes and genotypes. Antigens of the Dombrock system are located on a GPI-linked glycoprotein, a member of the ADP-ribosyltransferase family (ART4, CD297). Asn265Asp represents the Do^a/Do^b polymorphism.

DO (*ART4*) is located on chromosome 12p13.2-12.1.

14.2 The Dombrock glycoprotein, ART4, and the gene that encodes it

Dombrock system antigens are on a glycoprotein that is anchored to the red cell membrane through glycosylphosphatidylinositol (GPI) (see Chapter 19). PNHIII red cells, the complement-sensitive population of red cells from patients with paroxysmal nocturnal haemoglobinuria (PNH), lack GPI-linked proteins and are deficient in Dombrock system antigens [2–4]. Immunochemical analyses revealed that Do^a, Gy^a, Hy, and Jo^a are on an *N*-glycosylated membrane protein of apparent MW 46 750–57 500 Da, without substantial *O*-glycosylation [1–3]. A putative dimer was also detected by immunoprecipitation [2].

Family analyses with numerous markers, including the gene for von Willebrand factor gene (*VWF*), localised *DO* (*ART4*) to chromosome 12p13.2-12.3 [5,6].

Gubin *et al.* [7] screened a database of about 5000 expressed sequence tags (ESTs) derived from differentiating erythroid cells for genes localised to chromosome 12p and encoding a GPI-anchor motif. *ART4*, a gene encoding a member of a family of mono-ADP-ribosyltransferases, was identified as a candidate for *DO* [8]. Stable transfection of a K562 erythroleukaemic cell line with the candidate open reading frame led to expression of Do^a, Gy^a, Hy, and Jo^a at the cell surface [7]. *ART4* spans 14 kb and contains 3 exons that appear to encode a protein of 314 amino acids with five putative *N*-glycosylation sites and six cysteine residues (including one in the signal peptide). Exon 1 encodes residues 1–45, including a 44 amino acid signal peptide, exon 2 encodes residues 49–285, and exon 3 encodes residues 286–314, including a 17 amino acid GPI-anchor motif, producing a membrane-bound protein of 253 amino acids. Alternative suggestions are that the signal peptide is only 22 amino acids, with Met22 representing the translation-initiation codon, and that the C-terminal 30 amino acids form the GPI-anchor motif. This would give a mature protein of 240 amino acids [9].

Although ART4 belongs to a family of adenosine diphosphate (ADP)-ribosyltransferases that catalyse the transfer of ADP-ribose from nicotinamide adenine dinucleotide (NAD^+) to a protein substrate, no enzyme activity has been demonstrated for ART4 and its biological role remains unknown [10]. The product of *DO*B* contains an Arg-Gly-Asp motif, characteristic of adhesion molecules involved in cellular interaction, but this motif

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Table 14.1 Antigens of the Dombrock system.

Antigen				Molecular basis*		
No.	Name	Frequency	Antithetical antigen	Nucleotides	Exon	Amino acids
DO1	Do^a	66%†	DO2	793A	2	Asn265
DO2	Do^b	82%†	DO1	793G	2	Asp265
DO3	Gy^a	High		Various		Various
DO4	Hy	High		323G (T)	2	Gly108 (Val)
DO5	Jo^a	High		350C (T)	2	Thr117 (Ile)
DO6	DOYA	High		547T (G)	2	Tyr183 (Asp)
DO7	DOMR	High		431C (A), 432C (A)	2	Ala144 (Glu)
DO8	DOLG	High		674T (A)	2	Leu225 (Gln)

*Molecular basis of antigen-negative phenotype in parentheses.
†Northern Europeans; Do^b calculated from gene frequencies.

Table 14.2 Dombrock phenotypes and usual genotypes.

Do^a	Do^b	Gy^a	Hy	Jo^a	Genotype
+	–	+	+	+	*DO*A/A*
+	+	+	+	+	*DO*A/B*
–	+	+	+	+	*DO*B/B*
–	–	–	–	–	*DO*GY/GY*
–	w	w	–	–*	*DO*HY/HY*
w	–	+	w	–	*DO*JO/JO*
w	w	w	w	–*	*DO*HY/JO*
–	–	w	w	w	*DO*DOYA/DOYA*
–	+	–	w	w	*DO*DOMR/DOMR*
+	–	w	+	+	*DO*DOLG/DOLG*

w, weakened expression of antigen.
*Very weak Jo^a may be detected by adsorption and elution.

is disrupted in the product of *DO*A*, where it becomes Arg-Gly-Asn, suggesting that it is of little importance [7].

14.3 Dombrock antigens

14.3.1 Do^a and Do^b (DO1 and DO2)

In 1965, Swanson *et al.* [11] identified an antibody in the serum of Mrs Dombrock that defined a new antigen (Do^a) on the red cells of 64% of Europeans. Not until 1973 was the antithetical antibody, anti-Do^b, identified by Molthan *et al.* [12].

The Do polymorphism results from a SNP in the Dombrock gene: *DO*A* encodes Asn265; *DO*B* encodes Asp265 (Table 14.1) [7,13]. *DO*B* has a *BseRI* restriction site that is not present in *DO*A* [14]. There are two synonymous SNPs, 378C/T (Tyr126) and 624T/C (Leu208). *DO*A* is usually associated with 378C, 624T and *DO*B* with 378T, 624C, but *DO*A* with 378T (*DO*A-HA*), *DO*A* with 624C (*DO*A-SH*), and *DO*B* with 378C (*DO*B-SH*) have been found [15,16].

Molecular genotyping for purposes of predicting Dombrock phenotype is very valuable because Dombrock reagents are often unavailable and serological Dombrock typing is difficult and unreliable. In one set of genotyping tests, eight donors who had been serologically typed as Do(b–) were found to be *DO*A/B* heterozygotes and subsequently shown to have a weak Do^b antigen [13]. Consequently, despite the pitfalls of predicting phenotype from SNP testing, such as the presence of null allele, testing for the 793A/G *DO* SNP would generally be considered a more reliable method of determining Dombrock phenotype than serological typing.

There are only limited antigen frequency studies concerning Dombrock. Almost all data have been derived from investigations with anti-Do^a alone. Most of the information is summarised in Table 14.3. From the Northern European gene frequencies [17] the following genotype frequencies have been calculated: *DO*A/A* 0.1764, *DO*A/B* 0.4872, *DO*B/B* 0.3364 (assuming *DO*B* is the only allele of *DO*A*). The frequency of Do^a is somewhat lower in black people and substantially

Table 14.3 Incidence of Do[a] and calculated frequencies of *DO*A* and *DO*B* genes in various populations.

Population	Total tested	No. Do(a+)	Do[a] frequency	Gene frequencies		References
				*DO*A*	*DO*B*	
Northern Europeans	755	501	0.6636	0.4200	0.5800	[11,17]
White North Americans	1091	696	0.6379	0.3983	0.6017	[18,19]
African Americans	161	89	0.5528	0.3313	0.6687	[19]
African Americans	76	34	0.4474	0.2566	0.7434	[17]
Japanese	760	179	0.2355	0.1257	0.8743	[20,21]
Thais	423	57	0.1348	0.0698	0.9302	[22]

*DO*B* gene frequency assumes that *DO*B* is the only allele of *DO*A*.

Table 14.4 *DO*A/B* genotype frequencies on four populations of American blood donors, obtained by testing on the BeadChip array [23].

Ethnic group	No. tested	Genotypes		
		*DO*A/A*	*DO*A/B*	*DO*B/B*
Caucasians	1243	0.13	0.51	0.36
African Americans	690	0.09	0.41	0.50
Hispanic	119	0.13	0.53	0.34
Asian	51	0.06	0.31	0.63

Table 14.5 Some Dombrock alleles, the associated nucleotide changes encoding amino acid substitutions, and gene frequencies on four populations of American blood donors, obtained by testing on the BeadChip array [23].

Gene names		Nucleotide and amino acid						Gene frequency			
	ISBT	323 108	350 117	431/432 144	547 183	674 225	793 265	Caucasian	African American	Hispanic	Asian
*DO*A*	*DO*01*	G Gly	C Thr	CC Ala	T Tyr	T Leu	A Asn	0.37	0.278	0.36	0.21
*DO*B*	*DO*02*	G Gly	C Thr	CC Ala	T Tyr	T Leu	G Asp	0.63	0.647	0.61	0.79
*DO*HY*	*DO*02.–04*	T Val	C Thr				G Asp	0	0.040	0	0
*DO*JO*	*DO*01.–05*	G Gly	T Ile				A Asn	0	0.035	0.03	0
*DO*DOYA*	*DO*01.–06*	G Gly	C Thr	CC Ala	G Asp	T Leu	A Asn				
*DO*DOMR*	*DO*02.–07*	G Gly	C Thr	AA Glu		T Leu	G Asp				
*DO*DOLG*	*DO*02.–08*	G Gly	C Thr	CC Ala	T Tyr	A Glu	A Asn				

lower in eastern Asians (Table 14.3). These indications are supported by genotyping studies [23] (Tables 14.4 and 14.5). Genotypes obtained by molecular tests on 220 Chinese North Han were *DO*A* 0.1159, *DO*B* 0.8841 [24].

14.3.2 Gy[a] (DO3) and Gy(a–), the Do$_{null}$ phenotype

An American family of Czech origin, in which four of seven children from a second cousin mating lacked a new public antigen Gy[a], was described by Swanson *et al.* [25]

in 1967. The propositus and her sister had anti-Gy^a in their sera; the propositus was in her fifth pregnancy and her sister had two children. A second family, also of Czech descent, contained two Gy(a–) sisters, both of whom had been pregnant and had anti-Gy^a in their sera [26]. Six more Gy(a–) individuals were found in an English family, possibly of Romany stock [27]. The four multiparous sisters had anti-Gy^a, whereas their two Gy(a–) brothers did not. Six Gy(a–) propositi have been found in Japanese; all were female and all were ascertained through the presence of anti-Gy^a [28].

Gy(a–) phenotype, in which the red cells lack all antigens of Dombrock system (Do$_{null}$), results from inactivating mutations in the Dombrock gene.

*DO*01N.01*. An American patient, homozygous for 442C>T in exon 2 of a *DO*A* allele, converting the codon for Gln148 to a stop codon [29].

*DO*01N.02*. A blood donor from Réunion Island, homozygous for an 8 bp deletion (nucleotides 3423–350) in exon 2 of a *DO*A* allele, creating a frameshift, premature stop codon, and loss of the GPI-anchor motif [30].

*DO*02N.01*. The original Gy(a–) propositus and two Gy(a–) sisters from another family, all of Czech origin, were homozygous for a single nucleotide change in the acceptor splice site of intron 1 (IVS1–2a>g) of *DO*B*, which results in splicing out of exon 2, introducing a frameshift and premature stop codon [31].

*DO*02N.02*. A Canadian patient homozygous for a single nucleotide change in the donor splice site of intron 1 (IVS1+2t>c) of *DO*B*, which results in splicing out of exon 2, introducing a frameshift and premature stop codon [29].

*DO*02N.03*. Homozygosity for 185T>C in *DO*A*, encoding Phe62Ser in a highly conserved FDDQY motif near the C-terminus of ART family proteins. Expression analysis confirmed that Ser62 was responsible for the absence of the Dombrock protein and homology modelling suggested that the mutation disrupts important aromatic side chain interactions between Phe62 and His160 [32].

No Gy(a–) individual was found among 9350 Japanese blood donors [28] or 10 145 Americans, including 75 African Americans and 611 native Americans [25]. Gy(a–) has not been found in people of African origin.

14.3.3 Hy (DO4) and Jo^a (DO5)

The first anti-Hy was identified in the serum of an African American woman at the delivery of her third child [33]. Other examples of anti-Hy followed [34–36]; all were made by Hy– black propositi, most of whom had Hy– siblings. Hy– red cells are Do(a–) and have weakened expression of Do^b and Gy^a [1,34].

In 18 individuals, the Gy(a+^{w}) Hy– phenotype was associated with 323G>T, encoding Gly108Val, in exon 2 of *DO*B* that has 378C (*DO*B-SH*), referred to here as *DO*HY* (Table 14.5) [37]. In most of these samples there was also an 898C>G change in exon 3 encoding Leu-300Val, but this probably represents a polymorphism in people of African origin and does not affect Do^b expression [16].

Screening with anti-Gy^a, diluted so that it would not react with Gy(a+^{w}) Hy– cells, revealed no negatives among 4530 white North Americans, 735 Czechs, 683 white South Africans, 846 black North Americans, 1023 black South Africans, 633 South African Asian Indians, or 1679 Pima Native Americans [34]. Two of 597 Apache were Gy(a+^{w}) Hy– [34], the only individuals reported who are not of African origin, although some racial admixture was suspected.

Anti-Jo^a (Joseph) was first described when cells and sera of two African American patients with antibodies to high frequency antigens were found to be mutually compatible [38], though subsequent genotyping suggests that the eponymous antibody may actually have been anti-Hy [37]. Another example of anti-Jo^a was found in an African American sickle cell disease patient [39], though he was also subsequently found to have an unexpected genotype, being heterozygous for the *DO*JO* allele and a normal *DO*B* allele [37]. All three antibody makers had been transfused. Many examples of anti-Jo^a have been found since.

Gy(a+^{w}) Hy– red cells are Jo(a–) [40], or at least express very low levels of Jo^a as determined by adsorption and elution tests [41] (Table 14.2). Makers of anti-Jo^a are Gy(a+) Hy+ Jo(a–) [40–43]. There is some degree of weakening of Do^a and Hy antigens on Gy(a+) Hy+ Jo(a–) cells compared with Gy(a+) Hy+ Jo(a+) cells.

Hy+ Jo(a–) phenotype arises from 350C>T in exon 2, encoding Thr117Ile, in *DO*A* with 378T (*DO*A-HA*), referred to here as *DO*JO* (Table 14.5) [37]. It appears that whereas Gly108Val ablates expression of both Hy and Jo^a, and significantly weakens Do^b and Gy^a, Thr117Ile silences Jo^a, but has much less significant effects on Do^a and Hy. The proximity of residues 108 and 117 suggests that they are within range of an antigenic determinant [37]. Hy+ Jo(a–) usually results either from homozygosity for 350C>T (*DO*JO/JO*) or from heterozygosity for 350C>T and 323G>T (*DO*HY/JO*). A phenomenon that is not understood is that some individuals with the

genotypes *DO*B/HY* and *DO*B/JO* have made anti-Hy and -Jo^a, respectively [37,44].

Following tests on 27 226 African Americans, DNA from the 176 (0.28%) donors whose red cells gave weak or negative reactions with anti-Gy^a was analysed for *DO*HY* and *DO*JO*. Of those that had *DO*HY* or *DO*JO*, 8% were *DO*HY/HY*, 75% were *DO*HY/DO* (where *DO*DO* represents the 'wild type'), 7% were *DO*HY/JO*, 8% were *DO*JO/DO*, and 2% were *DO*JO/JO* [44]. In New York *DO*HY* was five times more common than *DO*JO*, whereas a similar study on Afro-Brazilians revealed that *DO*JO* is more than twice as common as *DO*HY* [44]. Table 14.5 shows the results of some frequency studies for Dombrock genes obtained by array technology [23].

14.3.4 DOYA (DO6), DOMR (DO7), and DOLG (DO8)

DOYA, DOMR, and DOLG are antigens of very high frequency absent from Gy(a–) red cells.

Anti-DOYA, which reacted weakly with Hy– and Jo(a–) cells, was found in a Turkish Kurd woman whose red cells were Do(a–b–) and had weak expression of Gy^a, Hy, and Jo^a. She was homozygous for 547T>G in exon 2 of *DO*A*, encoding Tyr183Asp. Her two children were both heterozygous 547T/G. Homology modelling predicted that Asp183 influences the formation or stability of the Cys182–Cys123 disulphide bond [45].

Anti-DOMR, found in a black Brazilian pregnant woman, reacted with Jo(a–) red cells, but did not react or reacted very weakly with Hy– cells. Her red cells were Do(a–b+), weak for Hy and Jo^a, and non-reactive with alloanti-Gy^a, but reactive with monoclonal antibodies to the Dombrock protein. DOMR– results from homozygosity for two nucleotide changes within the same codon, 431C>A and 432C>A, encoding Ala144Glu [46].

Anti-DOLG in the serum of a Sri Lankan woman reacted with Do(a+b–), Jo(a–), and DOYA– red cells, but gave variable reactions with Hy– cells. Her red cells were Do(a+b–) Hy+ Jo(a+) Gy(a+), but with Gy^a marginally reduced in strength. DOLG– results from homozygosity for 674T>A in exon 2, encoding Leu225Glu [47].

14.3.5 Development and distribution of Dombrock antigens

Do^a, Do^b, and Jo^a are fully expressed on cord red cells [11,12,38,40]. In contrast, it is reported that Gy^a and Hy are expressed only weakly on cord cells [27,34]. Rodent monoclonal antibodies to ART4 reacted strongly with red cells, weakly with peripheral blood monocytes and macrophages, and did not react with B- or T-lymphocytes [48]. *DO* mRNA was detected in spleen, lymph node, bone marrow, and fetal liver, but not in thymus or peripheral blood leucocytes [7].

14.3.6 Effects of enzymes and reducing agents

Dombrock system antigens are resistant to papain or ficin treatment of red cells, and an antiglobulin test with papain- or ficin-treated cells is often the optimal method for using Dombrock reagents, especially anti-Do^a and -Do^b. Dombrock system antigens are generally sensitive to trypsin, chymotrypsin, and pronase, which either destroy the antigens or cause a marked reduction in their expression. Sialidase treatment has no effect. The antigens are usually sensitive to the action of the reducing agents AET and DTT [1–3,43,46].

14.4 Dombrock system antibodies

14.4.1 Anti-Do^a and -Do^b

Although not common antibodies, many examples of anti-Do^a and -Do^b are known. They occur in approximately equal numbers suggesting that Do^a and Do^b do not differ markedly in immunogenicity, considering their similar frequencies. Anti-Do^a and -Do^b usually occur in sera containing mixtures of multiple antibodies to red cell antigens, though examples of pure anti-Do^a [49] and -Do^b [50] have been identified. No 'naturally occurring' Dombrock antibodies are reported.

Anti-Do^a and -Do^b generally react by an antiglobulin test, working best with papain-treated cells. They are usually IgG and unable to fix complement [19,51].

Dombrock antibodies have not been implicated in HDFN. Anti-Do^a [52–54] and -Do^b [50,53,55,56] have been responsible for acute and delayed HTRs and antigen-negative red cells should be selected for transfusion. *In vivo* red cell survival studies and *in vitro* monocyte monolayer assays confirm that anti-Do^a and -Do^b can cause accelerated red cell destruction [6,50,57]. Dombrock antibodies are often difficult to detect and are notorious for becoming undetectable. In one patient recurrent acute HTRs were caused by anti-Do^a that was not detectable by crossmatching [54]. In view of the lack of suitable Do^a and Do^b typing reagents, molecular genotyping is the best technology for selecting suitable blood for patients with anti-Do^a or -Do^b, and it has been claimed that provision of Do^a or Do^b compatible red cells selected on the basis of DNA testing has improved red

cell survival in transfusion-dependent patients with the corresponding antibodies [58].

Five monoclonal anti-Do^b were produced as the result of immunising mice with HEK cells transiently transfected with *DO* cDNA [59].

14.4.2 Antibodies to other Dombrock antigens

Gy^a appears to be very immunogenic as virtually all reported Gy(a–) women who have been pregnant have anti-Gy^a in their serum [25–27,34]. An elderly man, who had never been transfused, had transient anti-Gy^a, which disappeared after three months [60]. Unlike most Gy(a–) cells, his red cells could adsorb and elute anti-Hy, leading to the suggestion that this patient may have had an acquired Gy(a–) phenotype [61]. Apart from this one case, there is no reported example of 'naturally occurring' anti-Gy^a, -Hy, or -Jo^a.

Anti-Gy^a, -Hy, and -Jo^a are usually IgG [25,27,34–36,38,39,43,60,62], predominantly IgG1 [57], and react best by an antiglobulin test. One anti-Gy^a also contained some IgA and bound complement as determined by a two-stage antiglobulin test [27]. One anti-Hy, which directly agglutinated Hy+ cells, was IgM plus IgG [62].

Like anti-Do^a and -Do^b, the other Dombrock system antibodies have never been implicated in HDFN, despite numerous opportunities, though the baby of the woman with anti-DOMR had DAT-positive red cells and was jaundiced, but only required phototherapy [46]. One anti-Hy has been responsible for an HTR in a man who received two units of Hy+ blood [35]. A man with anti-Gy^a tolerated 10 units of Gy(a+) blood with no adverse effect [63] and the patient with anti-DOYA was transfused with 3 units of incompatible blood, without evidence of reduced survival [45]. Anti-Hy was responsible for shortened *in vivo* red cell survival [36] and anti-Jo^a in a sickle cell patient caused significant removal of radiolabelled Jo(a+) cells compared with Jo(a–) cells [64]. In monocyte monolayer assays, 4 of 6 anti-Gy^a, 5 of 8 anti-Hy, and 4 of 5 anti-Jo^a gave results suggestive of a likelihood of clinical signs of an HTR following transfusion of incompatible red cells [57].

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15 Colton Blood Group System

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15.1 Introduction

The Colton system contains a single polymorphism, with relatively high and low incidence alleles represented by Co^a and Co^b antigens, respectively. A third antigen, Co3, is present on all cells save those of the null phenotype, Co(a–b–). Absence of Co4 is associated with a Co(a–b–) Co:3 phenotype (Table 15.1).

The *CO* locus is on chromosome 7p and the Co(a–b–) phenotype is sometimes associated with acquired chromosome 7 monosomy. The Colton antigens are located on aquaporin-1 (AQP1), a water channel-forming protein.

15.2 The Colton glycoprotein, aquaporin-1, and the gene that encodes it

Thirteen members of the aquaporin family of water channels are found in humans; 2 of these, AQP1, the Colton glycoprotein, and AQP3, the Gill glycoprotein (Chapter 26), are present in human red cells (reviews in [1,2]). The MW of AQP1 is 28 kDa in its unglycosylated form and 40–60 kDa in its glycosylated form. There are between 120 000 and 160 000 molecules per red cell, arranged as tetramers, with each tetramer containing one glycosylated molecule [3]. PCR amplification of a human fetal liver cDNA template with degenerate oligonucleotide primers representing the amino acid sequence of the N-terminal region of AQP1 provided a probe for isolation. The sequence of the 807 bp open reading frame of *AQP1* cDNA from a human bone marrow cDNA library predicted a 269 amino acid polypeptide, which spans the membrane six times and has cytoplasmic N- and C-termini [4] (Figure 15.1). The two halves of AQP1 are sequence-related: each has three membrane-spanning domains and each has a loop, one extracellular (E in Figure 15.1) and one cytoplasmic (B), containing the Asn-Pro-Ala (NPA) motif characteristic of the aquaporin family. In accordance with several structural models these two NPA motifs may interact within the membrane to form a single aqueous channel spanning the bilayer [5–7]. The first extracellular loop may be *N*-glycosylated, the oligosaccharide resembling the *N*-glycan of band 3 and expressing ABH activity [8].

The 17 kb *AQP1* gene consists of 4 exons encoding amino acids 1–128, 129–183, 184–210, and 211–269, and has been localised, by *in situ* hybridisation, to chromosome 7p14 [9]. The *AQP1* promoter contains TATA and CCAAT boxes, Sp1, AP1, AP2, and E-box elements, and erythroid-specific CACCC and Krüppel-like (CACCCA) elements [10].

Localisation of *AQP1* to the same region of chromosome 7 as the Colton blood group gene led to the discovery that the Colton antigens are on AQP1 [11]. Smith *et al.* [8] found that AQP1 could be selectively precipitated with anti-Co^a and -Co^b from red cells of the appropriate Colton phenotypes. Anti-Co3 precipitated AQP1 from Co(a+b–) and Co(a–b+) cells.

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Table 15.1 Antigens of the Colton system.

Antigen				Molecular basis*		
No.	Name	Frequency	Antithetical antigen	Nucleotides	Exon	Amino acids
CO1	Co^a	High	CO2	134C	1	Ala45
CO2	Co^b	8.5%†	CO1	134T	1	Val45
CO3	Co3	High		Various		Various
CO4	Co4	High		140A (G)	1	Gln47 (Arg)

*Molecular basis of antigen-negative phenotype in parentheses.
†Northern Europeans.

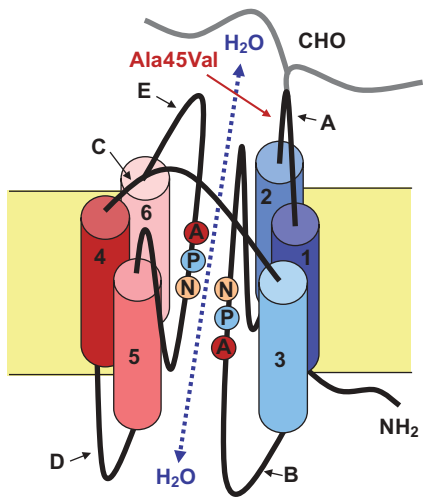


Figure 15.1 Three-dimensional model for AQP1 in the plasma membrane [5–7]. The six membrane-spanning domains are shown as cylinders and numbered from the N-terminus. A, C, and E represent the three extracellular loops; B and D, two cytoplasmic loops. B and E are extended loops that pass into the membrane to form a pore through which water molecules pass. NPA represents the Asn-Pro-Ala motifs in loops E and B. CHO, *N*-glycan at Asn42; Ala45Val, site of Colton polymorphism.

15.3 Co^a and Co^b (CO1 and CO2)

In 1967, Heistö *et al.* [12] gave the name anti-Co^a to three antibodies defining a new inherited public antigen. Three years later, Giles *et al.* [13] identified the antithetical anti-body, anti-Co^b, and a new blood group polymorphism was born.

From seven separate studies with anti-Co^a on a total of 13 460 white donors from Northern Europe and North America, 27 were Co(a–), giving a frequency for Co^a of 99.8% [12–17]. From five series of tests with anti-Co^b on 5186 white donors from England, Canada, Australia, and New Zealand, 443 or 8.5% were Co(b+) [13,17–19]. Gene and genotype frequencies calculated from these data (assuming that *CO*A* and *CO*B* are the only alleles present) are shown in Table 15.2; those calculated from the results of tests with anti-Co^a correlate remarkably well with those derived from tests with anti-Co^b. Of 1706 African Americans, all were Co(a+) [15]. The following Co^b frequencies were obtained: 4.6% in Miami Hispanics (799 tested) [20]; 2% in Cree Indians (100 tested) [21]; 0.58% in Japanese (2244 tested) [22].

The Colton polymorphism is associated with a 134C>T change in exon 1, the *CO*A* allele encoding alanine at position 45 and the *CO*B* allele encoding valine on the first extracellular loop of AQP1 (loop A), close to the site of *N*-glycosylation (Asn42) [8] (Figure 15.1). Altered glycosylation may prevent expression of Colton antigens; *Xenopus* oocytes expressing human AQP1 do not bind anti-Co^a [8]. A *Pfi*MI restriction site is created by the *CO*B* allele.

Co^a and Co^b are resistant to denaturation by the proteases papain, trypsin, chymotrypsin, and pronase, by sialidase, and by the disulphide bond reducing agent AET.

Co^a was not detected by flow cytometry on lymphocytes, monocytes, or granulocytes [23].

Table 15.2 Antigen, gene, and genotype frequencies in white people, determined from tests with anti-Co^a [12–17] and -Co^b [13,17–19].

		With anti-Co^a	With anti-Co^b
Antigens	Co^a	0.998	
	Co^b		0.085
Genes	*Co^a*	0.955	0.956
	Co^b	0.045	0.044
Genotypes	*CO*A/A*	0.912	0.914
	*CO*A/B*	0.086	0.084
	*CO*B/B*	0.002	0.002

15.4 Co3 and the Co$_{null}$ and Co$_{mod}$ phenotypes

In 1974 the awaited Co$_{null}$ phenotype, Co(a–b–), was identified in a French-Canadian woman and two of her four siblings [24]. Her parents and other two siblings were Co(a+b–). The serum of the propositus contained an antibody, anti-Co3, which reacted with all cells except those of the Co(a–b–) phenotype and could not be separated into anti-Co^a and anti-Co^b components. Subsequently other Co$_{null}$ individuals have been ascertained through the presence of anti-Co3, all of European extraction [25–31], with the exception of one Indian woman [32]. No negative was found as a result of testing 40 000 donors (29 000 North Americans, 9000 Australians, 2000 Finns) with anti-Co3 [28].

Molecular genetical analyses have been performed on Co(a–b–) Co:–3 propositi revealed the following mutations in *AQP1*.

1 Homozygosity for a deletion encompassing most or all of exon 1 [33] (*CO*N.01*). No AQP1 was detected by immunoblotting. Red cells had normal morphology, haematocrit, and haemoglobin levels, but a slightly reduced lifespan *in vivo* [34].

2 Homozygosity for a single base insertion at nucleotide 307 (exon 1), initiating a reading frameshift after Gly104, in the third membrane-spanning domain [33] (*CO*N.02*). No AQP1 was detected by immunoblotting.

3 Homozygosity for 576C>A in exon 3 of *CO*A*, encoding Asn192Lys [29] (*CO*01N.03*). This substitution converts the Asn-Pro-Ala motif in the third extracellular loop (E in Figure 15.1) to Lys-Pro-Ala. It is predicted that such a change in this important motif would result in failure of the protein to reach the membrane.

4 Homozygosity for a deletion of G232 in exon 1 of *CO*A*, introducing a reading frameshift after the codon for Ala78 (*CO*01N.04*), in an Indian woman from a small ethnic group and whose parents were first cousins [32].

5 Homozygosity for a deletion of 601G in exon 3 of *CO*A*, resulting in Val201Stop, (CO*01N.06) in two propositi [30,31].

A Co(a–) blood donor with weak Co^b was heterozygous 134C/T (*CO*A/B*), with 112C>T mutation in the *CO*A* allele (*CO*01N.05*). Encoded Pro38Ser was probably responsible for the absence of Co^a antigen expression [35].

Homozygosity for 113C>T in exon 1, encoding Pro-38Leu [33] (*CO*M.01*), resulted in a Co$_{mod}$ phenotype. Trace amounts of apparently normal AQP1 were detected on immunoblots of red cell membranes probed with monoclonal anti-AQP1 and the red cells reacted weakly with an extremely potent anti-Co3 [28]. *Xenopus* oocytes transfected with *AQP1* cDNA containing the Pro38Leu mutation had osmotic water permeabilities higher than those transfected with no *AQP1* cDNA, but substantially lower than those transfected with normal *AQP1* cDNA [33].

Red cells of a child with a unique form of congenital dyserythropoietic anaemia (CDA), but no *AQP1* mutation, had less than 10% of normal AQP1 levels and were Co(a–b–), but reacted with potent anti-Co3, and had very low osmotic water permeability [36,37]. Her red cells were also CD44-deficient, In(a–b–), and AnWj–, had weak LW^{ab}, but expressed normal Lutheran antigens. She, and two other patients with similar symptoms of CDA, were heterozygous for 973G>A in *KLF1*, the gene for the erythroid transcription factor EKLF (see Section 6.8.1) [38,39]. This mutation encodes the substitution of Glu325, which is predicted to contact DNA, by lysine in the second zinc finger domain. Since the disease phenotype occurs in the presence of a non-mutated allele, it is likely that the mutated protein actively interferes with EKLF-dependent processes by destabilising transcription complexes. Transfection experiments in K562 cells demonstrated that EKLF Glu325Lys has reduced ability to activate haemoglobin beta and CD44 gene expression [38].

Like Co^a and Co^b, Co3 is resistant to protease, sialidase, and AET treatment of red cells.

15.5 Co4 and the Co(a–b–) Co:3 phenotype

Anti-Co4, an antibody to a high frequency antigen, was found in a Co(a–b–) Turkish woman with two Co(a+b–)

children. Her phenotype was not Co_{null} as her red cells expressed normal levels of Co3, normal quantities of AQP1, and exhibited normal water permeability. Her antibody did not react with Co(a–b–) cells, but could not be anti-Co3 as it did not react with her own Co:3 red cells [40]. She was homozygous for 140A>G encoding Gln47Arg (*CO*01.–04*). As she was homozygous for *CO*A* (Ala45), it is probable that both Ala45 and Gln47 are required for Co^a expression. Transfection experiments in K562 cells demonstrated that Gln47 is also required for Co^b expression [40]. Two other Co(a–b–) individuals with the Gln47Arg are reported [40,41].

15.6 Colton antigens and monosomy 7

Monosomy 7 of the bone marrow, the loss of one chromosome 7 from haemopoietic stem cells, is a chromosomal abnormality occasionally associated with acute myeloid leukaemia and preleukaemic dysmyelopoietic syndromes. Monosomy 7 is often associated with Co(a–b–) Co:–3 phenotype or with weakening of Co^a and Co3 [42–44]. Of 35 monosomy 7 patients, eight had either Co(a–b–) Co:–3 or Co($a+^wb–$) Co3-weak red cells [44]. None of these eight had been recently transfused, whereas transfused red cells were present in the circulation of 21 of the remaining 27 Co(a+b–) patients. Zelinski *et al.* [45] suggested that absence of Colton antigens in some monosomy 7 patients results from loss of one allele, owing to the monosomy, and altered expression of the product of the other allele, resulting from the concomitant haematological disorder.

15.7 Colton antibodies

15.7.1 Anti-Co^a

Many examples of anti-Co^a have been identified. Like anti-Co^b and -Co3, they are generally IgG and react best by the antiglobulin test, especially if protease-treated cells are used, although an agglutinating IgM anti-Co^a has been reported [46].

Anti-Co^a has caused severe HDFN [47,48] and has been implicated in acute and delayed HTRs [49,50]. *In vivo* survival studies and monocyte monolayer functional assays also predict that anti-Co^a have the potential to cause HTRs [46,51] and Co(a–) red cells should be selected for transfusion to patients with anti-Co^a.

Anti-Co^a in a Co(a+b+) patient, shown to be *CO*A/B* by genomic analysis, was considered either to detect a partial Co^a antigen or to be an autoantibody [52].

15.7.2 Anti-Co^b

Anti-Co^b, a relatively rare antibody, was not detected in sera from 1430 transfused and non-transfused patients, or in sera from seven patients known to have been transfused with Co(a–) blood [12]. Anti-Co^b is often found in sera containing other blood group antibodies.

Anti-Co^b has been responsible for an acute HTR [53] and a mild delayed HTR [54]. *In vivo* survival studies demonstrated accelerated destruction of radiolabelled Co(b+) cells in patients with anti-Co^b [51,55,56]. Red cells compatible by IAT at 37°C should be selected for transfusion to patients with anti-Co^b. There is no report of serious HDFN caused by anti-Co^b.

15.7.3 Anti-Co3

Anti-Co3 has caused severe HDFN requiring neonatal transfusion [27,28]. Transfusion of Co(a+b–) blood to a patient with anti-Co3 resulted in a mild haemolytic reaction [29]. A very high titred anti-Co3 consisted of IgG1, IgG3, and some IgG2, was complement binding, and was haemolytic *in vitro* [28].

A 'mimicking autoanti-Co3' in a non-Hodgkin's lymphoma patient with Co(a–b–) Co:3 red cells directly agglutinated most red cells, but a papain antiglobulin test was required to demonstrate reactivity with the patient's own cells and with Co(a–b–) Co:–3 cells [57].

15.7.4 An antibody reactive only when Co^a and Co^b are both present

An antibody produced by a Co(a+b–) patient reacted by an antiglobulin test with 12 examples of Co(a+b+) red cells, but not with eight examples of Co(a–b+) or many examples of Co(a+b–) cells [58]. It is feasible that binding of this antibody to red cells of *CO*A/B* heterozygotes is dependent on the conformational effects of interactions between valine and alanine at position 45 of different molecules within AQP1 tetramers of the red cell membrane.

15.8 Functional aspects

AQP1 functions to form channels in the plasma membrane that enhance osmotically driven water transport. The extended loops B and E in Figure 15.1 form a channel through the membrane with a pore diameter of about 3 Å,

only slightly larger than the 2.8 Å diameter of a water molecule, so each unit of the AQP1 tetramers forms a separate channel. Interaction with the asparagine residues of the Asn-Pro-Ala motifs enhances transfer of water molecules, whilst preventing H^+ transport [6]. AQP1 may enable red cells to rehydrate rapidly after their shrinkage in the hypertonic environment of the renal medulla [59]. This would act in concert with the urea transporter, which also serves to reduce cell shrinkage in the renal medulla by enhancing the red cell's permeability to urea (Section 9.5).

AQP1 is strongly expressed in the proximal convoluted tubules and descending thin limbs of the kidney and has also been detected in various other epithelia and endothelia. AQP1 plays a role in reabsorption of water from the glomerular filtrate in the proximal tubule and thin descending loop of Henle. AQP1 has been detected in several other organs and tissues: lung, where it may be involved in maintaining water balance; brain, where it could play a part in regulation of cerebral-spinal fluid; and eye, where it might have a role in secretion and uptake of the aqueous humour [1,2].

Three Co_{null} propositi had about an 80% reduction in red cell osmotic water permeabilities and no AQP1 from renal tubules could be detected in their urinary sediment [33]; they were apparently healthy, but were unable to concentrate urine maximally when deprived of water [60]. AQP1 knockout mice are grossly normal, but become severely dehydrated compared with control mice after 36 hours of water deprivation [61]. It is likely, therefore, that AQP1 in the thin descending limb of Henle is required for the production of concentrated urine during times of water shortage [62]. AQP1 function in renal tubules may be shared with other members of the aquaporin family, in particular AQP2; in red cells the function may be shared with AQP3 (see Chapter 26).

Co_{null} red cells have about a 50% reduction in CO_2 membrane permeability compared with cells of normal phenotype [63]. Consequently AQP1 could also provide an important pathway for CO_2 in human red cells, though this has been disputed [64]. Transfection experiments in mammalian endothelial cells have also suggested that AQP1 facilitates transport of O_2 and NO across membranes [65,66].

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16 LW Blood Group System

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16.1 Introduction and history

A phenotypic relationship between LW and the Rh antigen D delayed recognition of LW as an independent blood group system for at least 20 years until 1963. Only in 1982 was LW resolved into a three-antigen system (Table 16.1).

The first anti-LW, described by Landsteiner and Wiener [1] in 1940, was called anti-Rhesus and resulted from immunising rabbits, and later guinea pigs, with blood from the monkey *Macacus rhesus*. This antibody appeared to be of the same specificity as a human alloantibody described, but not named, by Levine and Stetson [2] in 1939. Both human and animal antibodies were called anti-Rh.

As early as 1942, Fisk and Foord [3] demonstrated that guinea pig anti-Rh differed from human anti-Rh (later called anti-D) when they observed that red cells from all neonates, whether Rh-positive (D+) or Rh-negative (D–) as defined by the human anti-Rh, were positive with guinea pig anti-Rh. Anti-Rh could be produced by immunising guinea pigs with either Rh-positive or Rh-negative adult human red cells, or with heat extracts from those cells [4]. Levine *et al.* [5,6] repeated and confirmed this work and also showed that animal anti-Rh agglutinated D+ cells with the D antigen 'blocked' by non-agglutinating anti-D; effective blocking was demonstrated by the failure of these cells to be agglutinated by human anti-D. Furthermore, adsorption/elution tests demonstrated that the animal anti-'D-like', as it was now called, bound to D– cells, although it only agglutinated D+ cells.

Two D+ women made alloantibodies that appeared to be anti-D, but which behaved atypically since they were easily adsorbed by D– cells and, therefore, resembled animal 'D-like' antibodies [7]. The red cells of these women did not react with guinea pig anti-Rh, even by adsorption/elution [8]. Since the name Rh was firmly established in the literature and in common usage for the clinically important CDE groups, Levine *et al.* [8] suggested that the antigen defined by the animal and rare human 'D-like' antibodies be called LW in honour of Landsteiner and Wiener.

Although LW and D are different antigens, they are phenotypically related. D+ cells of adults express LW more strongly than D– cells, so anti-LW is easily mistaken for anti-D unless adsorption tests are done or rare D+ LW– cells are used. Rh_{null} cells, which lack all Rh antigens, also lack LW [8].

Swanson *et al.* [9] observed that red cells of LW– people with anti-LW in their serum were not always mutually compatible. A new low incidence antigen, Ne^a, present in 5–6% of the Finnish population [10], was found to have a phenotypic relationship with D similar to that of LW [11]. Sistonen and Tippett [12] observed that anti-Ne^a and most anti-LW were detecting the products of alleles. This led to the renaming of the LW system antigens: Ne^a became LW^b (LW7), the antithetical antigen LW^a (LW5), and those LW antibodies that reacted with LW(a–b+) red cells became anti-LW^{ab} (LW6) (Table 16.1). The LW^a, LW^b, LW^{ab} notation will be used in this chapter wherever possible, although this is sometimes difficult as many publications predate the discovery of anti-LW^b and it is not always possible to decide whether 'anti-LW' were really

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Table 16.1 Antigens of the LW system.

Antigen				Molecular basis*		
No.	Name	Frequency	Antithetical antigen	Nucleotides	Exon	Amino acids
LW5	LW^a	High	LW6	299A (G)	1	Gln100 (Arg)
LW7	LW^b	Low	LW5	299G>A	1	Arg100Gln
LW6	LW^{ab}	High		Various	1	Various

LW1 to LW4 are obsolete as they were previously used as phenotype designations.
*Molecular basis of antigen-negative phenotype in parentheses.

anti-LW^a or anti-LW^{ab}. In the ISBT numerical notation, the numbers LW1 to LW4 were avoided to prevent confusion with an obsolete phenotype designation.

LW antigens reside on an intercellular adhesion molecule ICAM-4 (CD242) (Sections 16.2 and 16.8). The LW^a/LW^b polymorphism is associated with a Gln70Arg substitution (Section 16.3.2).

The *ICAM4* (*LW*) locus is located on chromosome 19p13.3.

16.2 The LW glycoprotein (ICAM-4) and the gene that encodes it

Immunochemical analyses with alloanti-LW^{ab} and monoclonal anti-LW^{ab} demonstrated that LW antigens are located on a red cell membrane component of MW 40 kDa [13–16]. A broad band representing MW 37–47 kDa obtained by immunoblotting under non-reducing conditions was 'sharpened' to 36–43 kDa when sialidase-treated ghosts were used, suggesting that the size range results from heterogeneity of sialylation [13]. LW glycoprotein is *O*- and *N*-glycosylated: its MW was reduced by 2 and 17 kDa following treatment with *O*-glycanase and *N*-glycanase, respectively [16]. The presence of ethylenediaminetetra-acetic acid (EDTA) inhibits expression of LW^a, LW^b, and LW^{ab} on red cells [14]. Antigen expression could be restored to normal by Mg^{2+} ions, but not by Mn^{2+} or Ca^{2+}.

The product of PCR amplification from primers based on partial amino acid sequences from purified LW glycoprotein was used to screen a human bone marrow cDNA library [17]. The nucleotide sequence of an isolated cDNA clone predicted a polypeptide of MW 26.5 kDa. A rabbit antibody raised to a synthetic peptide with a sequence corresponding to the 15 N-terminal amino acids reacted with the purified LW glycoprotein on immunoblots and agglutinated, in an antiglobulin test, all red cells tested apart from those with the LW(a–b–) phenotype. D+ cells were more strongly agglutinated than D– cells. LW(a–b+) D+ cells reacted only weakly. The original *LW* sequence [17] contained errors involving three bases affecting the sequence of 16 amino acids in the signal peptide [18].

LW cDNA encodes a 271 amino acid protein with a 30-residue signal peptide, a 208 amino acid N-terminal extracellular domain, a 21 amino acid hydrophobic membrane-spanning domain, and a 12 amino acid C-terminal cytoplasmic domain [17]. There are potential *N*-glycosylation sites at Asn68, Asn78, Asn190, and Asn223 (counting from the translation-initiating methionine). Typical *N*-glycosylation at all four sites would produce a glycoprotein of 38–46 kDa. The proposed presence of three disulphide bonds at three pairs of cysteine residues (Cys69/Cys113, Cys153/Cys210, Cys73/Cys117) is supported by the sensitivity of LW antigens to thiol reducing agents.

The LW glycoprotein is a member of the immunoglobulin superfamily (IgSF), with two I-set IgSF domains (see Section 6.2.2). It is structurally related to the intercellular adhesion molecule ICAM-2, and to the first two IgSF domains of ICAM-1 and ICAM-3. Three-dimensional models of LW glycoprotein have been built, based on the crystal structure of ICAM-2 [19,20] (Figure 16.1). The potential function of LW glycoprotein, ICAM-4, is discussed in Section 16.8.

The LW glycoprotein, which appeared to be co-precipitated with an MW 31 kDa Rh protein [16], is part of the band 3 macrocomplex, which also contains the Rh proteins (see Section 10.7 and Figure 10.2). Red cells of

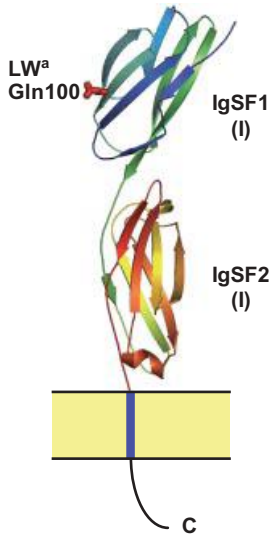


Figure 16.1 Ribbon model of the two extracellular I-set IgSF domains of the LW glycoprotein (ICAM-4), showing the position of Gln100 associated with LW^a expression (Based on Spring *et al.* [20]; model provided by N. Burton.)

a child with only trace quantities of band 3 had only about 6% of normal levels of LW glycoprotein [21].

The 2.65 kb *ICAM4* gene is organised into three exons [22]. Exon 1 encodes the 5′ untranslated sequence (96 bp), the signal peptide, and the first IgSF domain (amino acids 29 to 102). Exon 1 is separated by a 129 bp intron from exon 2, which encodes the second IgSF domain (amino acids 102–203) and is separated by a 147 bp intron from exon 3, which encodes the transmembrane domain, the cytoplasmic tail, and 3′ untranslated sequence (amino acids 203–241). The promoter region has no TATA- or CAAT-box, but includes potential binding sites for transcription factors, including those involved in erythroid and megakaryocytic expression [22]. LW has not, however, been detected on megakaryocytes.

When *ICAM4* was shown to be closely linked to *C3* and *LU*, it joined the chromosome 19 linkage group that also contained the fucosyltransferase genes *FUT1* (H), *FUT2* (secreted H), and *FUT3* (Lewis) [23,24] (see Section 6.2.4). Location of *ICAM4* on 19p13.3 was confirmed by *in situ* hybridisation [22].

A mouse homologue of human *ICAM4* encodes a protein with 68% identity to human ICAM-4 [25]. A secreted isoform of LW glycoprotein in mice results from the loss of the product of exon 3 and, therefore, loss of the transmembrane domain.

16.3 LW^a and LW^b (LW5 and LW7)

16.3.1 Frequency

In most populations, LW^a and LW^b are antigens of very high and low frequency, respectively. Polymorphism of LW was first observed in the Finnish population [10,12]. The highest frequency of LW^b has been found in Baltic Latvians and Lithuanians, and LW^b appears to be a Baltic marker, its presence in other populations being an indicator of the degree of Baltic genetic influence [26].

The calculated gene, genotype, and phenotype frequencies for the Finnish population are:

*LW*A*	0.971	*LW*A/A*	LW(a+b–)	0.9429
		*LW*A/B*	LW(a+b+)	0.0563
*LW*B*	0.029	*LW*B/B*	LW(a–b+)	0.0008

16.3.2 Inheritance LW^a and LW^b and the molecular basis of the LW polymorphism

Prior to the identification of anti-LW^b, all LW(a–) propositi with normal Rh groups were ascertained through their antibody. The inherited LW(a–) phenotype could only be distinguished from the acquired phenotype by family studies. Family studies have confirmed that *LW*A* and *LW*B* are co-dominant alleles and confirmed that *LW* is independent of the Rh genes [10,27].

The LW^a/LW^b polymorphism is associated with Gln100Arg in the first IgSF domain of the LW glycoprotein [18] (Table 16.1, Figure 16.1). This was confirmed by detection of LW^a and LW^b on COS-7 simian cells transiently transfected with cDNA from the corresponding alleles. *LW*B* lacks a *Pvu*I restriction site present in *LW*A*. Monoclonal anti-LW^{ab} bound more strongly to COS-7 cells transfected with *LW*A* cDNA, than with those transfected with *LW*B* cDNA [18].

16.4 LW(a–b–) and LW^{ab} (LW6)

Inherited LW(a–b–) phenotype is exceedingly rare. Of 10 552 Canadians tested with anti-LW^{ab}, none was negative [28]. The original propositus, a white Canadian antenatal patient (Mrs Big.) with anti-LW^{ab}, had an LW(a–b–) brother [28,12]. Red cells of her three children reacted with her anti-LW^{ab}, but the cells of two of them reacted only weakly. The LW-null phenotype of Mrs Big. results from homozygosity for a 10 bp deletion in exon 1 (codons 86–89) of an *LW*A* allele, which introduces a premature

stop codon and encodes a truncated protein lacking transmembrane and cytoplasmic domains [22].

Two other LW(a–b–) propositi had anti-LW^{ab}: one had a deletion of 46T introducing a premature stop codon; the other 2T>A, Met1Lys [83].

16.5 LW expression and effects of enzymes and reducing agents

16.5.1 Phenotypic relationship to D

Stronger reactions with D+ than D– cells have been noted for many anti-LW^a and -LW^{ab}, and for some sera this difference is so great that the antibody could be misidentified as anti-D. Estimation of antigen site density with monoclonal anti-LW^{ab} gave the following results: D+ adult, 4400; D– adult, 2835; D+ cord, 5150; D– cord, 3620 [13].

The strength of expression of LW on D+ red cells is not obviously influenced by the CcEe antigens or by D zygosity [30], but does reflect D antigen strength: DcE/DcE cells had more D and LW than DcE/dce, which had more than DCe/dce cells [31]. Red cells with weak D (D^u) gave similar strength reactions to D– cells in titrations of anti-LW^a [30]. LW^b has a similar relationship with D [11].

16.5.2 Development of LW

LW is expressed strongly on the red cells of neonates. Animal anti-LW react more strongly with red cells from cord blood samples, both D+ and D–, than with those of adults; human anti-LW do not always make this distinction so clearly. The strength of LW antigens, as judged by guinea pig anti-LW, decreases from birth until the adult level is reached at about five years of age [30]. During *ex vivo* erythropoiesis LW appears either at the CFU-E stage [32] or later at the proerythroblast stage [33].

16.5.3 Effects of enzymes and reducing agents

LW^a, LW^b, and LW^{ab} are unaffected by treatment of intact cells with the proteases papain, ficin, trypsin, or chymotrypsin, but are destroyed by pronase. Treatment of intact red cells with sialidase has no affect on their reaction with anti-LW^a or anti-LW^{ab}. The disulphide bond reducing agents DTT and AET either destroy or greatly reduce LW^a and LW^{ab} activity on red cells [34–36].

16.6 Acquired LW-negative phenotypes and transient anti-LW

The expression of LW on red cells can be affected by non-genetic factors. The acquired LW-negative phenotype is associated with loss of LW^a and possibly LW^{ab} and LW^b, and is generally found through the presence of anti-LW^a or -LW^{ab} in the patient's serum. Both antigen loss and antibody production may be temporary.

The first and fullest description of this phenomenon is the report by Giles and Lundsgaard [37] of transient anti-LW in the serum of a D– woman during her first pregnancy. Just before delivery her serum contained anti-C+D and -LW, and her cells were considered LW–, although they gave a weakly positive DAT. A year after delivery her red cells were LW+ and her anti-LW had disappeared. Chown *et al.* [38] suggested that transient production of anti-LW may not be very rare when they reported three more examples, two in pregnant D– women and one in a transfused D+ patient. They proposed that the red cells had genuinely lost their LW antigens and that the phenotype did not result from blocking of antigen sites by anti-LW.

Eleven of 18 D– men immunized with D+ red cells transiently produced an antibody resembling anti-LW, suggesting that anti-LW may be an antecedent in the immune response leading to production of anti-D [38]. Three months after transplantation of a D+ boy with bone marrow from his D– sister, anti-LW and anti-D were present, presumably resulting from a primary response of transplanted lymphocytes [39]. After two years the anti-LW had disappeared and very weak anti-D remained.

The expression of LW on red cells may be depressed during some diseases and re-expressed at normal strength in remission. Several similar examples are known in patients with lymphoma, leukaemia, sarcoma, and other forms of malignancy [40–43]. Two cycles of relapse associated with LW(a–) phenotype and production of anti-LW^a, followed by regaining of LW^a antigen and disappearance of antibody during chemotherapy-induced remission, occurred in a Japanese patient with malignant lymphoma [43]. Occasionally transient LW-negative phenotype occurs in the absence of malignancy, apparent immunological disorder, or pregnancy [44,45].

There appears to be a reciprocal relationship between the amount of LW antigen expressed on red cells and the broadness of the specificity of anti-LW in the serum [38]. Red cells of some LW(a–b–) patients are LW^{ab}+ and their

transient antibodies behave as anti-LW^{a}; others are LW^{ab}-negative and make anti-LW^{ab}. Many transient 'anti-LW' cannot be fitted neatly into anti-LW^{a} or anti-LW^{ab} specificity, presumably reflecting an intermediate stage.

Red cells of a patient with anti-LW^{a} in his serum were LW(a–) LW^{ab}+, but later became LW^{ab}-negative during terminal illness [27]. They were LW(b–) by direct testing, but adsorbed anti-LW^{b}, suggesting that LW^{b} may be lost with the other LW antigens. A brother and two daughters of the patient, who was married to his cousin, were LW(a–b+) LW^{ab}+.

16.7 LW antibodies

16.7.1 Alloantibodies

16.7.1.1 Anti-LW^{a}

Alloanti-LW^{a} are found in the sera of immunised LW(a–b+) individuals. Unless the red cells of the antibody maker are tested with anti-LW^{b}, other LW(a–) individuals are present in the family, or the antibody maker is shown to be *LW*B/B* by genomic testing, it is almost impossible to distinguish true alloanti-LW^{a} from that associated with an acquired LW(a–) phenotype. Most examples of alloanti-LW^{a} have probably been stimulated by transfusion; one is attributed solely to pregnancy [40], another to immunisation of a male volunteer for production of anti-D [46].

16.7.1.2 Anti-LW^{ab}

There are only four examples of alloanti-LW^{ab}, the antibodies of the only four propositi known to have an inherited LW(a–b–) phenotype [28,29,83]. The first propositus (Mrs Big.) had been pregnant three times, but never transfused. Initially, the anti-LW^{ab} was very potent, reacting much more strongly with D+ (1:32 000) than with D– cells (about 1:1000). When the antibody decreased in titre it no longer distinguished D+ from D– cells [28].

16.7.1.3 Anti-LW^{b}

Several anti-LW^{b} have been found, all in Finnish multitransfused patients [47]. Although the original anti-LW^{b} serum did not contain any other irregular antibodies, other reagents have contained additional antibodies such as anti-K, -Kp^{a}, and -Ul^{a}.

16.7.1.4 Transient antibodies

Transient antibodies should probably be considered autoantibodies since they are produced by genetically LW+ individuals. Although red cells of people with transient LW antibodies often give a positive DAT, in some cases the red cells have an acquired LW– phenotype and the anti-LW behaves as an alloantibody (Section 16.6). These antibodies are difficult to distinguish from true alloantibodies and, from a transfusion point of view, are generally managed in the same way. True alloantibodies, transient antibodies, and those of undetermined status will be considered together for clinical significance.

16.7.1.5 Clinical significance

No LW antibody has been responsible for an HTR or for HDFN. Many patients with anti-LW^{a} or -LW^{ab} have been successfully transfused with crossmatch-incompatible D– red cells [41,43–45,48,49] and the very potent anti-LW^{ab} of Mrs Big. caused no more than minimal evidence of HDFN in her D– third baby [28].

LW antibodies are mostly IgG, with IgG1 the main component [44,46,48], though one anti-LW^{a} was inactive by an antiglobulin test and was probably IgM [50]. In most patients with anti-LW^{a} or -LW^{ab}, where *in vitro* phagocytosis assays or *in vivo* red cell survival studies have been carried out, the results predicted that transfusion with D– cells would be efficacious [43,44,46,48,49]. Exceptions were two examples of IgG3 anti-LW^{ab} [42,51]: both antibodies produced high scores in mononuclear phagocyte assays and in one patient only 53% of radiolabelled D– LW+ red cells remained one hour after injection. *In vivo* red cell survival tests in a patient with potent anti-LW^{b} resulted in a rapid elimination of radiolabelled LW(b+) (D type not specified) cells, with a half-life of 2–5 hours [10].

16.7.2 Autoantibodies

16.7.2.1 Cold autoanti-LW

Ten examples of autoanti-LW were found by screening 45 000 blood samples, but the antibodies could only be detected by a low-ionic strength polybrene method in an AutoAnalyser at temperatures below 37°C; they were not detectable by manual techniques [20]. Most of the antibody makers were healthy blood donors or were pregnant and the antibodies were not associated with any increased red cell destruction.

16.7.2.2 Autoimmune haemolytic anaemia (AIHA) and HDFN

Levine [52] suggested that anti-LW is the most frequent antibody in cases of AIHA with a positive DAT. In two surveys of red cell eluates from 14 patients with AIHA, 12 eluates contained anti-LW, one of which contained

only anti-LW [53,54]. Severe AIHA has been associated with anti-LW as the sole autoantibody [55].

Autoanti-LW^{a} implicated in mild HDFN was treated successfully with phototherapy [56].

16.7.3 Animal antibodies

Anti-LW was first made in rabbits [1] and later, more successfully, in guinea pigs. Anti-LW has been stimulated in these animals by injections of red cells from rhesus monkeys (*Macaca mulatta*), from baboons, and from D+ or D– humans [1–6,30,57–59]. Heat extracts of human D+ and D– cells also stimulate anti-LW [4–6].

LW(a–b+) and LW(a–b–) (Mrs Big.) red cells are able to stimulate anti-LW in guinea pigs [54,57]; only Rh_{null} cells have failed to elicit any such response in animals [52,57,60]. The response to red cells of Mrs Big. is surprising considering the nature of the mutation responsible for her LW-null phenotype, as no LW glycoprotein would be expected to be present in her red cell membranes (Section 16.4).

16.7.4 Monoclonal anti-LW^{ab}

Of four monoclonal antibodies identified as anti-LW^{ab}, three (IgG1) were derived from mice immunised with human red cells [61,62], and one (IgM) from a mouse immunised with rhesus monkey red cells [63]. Binding of the murine anti-LW^{ab} to red cells could be totally blocked by human anti-LW^{ab} and partially blocked by human anti-LW^{a}; anti-D did not inhibit the reaction [61]. Domain-deletion experiments suggested that the epitopes for the three IgG antibodies are on the first IgSF domain [19]. Five other monoclonal antibodies to the LW glycoprotein, five binding to domain 1 and one to domain 2, were produced by immunising mice with a recombinant chimeric protein consisting of the two IgSF domains of LW and the Fc fragment of IgG1 [64].

16.8 Functional aspects and disease association

ICAMs are intercellular adhesion molecules, a group of five related structures belonging to the immunoglobulin superfamily (IgSF, see Section 6.2.2) [65,66]. The N-terminal domain of ICAM-4, the LW glycoprotein, is an I-set IgSF domain that shares about 30% sequence identity with that of the other ICAMs [17]. ICAM-2 (CD102), like ICAM-4, has two IgSF domains. ICAM-1 (CD54) and ICAM-3 (CD50) each have five IgSF domains, ICAM-5 has nine domains [66].

ICAMs are ligands for integrins, adhesion molecules consisting of heterodimers for α and β transmembrane subunits. Eighteen different α subunits combine with eight different β subunits to form over 24 different heterodimers [67]. ICAMs bind the αLβ2 integrin (LFA-1), which is present on lymphocytes, granulocytes, monocytes, and macrophages [66]. ICAM-4, however, differs from other ICAMs as it interacts with a variety of integrins in addition to αLβ2: αMβ2 (Mac-1), αXβ2, αVβ1, αVβ3, αVβ5, α4β1 (VLA-1), and αIIbβ3 the platelet fibrinogen receptor [19,20,66,68–73], though the α4β1 binding is disputed [71]. A glutamic acid residue critical for binding of other ICAMs to integrin is replaced by arginine in ICAM-4, demonstrating that another mechanism must be involved. Domain deletion and single amino acid site-directed mutagenesis experiments revealed that the first IgSF domain is most important to binding, but that the second domain also contributes, with the possible exception of binding to αLβ2 [19,70,71,74] (reviewed in [75]).

ICAMs 1, 2, and 3 are adhesion molecules of lymphocytes, granulocytes, and monocytes, and may be more widely expressed, but ICAM-4 appears to be restricted to erythroid cells and possibly placenta [66]. During *ex vivo* erythropoiesis LW is detected around the CFU-E to proerythroblast stage [32,33]. During the latter stages of erythropoiesis erythroblasts cluster around bone marrow macrophages to form erythroblastic islands, where the erythroblasts extrude their nuclei, which are ingested by the macrophage [76]. Adhesive interactions between ICAM-4 and α4β1 integrin on adjacent erythroblasts and between ICAM-4 on erythroblasts and αV integrins on macrophages may assist in maintaining the stability of the erythroblastic islands [20,66,77]. This supposition is supported by experiments on mice: ICAM-4 knockout mice have 64% less islands than wild-type mice and peptides that block ICAM-4 to αV binding decreased *in vitro* reconstitution of islands from single cell suspensions of wild-type mouse marrow [77]. A secreted isoform of ICAM-4 in mice is upregulated late in terminal erythroid differentiation and could assist young reticulocytes in detaching from the erythroblastic islands [25,77]. Downregulation of α4β1 integrin once the cells have enucleated may also aid in their release from the erythroblastic islands.

Binding of ICAM-4 on red cells to αXβ2 integrin on macrophages in the spleen may play a role in the removal of senescent red cells [70,75]. Through binding to the platelet integrin αIIbβ3, ICAM-4 could be involved in interactions between platelets and red cells during

coagulation [73]. As part of the band 3/Rh red cell surface macrocomplex, ICAM-4 might also assist in facilitating transient adhesive interactions between the red cell and the vascular endothelium to maximise gas transfer [21]. The functional importance of ICAM-4, however, must be considered in light of the absence of any obvious pathology associated with its absence in the rare inherited LW-null and Rh_{null} phenotypes, and in ICAM-4 knockout mice.

Like the Lutheran glycoprotein, expression of ICAM-4 may be elevated on sickle red cells, and antibodies to ICAM-4 partially inhibit adhesion of sickle red cells to activated endothelium [66,72,78]. Interactions between ICAM-4 on red cells and αVβ3 integrin on the endothelial cells of vessel walls may be involved in the microvascular occlusions that produce the painful crises of sickle cell disease. Peptides or mimetics representing ICAM-4 reduce adhesion and vessel blockage, and may have therapeutic potential [78]. Stimulation of β_2-adrenergic receptor by the physiological stress mediator epinephrine induces increased levels of cyclic adenosine monophosphate (cAMP) in SS red cells. This elevation of cAMP may induce serine phosphorylation of the cytoplasmic domain of ICAM-4, through abnormal activation of extracellular signal-regulated kinase-1/2 (ERK1/2), and could induce conformational changes to the external domain of ICAM-4, modulating its attraction to αVβ3 on endothelial cells [72,79,80].

16.9 LW antigens in animals

Summarising work with animal anti-LW from several laboratories shows that LW antigen has been detected on red cells of all primate species tested, including chimpanzee, gorilla, orangutan, baboon, and a variety of other species of monkey [1,6,58,81,82]. LW has not been found on the red cells of any of the non-primate species tested: rabbit, mouse, rat, sheep, goat, horse, and cattle.

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17 Chido/Rodgers Blood Group System

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17.1 Introduction

Chido and Rodgers antigens are not located on intrinsic red cell structures, but on the fourth component of complement (C4), which becomes bound to the red cells from the plasma. As Chido/Rodgers antigens are readily detected on red cells by conventional blood grouping methods and were considered to be blood group antigens before the association with C4 was disclosed, they have been adopted as the seventeenth blood group system. Currently nine Chido/Rodgers antigens have been defined [1]: Ch1 to Ch6, Rg1, and Rg2 have frequencies greater than 90%; W.H. has an incidence of about 15%. A complex relationship exists between these nine determinants and polymorphic variation of the C4 α-chain.

According to the ISBT terminology, Ch1 to Ch6 are CH/RG1 to CH/RG6, WH is CH/RG7, Rg1 is CH/RG11, and Rg2 is CH/RG12. Numbers CH/RG8 to CH/RG10 are available for additional Ch antigens.

In Section 17.2, anti-Ch and -Rg will be considered as simple, monospecific antibodies, their complexities being discussed in Section 17.5.

17.2 Basic serology

Antibodies to a relatively high frequency antigen, called anti-Chido (anti-Ch) by Harris *et al.* [2], were described as 'nebulous' because antigen strength was variable and difficulty in distinguishing weakly positive from negatively reacting red cells could not be resolved by adsorption experiments. All seven of the original anti-Ch sera were found in multiply transfused patients; in six of them no other atypical antibody was detected [2]. Middleton and Crookston [3] found that the reaction of anti-Ch with Ch+ red cells was inhibited by plasma from Ch+, but not Ch–, individuals. Furthermore, plasma of people with weak Ch expression on their red cells was as effective in inhibition tests as plasma from strongly Ch+ individuals. Inhibition techniques, therefore, are more effective than testing of red cells for phenotype determination. Ch– red cells can be converted to Ch+ by incubation in Ch+ plasma [4]. About 97% of white donors are Ch+ [3,5]. The gene controlling Ch production is inherited as an autosomal dominant character and the locus is strongly linked to *HLA* [6].

When Longster and Giles [7] described anti-Rg (Rodgers), the resemblance to anti-Ch was patent: the antibody reacted with red cells of about 97% of white people, strength of red cell Rg expression was variable, and the reaction with Rg+ red cells was specifically inhibited by Rg+ plasma. The high frequencies of both Ch and Rg precluded an allelic relationship, yet the excessive rarity of the Ch– Rg– phenotype suggested a genetic association. The locus controlling Rg expression is also very tightly linked to *HLA*, with strong association between Rg– and HLA-B8 [8,9]. The gene

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producing Rg antigen is inherited in an autosomal dominant fashion [7,8].

About 2.5% of Rg+ plasmas were partially effective in inhibiting anti-Rg and initially appeared to represent a quantitative variant [7]. Partial inhibition of anti-Ch and -Rg was later shown to be due to some Ch/Rg antisera containing antibodies to more than one determinant [5,10–13]. This polyspecificity became the basis of much of the complexity described in Section 17.5.

Reliable results can be obtained by testing red cells with anti-Ch or -Rg if a suitable technique is used [14,15]. Ch and Rg antigens are expressed less strongly on cord cells than on red cells of adults, although cord and adult plasma are equally effective at inhibiting Ch/Rg antibodies [3,16]. The effect of enzymes on Ch and Rg antigens will be described in Section 17.3.

17.3 Ch and Rg antigens are located on C4

C4 is the fourth component of complement, involved in the classical pathway of complement activation. Activation of C1 by binding to IgG or IgM molecules, usually on a cell surface, results in the cleavage of C4 into a small fragment, C4a, and a large fragment, C4b. C4b immediately becomes covalently bonded to the cell surface as the result of the breaking of an intramolecular thioester bond, a rare type of bond involving the side chains of cysteine and glutamine. When broken, the thioester bond generates a very reactive carbonyl group, which can couple instantaneously to a membrane-bound macromolecule. C4b can then bind C2. The activated C4b,2a complex cleaves C3 and brings about a cascade reaction involving C5–C9 culminating in the puncturing of the cell membrane.

The product of the C4 gene is a pro-C4 molecule, a single polypeptide chain of MW 200 kDa, which is subsequently cleaved into α (95 kDa), β (75 kDa), and γ (30 kDa) chains. These three polypeptides are glycosylated and linked together by disulphide bonds (Figure 17.1). The α-chain occupies most of the molecular surface and is responsible for the majority of the molecule's biological activities. C4b represents the whole molecule minus a short N-terminal fragment, C4a. Degradation of membrane-bound C4b, primarily by factor I, releases most of the molecule and leaves C4d covalently bound to the membrane. A similar effect is produced by trypsin treatment of membrane-bound C4b. For reviews on complement and C4 see [17,18].

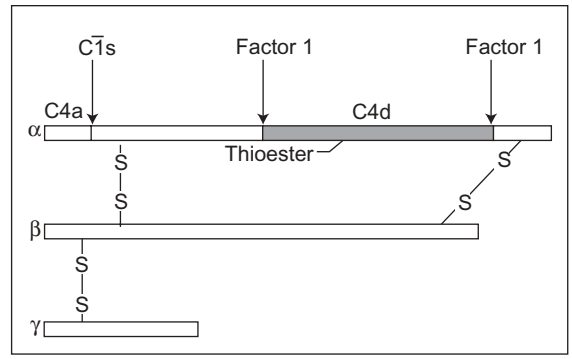


Figure 17.1 Diagram of C4 molecule, showing the three polypeptide chains linked by disulphide bonds, the C4a fragment, which is cleaved by the action of C1s, the site of the thioester bond, which causes the molecule to become covalently bonded to a cell surface, and the C4d region, which carries the Ch and Rg determinants and remains bound to the cell after cleavage of the remainder of the molecule by factor I. After [17], reproduced with permission from Elsevier.

Immunofixation electrophoresis reveals structural polymorphism of C4. C4 of most people falls into one of three patterns:

1 four rapidly migrating bands (C4A);
2 four slowly migrating bands (C4B); or
3 both sets of bands together [19,20] (Figure 17.2).

C4A and C4B represent genes at two very closely linked loci, *C4A* and *C4B*, with common silent alleles at each locus [20,21]. People who only have the faster migrating bands are homozygous for the silent allele at the *C4B* locus (*C4B*Q0*, quantity zero) and those who only have the slower bands are homozygous for a silent gene at the *C4A* locus (*C4A*Q0*). People with both sets of bands have at least one active allele at each locus. The excessive rarity of C4 deficiency arising from homozygosity for silent alleles at both loci is explained by a high level of linkage disequilibrium between the two loci, so that the haplotype *C4A*Q0 B*Q0* very seldom occurs. (For those wishing to read the original papers, it should be pointed out that O'Neill *et al.* [20,21] used the notation C4F (fast) and C4S (slow) for C4A (acidic) and C4B (basic), respectively.)

O'Neill *et al.* [21] showed that the Ch and Rg antigens are associated with C4. Ch+ Rg+ plasma has both C4A and C4B isotypes, Ch+ Rg– plasma has only C4B, and Ch– Rg+ plasma has only C4A (Figure 17.2). Ch and Rg antigens, therefore, appeared to be located on the products of the *C4B* and *C4A* loci, respectively. C4-deficient

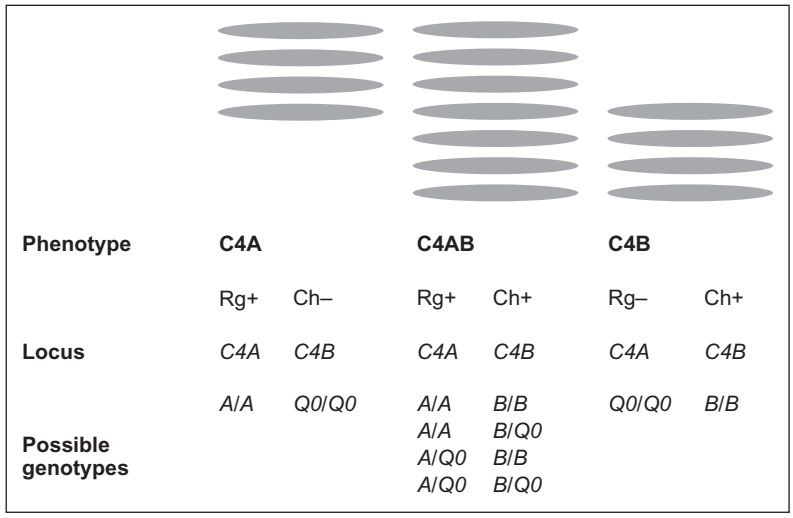


Figure 17.2 Diagram of common C4 electrophoretic patterns showing C4 and Ch/Rg phenotypes, and suggested C4 genotypes [21]. The extremely rare *C4A*Q0 B*Q0* haplotype is not included.

plasma lacked both Ch and Rg activity, whether the C4-deficient plasma was obtained from rare C4-deficient individuals [21–23] or was produced by removal of C4 from normal plasma by goat anti-C4 on an affinity column [21]. Red cells coated with C4 *in vitro* are directly agglutinated by anti-Ch and -Rg; antibodies that would normally require anti-human globulin to agglutinate the same red cells when uncoated [24]. C4-coated red cells acquire the Ch/Rg phenotype of the serum donor. Ch and Rg determinants on C4-coated red cells are relatively resistant to trypsin cleavage and must reside on the C4d fragment remaining bound to the red cell after trypsin cleavage of C4b [24]. Ch/Rg activity of uncoated red cells probably results from low level adsorption of C4 *in vivo*, either *via* the classical pathway or by spontaneous cleavage of the thioester [25].

The usual technique for coating red cells with C4 for serological purposes involves dropping freshly drawn citrated blood into a low ionic strength 10% sucrose solution [24]. These red cells are coated with C4 and C3 and are useful either for Ch/Rg typing by direct agglutination with appropriate antibodies or as indicator cells in Ch/Rg plasma inhibition tests. If red cells coated with C4 from another person are required, washed cells are mixed with the required fresh plasma (ABO compatible) and added to the sucrose solution. Trypsin treatment of cells coated with C4 and C3 cleaves the C3 and C4b leaving C4d coated cells. Red cells can also be coated with C4 *in vitro* or *in vivo* by complement fixing IgM antibodies [24].

Serological tests suggest that Ch and Rg antigens on native, 'uncoated' red cells, but not on cells coated with C4 by the 10% sucrose method, are denatured by the proteases trypsin, chymotrypsin, papain, ficin, and pronase [3,7,16,24]. Trypsin treatment reduces the number of detectable C4d molecules by about 50%, which reduces the number of C4d molecules on native cells, but not on coated cells, to a level below the threshold required for detection by agglutination tests with anti-Ch and -Rg [26].

There are four basic serological methods for determining Ch/Rg phenotypes:

1 testing of the subject's red cells with anti-Ch and -Rg;
2 inhibition of anti-Ch and -Rg with the subject's plasma;
3 testing of the subject's red cells coated with C4 from their own plasma;
4 testing of homologous red cells coated with the subject's C4.

17.4 Further complexities of C4

17.4.1 The complex polymorphisms of C4

Following the detection of two structural loci for C4 [20,21], a host of C4 variants were recognised and a variety of different notations used. In 1983 a single

nomenclature for C4 was agreed [27]. Although this has subsequently been modified [28,29], the 1983 nomenclature is used in most publications on the complexities of Ch/Rg, so that nomenclature will be used in this chapter. The protein products of *C4A* generally have the most acidic (anodal) migration on agarose gel electrophoresis and the products of *C4B* generally have the more basic (cathodal) migration. The C4 variants or allotypes are numbered, the most common being C4A 3 and C4B 1. Their genes are designated *C4A*3* and *C4B*1*, respectively. C4A Q0 and C4B Q0 represent unexpressed allotypes. There are at least 24 alleles at the *C4A* locus and 27 at the *C4B* locus, including a silent allele, *C4A*Q0* and *C4B*Q0*, at each [29]. In white people there are four *C4A* and three *C4B* alleles occurring with frequencies greater than 1% and producing variants with different electrophoretic mobilities: *C4A*2*, *C4A*3*, *C4A*4*, *C4A*6*, *C4B*1*, *C4B*2*, and *C4B*3*.

In most cases, C4A expresses Rg and C4B expresses Ch. Exceptions to this rule represent 'reversed antigenicity' [30,31]. For example: C4A 1 reacts with anti-Ch but not anti-Rg; C4B 5 (now called C4B 45) reacts with anti-Rg and with only some anti-Ch. The complexities of Ch and Rg are described in Section 17.5.

Protein modelling from the X-ray crystal structure of the C4d fragment showed that the residues associated with the major Ch/Rg epitopes are proximally located and accessible on the concave surface [32].

17.4.2 Molecular genetics of C4

C4A and *C4B* each consist of 41 exons [33] and are either 20.6 kb or 14.2 kb in length, the longer form having the endogenous retrovirus HERV-K(C4) integrated into exon 9 [34]. Both coding and non-coding regions of *C4A* and *C4B* are highly conserved. There is over 99% identity between the DNA nucleotide sequences of the two genes and between the amino acid sequences of the two proteins [35]. Eight amino acid changes within a region of the C4d fragment of the α-chain, close to the thioester bond, account for differences between isotypes (C4A and C4B) and allotypes (variants of these proteins) [35–38]. Four amino acid residues encoded by exon 26 determine isotype: the sequence for residues 1101–1106 of the pro-C4 molecule is *Pro-Cys*-Pro-Val-*Leu-Asp* in C4A and *Leu-Ser*-Pro-Val-*Ile-His* in C4B. *C4A* and *C4B* are distinguished by an *N1a*IV restriction fragment-length polymorphism (RFLP) [39]. Amino acid substitutions at positions 1054, 1157, 1188, and 1191, encoded by exons 25 and 28, account for the C4 allotypes and the various Ch and Rg determinants (Table 17.1). A detailed structural model to explain the location of Ch/Rg antigenic determinants and their correlation with the C4A and C4B isotypes [40] is described in Section 17.5.

Other complications occur that affect the C4 haplotype. Duplicated C4 genes appear to be quite common [11,42–45]: *C4A*3 A*2* and *C4B*2 B*1* both have frequencies close to 1% in Caucasians. There is also a high incidence of null alleles, about half of which result from a 28 kb DNA deletion [46,47]. Gene duplications and deletions probably arose by gene misalignment and unequal crossing-over [46]. *C4A*Q0* also results from a 2 bp insertion in exon 29 generating a stop codon in exon 30 [48]. Alternatively a *C4B* gene may appear to be absent because it has been changed to an identical copy of its neighbouring *C4A* homologue by a process of gene

Table 17.1 Sequence analysis of different C4 allotypes of known antigenic status (from [40,41]).

	Amino acid residues from N-terminus								Ch/Rg expression								
Allotype	1054	1101	1102	1105	1106	1157	1188	1191	Rg1	Rg2	Ch1	Ch2	Ch3	Ch4	Ch5	Ch6	WH
C4A 3	Asp	Pro	Cys	Leu	Asp	Asn	Val	Leu	+	+	–	–	–	–	–	–	
C4A 1	Gly	Pro	Cys	Leu	Asp	Ser	Ala	Arg	–	–	+	–	+	–	+	+	
C4B 3	Gly	Leu	Ser	Ile	His	Ser	Ala	Arg	–	–	+	+	+	+	+	+	
C4B 1	Gly	Leu	Ser	Ile	His	Asn	Ala	Arg	[–	–	+	+	–	+	+	–]*	
C4B 2	Asp	Leu	Ser	Ile	His	Ser	Ala	Arg	[–	–	+	–	+	+	–	–]*	
C4B 5†	Asp	Leu	Ser	Ile	His	Ser	Val	Leu	+	–	–	–	–	+	–	+	+

*Assumed phenotype.
†Now called C4B 45 [28].

conversion [49,50]. Intragenic unequal crossing-over, resulting in *C4A/B* hybrid genes, could explain reversed antigenicity – C4A variants that express Ch determinants and C4B variants that express Rg [30,51]. (The mechanisms of unequal crossing-over and gene conversion are discussed in Section 3.9.)

The C4 genes are located within the class III region of the major histocompatibility complex on chromosome 6 [52]. They form part of a discrete genetic unit or module known as RCCX comprising four consecutive genes or fragments and inactive mutants of those genes [53]. RCCX stands for serine/threonine nuclear protein kinase **R**P, **C**4, steroid 21-hydroxylase **C**YP21, and extracellular matrix protein tenascin TN**X**. Haplotypes containing 1, 2, and 3 RCCX modules, and therefore 1, 2, and 3 C4 genes, have frequencies of 17%, 69%, and 14%, respectively. Quadruplication of the module is also known, so the diploid contribution of C4 genes can vary between zero and eight.

17.5 Further complexities of Chido and Rodgers

Detection by plasma inhibition methods of three Rodgers phenotypes, Rg+, Rg–, and Rg partial inhibitor [7], and of four Chido phenotypes, Ch+, Ch–, and two types of Ch partial inhibitor [5,10], led Giles [5,12,13] to isolate two Rodgers and three Chido antibodies. Anti-Rg1, -Rg2, -Ch1, -Ch2, and -Ch3 define antigens of relatively high incidence. Rg+ plasmas are Rg:1,2; Rg– are Rg:–1,–2; and Rg partial inhibitors are Rg:1,–2. Rg:–1,2 has not been found and if the model of Yu *et al.* [40] is correct (see below), does not exist. Ch+ plasmas are Ch:1,2,3, Ch– are Ch:–1,–2,–3, and the two types of Ch partial inhibitors are Ch:1,–2,3 and Ch:1,2,–3. Ch:–1,2,–3 and Ch:1,–2,–3 are extremely rare variants [45,54]; Ch:–1,2,3 and Ch:–1,–2,3 have not been detected and may not exist [40]. The frequencies of these phenotypes are shown in Table 17.2. All C4 molecules express either Rg1 or Ch1; no C4 molecule expresses both.

Ch and Rg phenotypes do not correlate with specific C4 allotypes in a straightforward manner (Table 17.3), although a few generalisations can be made [57]. Of the partial inhibitor phenotypes, Rg:1,–2 is found predominantly with the haplotype *C4A*3 A*2 B*Q0*, there is a strong association between Ch:1,–2,3 and *C4B*2*, and Ch:1,2,–3 is most frequently associated with *C4A*6 B*1*, but also with *C4A*3 B*1*.

Table 17.2 Ch (1–3) and Rg phenotypes and frequencies in English (309 tested) and Japanese (89 tested) donors [1,5,55].

Phenotype	English (%)	Japanese (%)
Rg:1,2	95	100
Rg:1,–2	3	0
Rg:–1,–2	2	0
Ch:1,2,3	88	75
Ch:1,–2,3	5	24
Ch:1,2,–3	3	0
Ch:–1,–2,–3	4	1
Ch:–1,2,–3	Very rare	
Ch:1,–2,–3	Very rare	

Further complexities arose with the detection of another three high frequency Ch determinants, Ch4, Ch5 and Ch6 [58]. Antibodies to these specificities can only be reliably detected by testing polyspecific anti-Ch reagents with Ch:–1,–2,–3 red cells coated with C4 of various allotypes, especially those of reversed Ch/Rg antigenicity. Ch4 was detected on all C4B allotypes and Ch4 is not produced by any haplotypes with *C4B*Q0*, including *C4A*1 B*Q0*, which produces Ch1 and Ch3 in the absence of Rg1 and Rg2. Ch5 associates with Ch2 on C4B; Ch2 is only present on C4B, but Ch5 is also detected on C4A 1. Ch6 is associated with Ch3 on C4B, but, unlike Ch3, Ch6 is always detected on Rg:1,–2 C4 allotypes.

Another Ch/Rg antibody, W.H., was identified in the serum of a multiply transfused man, which also contained anti-Ch1 and -Ch4 [41]. W.H. expression is associated with allotypes producing Ch6 and Rg1 in the absence of Rg2 [41,59].

All Rg antisera contain anti-Rg1 and anti-Rg2; with very rare exceptions [60] all Ch antisera contain anti-Ch1, which is often the only specificity [12,13]. The approximate frequencies with which each of the other specificities have been found in anti-Ch reagents are as follows: anti-Ch2, 25%; anti-Ch3, 10%; anti-Ch4, 75%; anti-Ch5, 16%; and two examples each of anti-Ch6 and W.H. antibody [1]. Rg antibodies are only found in Rg:–1,–2 individuals. Ch antibodies are usually found in people with the Ch:–1,–2,–3,–4,–5,–6 phenotype, but a few exceptions are reported: anti-Ch2 plus -Ch5 in a Ch:1,–2,3,4,–5,6 person [60]; anti-Ch2 plus -Ch4 in a Ch:1,–2,3,–4,5,6 person [61]; anti-Ch1 in a Ch:–1,–2,–3,

Table 17.3 Nine antigenic determinants and one hypothetical determinant (Rg3) in 16 combinations predicted by Yu *et al.* [40] and Giles and Jones [41], 13 of which have been detected (from Giles [56]). The 1983 allotype nomenclature is used.

No.	Rg1	Rg2	(Rg3)*	Ch1	Ch2	Ch3	Ch4	Ch5	Ch6	WH	C4 isotype	Associated C4 allotype(s)
1	+	+†	+	–	+	–	+	+	–	–	B	B3
2	+	+	+	–	–	–	–	+	–	–	A	A3
3	+	+	+	–	–	–	+	–	–	–	B	B5
4	+	+	+	–	–	–	–	–	–	–	A	A1‡ A2 A3 A4 A5 A6
5	+	–†	–	–	+	–	+	+	+	+	(B)	Not found
6	+	–	–	–	–	–	–	+	+	+	A	A3 A(3,2)§
7	+	–†	–	–	–	–	+	–	+	+	B	B5
8	+	–	–	–	–	–	–	–	+	+	A	A3
9	–	–†	+	+	+	–	+	+	–	–	B	B1 B3
10	–	–†	+	+	–	–	–	+	–	–	(A)	Not found
11	–	–†	+	+	–	–	+	–	–	–	(B)	Not found
12	–	–†	+	+	–	–	–	–	–	–	A	A1‡
13	–	–	–	+	+	+	+	+	+	–	B	B1 B3
14	–	–	–	+	–	+	–	+	+	–	A	A1‡
15	–	–	–	+	–	+	+	–	+	–	B	B2 B3 B5 B6
16	–	–†	–	+	–	+	–	–	+	–	A	A1‡

*Hypothetical determinant.
†Predicted Rg phenotype not detected by haemagglutination inhibition.
‡C4 A1 is a heterogeneous electrophoretic group of allotypes.
§Duplicated C4A haplotypes.

–4,5,6 person [62]; and anti-Ch1, -Ch3, and -Ch4 in a Ch:–1,–2,–3,–4,5 person [62]. Serum of a Ch– Rg– C4-deficient patient contained an antibody to C4d not recognisable as any Ch or Rg antibody, although traces of anti-Ch and -Rg may also have been present [23].

From serological data on the two Rg and six Ch determinants, and from derived amino acid sequences of several C4 allotypes, Yu *et al.* [40] devised a model to explain Ch/Rg antigenicity. The model is represented in the diagram in Figure 17.3 and in some of the data presented in Table 17.1. The model involves sequential epitopes, which are dependent on one or more amino acid residues within a single sequence of a few residues, and conformational epitopes, which are more dependent on the shape of the molecule and require the presence of more than one sequential epitope. Ch1 and Ch6 are sequential epitopes. Ch1 requires Ala1188 and Arg1191; Ch6 requires Ser1157. Ch3, a conformational epitope, requires the presence of both Ch1 and Ch6. Ch4 and Ch5 are sequential epitopes. Ch4 requires Leu1101, Ser1102, Ile1105, and His1106 (defining the C4B isotype). Ch5 requires Gly1054. The conformational epitope Ch2 requires the presence of both Ch4 and Ch5. Rg1, a sequential epitope, requires Val1188 and Leu1191. Rg2, a conformational epitope, requires the expression of Rg1 and Asn1157. Asn1157 was assumed to represent a sequential epitope called Rg3, although no anti-Rg3 has been found. W.H. represents a conformational epitope expressed when valine and leucine occupy 1188 and 1191, respectively (Rg1) and when Ser1157 (Ch6) is present [41]. A study of 325 families supported the extended model, without exception [56]. The model allows for the 16 possible combinations shown in Table 17.3, 13 of which have been recognised.

In addition to anti-Rg1 and -Rg2, two of 10 Rg antisera contained an antibody specific for an epitope on the β-chain of C4 [63]. The antibody could not be separated from anti-Rg2 and a strong, but incomplete, association exists between Rg2 and the β-chain epitope.

C4 genes producing Ch1 or Rg1 determinants can be distinguished by an *Eco*O 109 RFLP, regardless of C4A or C4B isotype [39]. PCR-based methods combining

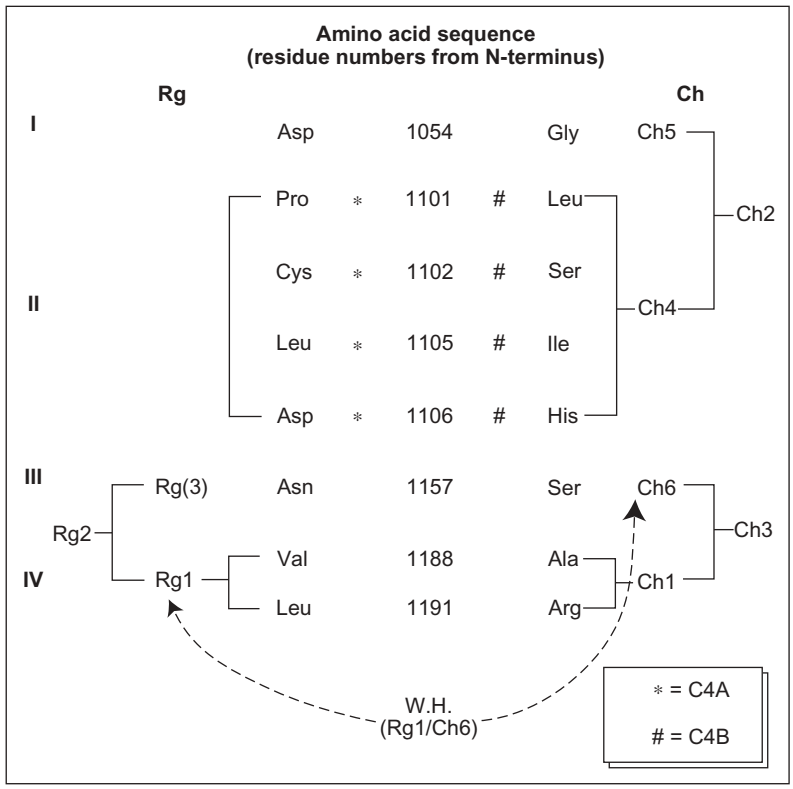


Figure 17.3 A structural model for the location of Ch/Rg determinants on C4 [40,56]. Ch1, Ch4, Ch5, Ch6, and Rg1 represent sequential epitopes; Ch2, Ch3, Rg2, and W.H. represent conformational epitopes involving two sequential epitopes; Rg(3) represents a hypothetical sequential epitope.

isotype- and allele-specific amplification with sequence-specific primers or direct sequencing enable the prediction of all Ch and Rg determinants [64–66].

17.6 Chido/Rodgers antibodies: clinical significance

Ch/Rg antibodies are IgG, mostly IgG2 and IgG4 [67], and they are not considered clinically significant from the red cell transfusion aspect. Ch/Rg antibodies have not caused any obvious signs of an HTR and radiolabelled Ch+ cells transfused to patients with anti-Ch survive normally [2,68–73]. Anti-Ch and -Rg have, however, been implicated in severe anaphylactic reactions following infusion of fresh frozen plasma, plasma fraction, or platelet concentrates containing plasma [74–76], though these events are exceptional.

Ch/Rg autoantibodies, responsible for a DAT, appeared in a woman at the 35th week of pregnancy, and disappeared a few months after delivery [77]. This occurred in two pregnancies and in both cases the red cells of the baby gave a negative DAT.

17.7 Associations with disease

C4 deficiency and its associated Ch/Rg-null phenotype is rare and is usually accompanied by autoimmune or immune complex disorder, typically systemic lupus erythematosus (SLE) [78]. This association may result from ineffective dissolution and removal of immune aggregates in the absence of C4 [17]. There is a significantly greater susceptibility to SLE in individuals with a *C4A* gene deletion (*C4A*Q0*) than in the general population [79–81]. In European Americans, absence or just a single

copy of *C4A* in the diploid genome are risk factors for SLE, whereas three or more copies appear to be protective [82]. It is not surprising, therefore, that symptoms of SLE have been diagnosed in substantially more Rg– individuals, mostly homozygous for *C4A*Q0*, than Rg+ people [83]. *C4A*Q0* has also been associated with several other autoimmune diseases, including Graves' disease and rheumatoid arthritis [84].

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18 Gerbich Blood Group System

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18.1 Introduction

The Gerbich system consists of 12 antigens, seven of very high frequency and five of low frequency (Table 18.1). They are located on either or both of the red cell membrane sialoglycoproteins glycophorin C (GPC, CD236C) and glycophorin D (GPD, CD236D), or on closely related glycoproteins. GPD is a truncate version of GPC. GPC and GPD are produced by the same gene, *GYPC*, as a result of initiation of mRNA translation at two sites. *GYPC* consists of four exons.

There are three rare 'Ge-negative' phenotypes in which the red cells lack one or more of the high frequency antigens, Ge2, Ge3, and Ge4. Ge:–2,3,4 (Yus phenotype) and Ge:–2,–3,4 (Gerbich phenotype) result from deletions of *GYPC* exon 2 and exon 3, respectively. Ge:– 2,–3,–4 (Leach or Ge-null phenotype) usually results from a deletion of exons 3 and 4, although a single nucleotide deletion was involved in one case. Ls^a arises from a duplication or triplication of *GYPC* exon 3. The remaining four low frequency antigens and absence of the other four high frequency antigens result from point mutations in *GYPC*.

GYPC is located on chromosome 2q14-q21.

18.2 Glycophorin C (GPC) and glycophorin D (GPD), and *GYPC*, the gene that encodes them

18.2.1 Red cell membrane sialoglycoproteins

Glycophorin is a name given to several sialic acid-rich glycoproteins of the red cell membrane that are detected after SDS PAGE by periodic acid-Schiff (PAS) staining. The major sialoglycoproteins are glycophorin A (GPA) and glycophorin B (GPB), homologous structures carrying the antigens of the MNS system (Chapter 3). The minor sialoglycoproteins, glycophorin C (GPC) and glycophorin D (GPD), are another pair of homologous structures, which represent about 6% and 1% of PAS staining material, respectively [1]. GPA and GPB are not genetically related to GPC and GPD, which carry the Gerbich system antigens.

Several synonyms have been used for GPC (CD236C, β-sialoglycoprotein, component D, PAS–2′) and GPD (CD236D, γ-sialoglycoprotein, component E) (Table 3.3).

18.2.2 GPC and GPD

The apparent MWs of GPC and GPD on SDS PAGE are 40 kDa and 30 kDa, respectively (reviews in [2–4]). By the

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Table 18.1 Antigens of the Gerbich system.

Antigen			Molecular basis*		
No.	Name	Frequency	Nucleotides	Exon	Associated glycoprotein
GE2	Ge2	High	(Deletion exon 2)	2	GPD N-ter region
GE3	Ge3	High	(Deletion exon 3)	3	GPC 42–50, GPD 21–29
GE4	Ge4	High	(Deletion exon 3 & 4) or (131G>T, 134delC)	2 & 3 or 3	GPC N-ter region
GE5	Wb	Low	23A>G	1	GPC Asn8Ser
GE6	Ls[a]	Low	Duplicated or triplicated exon 3		GPC/GPD, junction of product of 2 exons 3
GE7	An[a]	Low	67G>T	2	GPD Ala2Ser
GE8	Dh[a]	Low	40C>T	1	GPC Leu14Phe
GE9	GEIS	Low	95C>A	2	GPC Thr32Asn, GPD Thr11Asn
GE10	GEPL	High	134C (T)	3	GPC Pro45 (Leu), GPD Pro24 (Leu)
GE11	GEAT	High	56A (T)	2	GPC Asp19 (Val)
GE12	GETI	High	80C (T)	2	GPC Thr27 (Ile), GPD Thr6 (Ile)
	GERW	High	173A (T)	3	GPC Asp58 (Val), GPD Asp37 (Val)

*Molecular basis of antigen-negative phenotype in parentheses.
Obsolete: GE1.

use of Fab fragments of monoclonal antibodies, the number of molecules per red cell has been estimated as 143 000 for GPC and 225 000 for GPC plus GPD [5].

The first 47 amino acid residues of GPC were determined by manual sequencing [6]. Colin *et al.* [7] used a mixture of 32 synthetic oligonucleotides, each of which represented the sequence of amino acid residues 19–23 of GPC, as radioactive hybridisation probes in order to isolate *GYPC* cDNA from a human reticulocyte cDNA library.

GPC has three domains: a glycosylated N-terminal extracellular domain (residues 1–57) containing one *N*-linked oligosaccharide at Asn8 and sites for 12 *O*-linked oligosaccharides; a hydrophobic membrane-spanning domain (58–81); and a C-terminal cytoplasmic domain (82–128) [7,8]. The cytoplasmic domain of GPC interacts with the red cell membrane skeleton (Section 18.7). An N-terminal signal peptide is often associated with nascent transmembrane glycoproteins, including GPA and GPB, and cleaved from the mature protein. No such signal peptide is encoded by *GYPC* [8].

For reasons discussed below (Section 18.2.3), GPD is a truncate version of GPC, lacking the N-terminal 21 amino acid residues of GPC and identical to residues 22–128 of GPC. This is consistent with the following details:

1 GPD has no *N*-glycosylation;
2 GPD lacks epitopes present on the N-terminal domain of GPC [9];
3 Ge3 antigen, which represents a region around amino acid residues 40–50 of GPC, is also present on GPD [10] (Section 18.3.2.2);
4 antigenic determinants detected by monoclonal and polyclonal antibodies produced in animals are present on the cytoplasmic domains of both GPC and GPD [11–13] (see Figure 18.2).

GPC is part of the putative 'junctional' membrane-protein complex [14] (see Sections 10.7 and 18.7, and Figure 10.2).

18.2.3 GPC and GPD are encoded by the same gene

Despite the high level of homology between GPC and GPD, no homologous gene could be detected in genomic DNA using *GYPC* cDNA as a probe. This led to proposals that a separate GPD gene does not exist and that GPC and GPD are both produced by *GYPC* [15,16]; the result of initiation of translation of *GYPC* mRNA at two different sites, to produce two polypeptides.

The process of protein synthesis, in which the nucleotide sequence of mRNA is translated into an amino acid sequence, commences at an AUG codon (a methionine

codon, ATG in DNA), although a 10-nucleotide consensus sequence including the AUG codon is also important for effective initiation of translation. Newly processed polypeptides have methionine at their N-terminus, although this is usually cleaved from the mature protein. The RNA sequence around the start codon for GPC (CCAGGA **AUG** U) does not conform closely to the consensus start sequence (CC(A/G)CC **AUG** G) found in eukaryote transcripts; the sequence around a downstream AUG triplet (CCG GGG **AUG** G), the codon for Met22 of GPC, is a closer fit to the consensus sequence [16]. A process referred to as 'leaky initiation of translation' occurs in which the initiation site at the codon for Met1 of GPC is sometimes missed during the scanning of *GYPC* mRNA. Scanning continues along the mRNA until the second initiation site at Met22 is reached, where translation begins to produce GPD, a shorter molecule comprising amino acid residues 22–128 of GPC (Figure 18.1). *GYPC* cDNA transfected into COS-7 cells produced GPC and GPD, cDNA with a deletion of ATG at position 1 produced only GPD, cDNA with an ATG to ACG mutation at position 22 produced only GPC, and cDNA with ATG to ACG mutations at positions 1 and 22 produced neither glycoprotein [17]. A mutation at nucleotide 4 (ATG **T** to ATG **G**), creating a more consensus motif around the first ATG codon, resulted in a doubling of expression of GPC compared with GPD [17].

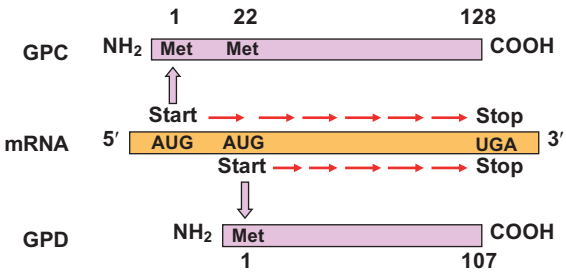


Figure 18.1 Production of GPC and GPD from a single gene by a process of leaky initiation of mRNA translation. When translation commences at the first AUG start codon a 128 amino acid GPC polypeptide is produced (above). If the first AUG codon is missed, then translation may commence at a second AUG site and a shorter 107-residue GPD polypeptide is produced (below).

18.2.4 Organization of *GYPC*

The 13.5 kb *GYPC* gene is organised into four exons (Table 18.2) [10,18]. Exons 1 to 3 encode the extracellular domain of GPC, exon 4 the membrane-spanning and cytoplasmic domains. Exons 2 and 3 show a high level of homology, probably arising from exon duplication, although exon 3 contains an insert of 27 nucleotides not present in exon 2, encoding amino acid residues 42–50 of GPC. The intronic flanking regions of exons 2 and 3 demonstrate an even higher level of homology than do the coding regions.

The upstream promoter region of *GYPC* contains the ubiquitous *cis*-acting elements CACC, TATA, and Sp1, plus three erythroid-specific GATA-1 binding sites and a binding site for an erythroid/megakaryocyte-specific factor, NF-E6 [19,20].

GYPC was localised to 2q14-q21 by *in situ* hybridisation [21].

18.3 The high frequency antigens Ge2, Ge3, and Ge4, and the Gerbich-negative phenotypes

18.3.1 Serological history

Gerbich began as a simple, inherited blood group antigen of very high frequency when, in 1960, Rosenfield *et al.*

Table 18.2 Organisation of *GYPC*.

	Amino acid residues		
Exon	GPC	GPD	Characteristics
1	1–16		N-terminus and part of extracellular domain of GPC; *N*-glycan; Ge4.
2	17–35	1–14	GPC Met22 (translation initiation site for GPD); part of extracellular domain of GPC and GPD including N-terminus of GPD; Ge2 on GPD.
3	36–63	15–42	Part of extracellular domain of GPC and GPD; Ge3 on GPC and GPD; trypsin cleavage site on GPC and GPD.
4	64–128	43–107	Membrane-spanning and cytoplasmic domains of GPC and GPD.

[22] described antibodies of apparently identical specificity in three women (including Mrs Gerbich). The simplicity of the Gerbich system was short-lived: in 1961 Cleghorn [23,24] found that the red cells of Mrs Yus., a Turkish Cypriot woman, failed to react with two of the original anti-Gerbich sera, but did react with the other, the antibody of Mrs Gerbich herself. The serum of Mrs Yus. contained an antibody that reacted with all cells tested apart from her own and those of the original three Gerbich-negatives. Adsorption of Mrs Gerbich's serum with Mrs Yus.'s cells removed all antibody. Although the Yus type is not strictly Gerbich-negative, as the cells react with the antibody of Mrs Gerbich, it is generally considered Gerbich-negative because the cells fail to react with the majority of antibodies made by Gerbich-negative people [25]. Both of these Gerbich-negative phenotypes are exceedingly rare in most populations.

Further complexities of the Gerbich system arose from studies of the Melanesians of Papua New Guinea. Not only was Gerbich found to be polymorphic among some populations of the north-east coast of New Guinea [26,27], but another Gerbich-related antibody was found in a Ge+ Melanesian [27]. This antibody failed to react with Gerbich-negative cells of Gerbich and Yus types, but also did not react with red cells of up to 15% of Ge+ Melanesians. Booth and McLoughlin [27] proposed a numerical notation for Gerbich phenotypes, shown in modified form in Table 18.3. Red cells of the Melanesian phenotype (Ge:–1,2,3) and the two examples of anti-Ge1 [27,28] have not been widely used and are not available for further investigation. Consequently, Ge1 has been declared obsolete and the Melanesian phenotype is omitted from Table 18.3.

Some monoclonal antibodies were shown to be related to the Gerbich system because they agglutinated all red cells except those of the Ge:–2,–3 (Gerbich) and Ge:–2,3 (Yus) phenotypes, although they reacted with these cells by an antiglobulin test [29,30]. Anstee *et al.* [30,31] found that red cells of two unrelated Ge:–2,–3 women failed to react with the Gerbich-related monoclonal antibodies by any technique. This new Gerbich-negative phenotype was called the Leach phenotype. An alloantibody in the serum of a Leach phenotype patient [32] behaved in a very similar manner to the monoclonal antibodies and became anti-Ge4. Leach phenotype red cells are Ge:–2,–3,–4, the Gerbich-null phenotype; all other red cells have Ge4 (Table 18.3).

Table 18.3 Gerbich-negative phenotypes.

Ge:–2,3,4	Yus phenotype
Ge:–2,–3,4	Gerbich phenotype
Ge:–2,–3,–4	Leach phenotype

18.3.2 High frequency antigens Ge2, Ge3, and Ge4

18.3.2.1 Ge2

Anti-Ge2 is the antibody characteristic of the Ge:–2,3,4 phenotype, but is also the most frequently encountered antibody in the Ge:–2,–3,4 and Ge:–2,–3,–4 phenotypes. Of 17 antibodies from Ge:–2,–3 people, only four were anti-Ge3, the other 13 were anti-Ge2 [25]; of the six Leach phenotype individuals with antibody, four had anti-Ge2, one anti-Ge3, and one anti-Ge4 [30,32–35].

Immunoblotting with numerous examples of alloanti-Ge2 demonstrated that the Ge2 antigen is located on GPD, but not on GPC [12,36]. It is on the N-terminal tryptic peptide of GPD (residues 1–27) [36]. Treatment of intact red cells with trypsin or papain destroys Ge2; chymotrypsin and pronase do not. About 50% of anti-Ge2 show a reduction in strength of reaction with sialidase-treated cells [25].

GPD is a shorter version of GPC and does not have any amino acid sequence that is not present in GPC. Ge2 is usually at the N-terminus of GPD. Anti-Ge2 might recognise an amino acid sequence only when it is in the conformation of the N-terminus of GPD and not when it is an internal sequence within GPC. Alternatively, the Ge2 determinant could involve the free amino group of GPD and the adjacent amino acid sequence. Some anti-Ge2 do not react with red cells after acetylation of membrane protein with acetic anhydride, suggesting that a free amino group is involved in the epitope detected by those antibodies [37]. Anti-Ge2 probably represents a heterogeneous collection of antibodies that react with epitopes at the N-terminal region of GPD.

18.3.2.2 Ge3

Anti-Ge3 has been found in immunized Ge:–2,–3,4 and Ge:–2,–3,–4 individuals, but is far less common than anti-Ge2 [25,33]. Like Ge2, Ge3 is destroyed by trypsin and not by chymotrypsin or pronase. Unlike Ge2, Ge3 is resistant to treatment of intact red cells with papain [25]. Consequently, papain-treated cells can be used for distinguishing anti-Ge2 and -Ge3 in the absence of the very rare Ge:–2,3,4 cells (although one anti-Ge3 in a Ge:–2,–3,4 patient detected a papain-sensitive Ge3 antigen [38]).

Table 18.4 Frequency studies performed by testing red cells of random individuals with Gerbich antibodies.

Antibody	Population tested	No. tested	No. negative	References
Anti-Ge2	English, Danes, New Zealanders, Californians	28 331	0	[22,23,42,43]
Anti-Ge3	New Yorkers*	11 000	0	[22]
Anti-Ge3	French	5912	1	[43,44]
	Total	45 943	1	
Anti-Ge2	PNG† Sepik Region	748	182	[27]
Anti-Ge2	PNG† Morobe Region	1014	517	[27]
Anti-Ge2	PNG† Highlands	1348	1	[27]
Anti-Ge2‡	PNG† Port Moresby donors	116	13	[45]
Anti-GPC‡	Ethiopean Jews	980	1	[46]
Anti-Ge2	Japanese	22 000	0	[47]

*Including at least 1500 black people and 100 Asiatics, mostly Chinese.
†Melanesians of Papua New Guinea.
‡Monoclonal antibody.

Immunoblotting with human alloantibodies and rodent monoclonal antibodies showed that Ge3 is present on both GPC and GPD [5,12,36,39,40]. Alloanti-Ge3 eluted from either GPC or GPD on an immunoblot detected both GPC and GPD on a separate immunoblot, confirming that anti-Ge3 is a single antibody to an antigen common to both proteins [12].

Ge3 is encoded by exon 3 of *GYPC*. Ge3 is missing in those rare phenotypes resulting from deletion of exon 3, but not from those resulting from a deletion of exon 2 (Section 18.3.3). Exon 2 and exon 3 are very similar apart from a 27 nucleotide insert in exon 3, representing amino acids 42–50 of GPC (21–29 of GPD) (Section 18.2.4), so Ge3 must be in this region, as confirmed by haemagglutination inhibition with fragments of GPC [36].

18.3.2.3 Ge4

The only example of alloanti-Ge4 was identified in the serum of a woman with the Ge:–2,–3,–4, Leach phenotype [32]. Anti-Ge4 was also found in the serum of a patient with a transient GPC deficiency [41]. Numerous monoclonal antibodies to GPC have been produced that behave serologically as anti-Ge4 (Section 18.5.3).

Ge4 is usually situated near the N-terminus of GPC and, therefore, is not on GPD [9,30,31,41]. Detailed analyses of monoclonal antibodies specific for GPC have shown that some require the amino group of Met1 of GPC for binding, whereas others detect other epitopes within the first 21 amino acids of GPC, but not involving Met1 [9,40]. Most required normal *O*-glycosylation of the GPC for effective binding. Ge4 is destroyed by trypsin and papain treatment of the red cells.

18.3.3 Gerbich-negative phenotypes

Outside Papua New Guinea (PNG), Gerbich-negative phenotypes are very rare. Screening of over 44 000 blood samples from white populations with anti-Ge2 or -Ge3 produced only one Gerbich-negative (Table 18.4). Molecular genotyping revealed frequencies for *GYPC* with a deletion of exon 3 (*GYPC.Ge*) of 0.46 and 0.18 in the Wosera and Liksul areas of PNG, respectively, with 22% and 3% homozygous for *GYPC.Ge*, predicting the Ge:–2,–3,4 phenotype [48].

18.3.3.1 Ge:–2,3,4: the Yus phenotype

Ge:–2,3 has been found in people of European origin, Middle Eastern Jews and Arabs, and in people of African origin, including Ethiopian Jews (Table 18.4); it has not been found in PNG.

Ge:–2,3,4 red cells contain no GPC or GPD, but they do have a GPC-like structure (GPC.Yus), represented on SDS PAGE by a broad, diffusely staining band of apparent MW 32.5–36.5 kDa, situated between the positions for GPC and GPD [12,30,49]. This structure carries Ge4 and Ge3 [12,30] (Figure 18.2). GPC, GPD, and GPC.Yus are

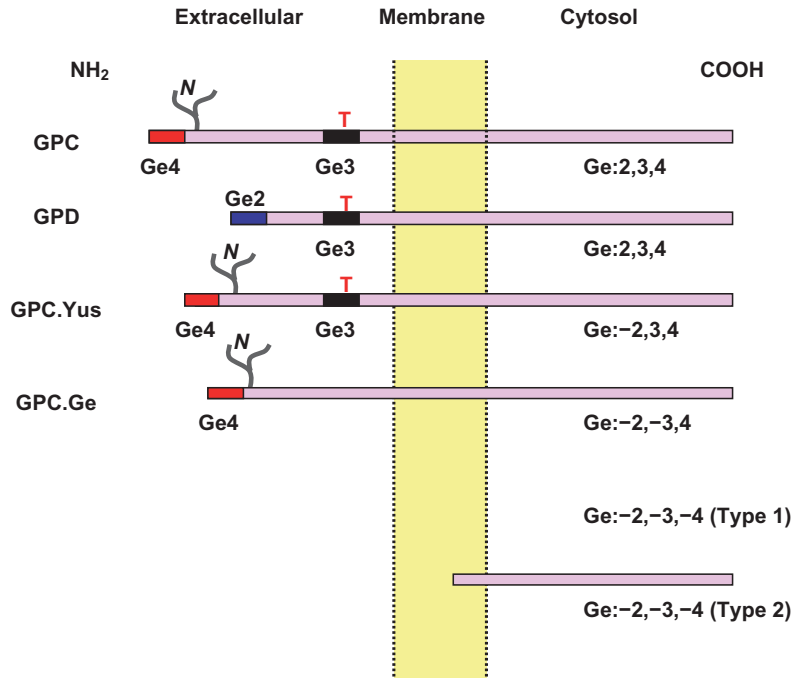


Figure 18.2 Diagram representing GPC, GPD, and related structures characteristic of Gerbich-negative phenotypes. Ge:2,3,4 cells have both GPC and GPD; Ge:–2,3,4 and Ge:–2,–3,4 cells have GPC.Yus and GPC.Ge, respectively, but no GPC or GPD. In most Ge:–2,–3,–4 (Leach phenotype) cells, no GPC, GPD, or related molecule is present (Type 1), but in one example a GPC/D C-terminal fragment was detected (Type 2). T, trypsin cleavage site at Arg48.

present in red cell membranes from individuals heterozygous for *GYPC* and *GYPC.Yus* [50].

Ge:–2,3,4 individuals are homozygous for a *GYPC* gene (*GYPC.Yus*, *GYPCΔex2*, or *GE*01.–02*) in which the second exon is deleted [10,51,52] (Figure 18.3). The protein product is a GPC-like molecule (GPC.Yus) lacking amino acid residues 17–35, with no loss of Ge4 or Ge3 (Figure 18.2). The second translation initiation site (Met22) is lost, so no GPD is formed and, therefore, no Ge2 is expressed.

Ge:–2,3,4 may also result from heterozygosity for *GYPC.Yus* and *GYPC.Ge* [55,56]. Five of 10 Ge:–2,3 propositi were found to have both GPC.Yus and GPC.Ge [55]. Heterozygosity for *GYPC* with 80C >T (*GE*01.–12*) and for *GYPC* with g >a in the 3′ nucleotide of intron 1 (IVS2−1g >a) was found in a brother and sister with the GETI– phenotype (Section 18.4.6.3) [57]. IVS2−1g>a would cause splicing out of exon 2, encoding GPC.Yus.

18.3.3.2 Ge:–2,–3,4: the Gerbich phenotype

Ge:–2,3,4 is the typical Gerbich-negative phenotype, the probable phenotype of Mrs Gerbich, although her cells were never tested with anti-Ge4. Ge:–2,–3 is polymorphic in certain regions of PNG [27,45,48] (Table 18.4). Ge:–2,–3 is rare in all other populations tested, but has been found among people of European and African origin, Iraqi Jews, Native Americans, Japanese, and Polynesians.

Like the Ge:–2,3,4 phenotype, Ge:–2,–3,4 cells have no GPC or GPD, but have a diffusely staining abnormal GPC-like structure (GPC.Ge) of mobility between that of GPC and GPD [12,30,49]. The apparent MW of GPC.Ge is 30.5–34.5 kDa, slightly less than that of GPC.Yus. GPC.Ge carries Ge4, but no Ge3 [12,30] (Figure 18.2). GPC.Ge is trypsin-resistant, unlike GPC, GPD, and GPC.Yus [30]. The composition of the *N*-glycan of GPC.Ge differs from that of GPC, with a higher mannose content [58].

Ge:–2,–3,4 results from a *GYPC* gene (*GYPC.Ge*, *GYPCΔex3*, or *GE*01.–03*) with a deletion of exon 3 [10,18,51,56,59] (Figure 18.3), encoding a GPC-like structure (GPC.Ge) lacking amino acid residues 17–35 of GPC. GPC.Ge is slightly smaller than GPC.Yus because exon 3 is larger than exon 2 owing to a 27 nucleotide insert. Loss of exon 3 also explains absence of Ge3 and of

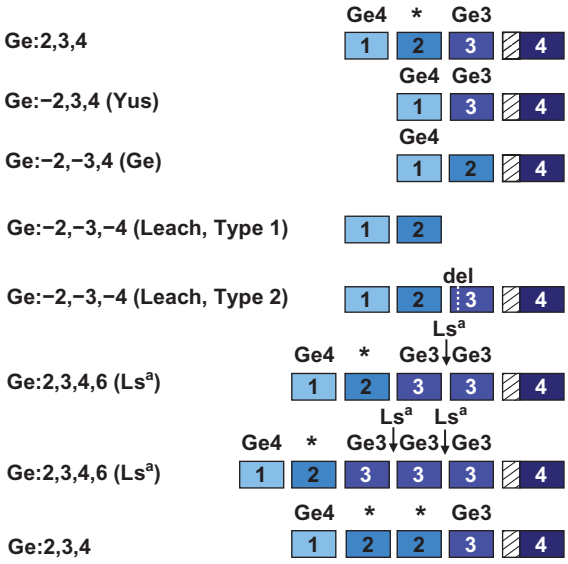


Figure 18.3 The genomic organisation of *GYPC* and variants of *GYPC* responsible for rare Gerbich phenotypes. The boxes represent exons and the shaded area in exon 4 represents the region encoding the membrane-spanning domain. *Second translation start codon (Met22). *GYPC* with duplication of exon 2 does not result in any qualitative change to the Gerbich phenotype [53,54]; triplication and quadruplication of exon 2 is not shown.

the trypsin cleavage site at Arg48 (Figure 18.2). It does not, however, explain the absence of GPD and, therefore, of Ge2. Colin *et al.* [18] suggested that a GPD molecule lacking most of its extracellular domain might not be transported to the membrane or might be unstable and rapidly degraded. GPC.Ge and GPC.Yus carry *N*-glycans with variable numbers of repeating lactosamine units, explaining their diffuse appearance on electrophoresis gels [12]. They may also have altered *O*-glycosylation [60].

It is probable that *GYPC* genes with deletions of exon 2 or exon 3 arose from intragenic unequal crossing-over, an event that would have simultaneously produced genes with duplications of exons 2 or exon 3 [10,18] (see Figure 18.4). This topic is returned to in the section on Ls^a (Section 18.4.2).

18.3.3.3 Ge:–2,–3,–4: the Leach phenotype

The Leach phenotype is the null phenotype of the Gerbich system. Ge:–2,–3,–4 red cells do not react with any Gerbich antibodies or related monoclonal antibodies. Six propositi with the Ge:–2,–3,–4 phenotype have been reported, all white English or North Americans [30, 32–35]. Ge:–2,–3,–4 red cells are totally devoid of GPC and GPD [10,12,30,33,34] (Figure 18.2).

Ge:–2,–3,–4 has at least two genetic backgrounds. Five unrelated Ge:–2,–3,–4 individuals were homozygous for a deletion of exons 3 and 4 of *GYPC* (*GE*01N.01*) [10,52,61,62] (Figure 18.3). Surprisingly, mRNA derived from this deleted gene was detected in reticulocytes from Ge:–2,–3,–4 individuals, despite the gene lacking the normal polyadenylation signal [62]. If any protein were produced by this gene, it would lack the membrane-spanning and cytoplasmic domains and could not be inserted into the membrane. One other Ge:–2,–3,–4 individual [32] was homozygous for *GYPC* with a single nucleotide deletion within codon 45 in exon 3 (134delC), resulting in a frameshift and premature generation of a stop codon after codon 55 (*GE*01N.02*) [61] (Figure 18.3). Most of exon 3 and all of exon 4 would not be translated and so viable GPC could not be produced. A 12 kDa component was detected, which bound a monoclonal antibody to an epitope on the cytoplasmic domains of GPC and GPD and appeared to represent the C-terminal domain of GPC/D [63] (Figure 18.2). This led to speculation that translation is reinitiated at an alternative start sequence overlapping the premature stop codon.

GPC and GPD are associated with the membrane skeleton, acting as a link between the membrane and the skeletal proteins (Section 18.7). One characteristic of the Ge:–2,–3,–4 phenotype is elliptocytosis [12,30,31,33,34]. Between 20 and 61% of the red cells of five Ge:–2,–3,–4 phenotype individuals were classed as elliptocytes [33]. Two Ge:–2,–3,–4 brothers had a longstanding history of mild anaemia [35]. Ge:–2,–3,–4 phenotype membranes have reduced mechanical stability [64,65] and may be released into the circulation as normal discocytes, but become distorted when exposed to shear stress and elongation [66]. Elliptocytes were present in a patient with a temporary reduction in red cell membrane GPC content and normal GPD content, but were not present when her GPC levels returned to normal [41]. Ge:–2,–3,4 and Ge:–2,3,4 cells show no sign of elliptocytosis [30] and have normal membrane stability [65], despite GPC and GPD deficiency, presumably because of the presence of the GPC-like molecules, GPC.Ge and GPC.Yus.

18.3.4 Associations with other blood group systems

Several reports link the Ge:–2,–3 phenotype with a depression of Kell-system antigens, although the effect is variable [25,30,32,33,44,47]. The phenomenon may

Table 18.5 Frequencies of low frequency Gerbich antigens.

Antigen	Population	No. tested	No. positive	Antigen frequency	References
Wb GE5	White Australians	3550	2	0.0006	[70]
	English	15 815	3	0.0002	[43]
	Welsh	10 117	8	0.0008	[71]
Ls^a GE6	Finnish	1113	18	0.0162	[43,72]
	Norwegians	7151	8	0.0011	[73]
	African Americans	110	1	0.0091	[43]
	Black West Indians	878	9	0.0103	[43]
	West Africans	81	2	0.0204	[43]
	Japanese	200 000	8	<0.0001	[74]
	English	5887	0		[43]
An^a GE7	Finnish	10 000	6	0.0006	[75]
	Swedish	3266	2	0.0006	[75]
Dh^a GE8	Danish	2493	0		[76]
GEIS GE9	Japanese	32 852	3	0.0001	[77]

affect all high frequency Kell antigens and K, if present [44]. Alternatively, K11 antigen may be the only antigen to show weakness [25]. Nine of 11 Ge:–2,–3 samples showed different degrees of weakening of Kell system antigens, whereas none of six Ge:–2,3 samples showed any such Kell depression [25]. At least four Ge:–2,–3, –4 propositi have depression of Kell system antigens [30,32,33]. One murine monoclonal antibody (MIMA-9) that did not react with K_o or Kp(a+b–) red cells, reacted only weakly with K+k–, McLeod phenotype, and Ge:–2, –3 cells [67].

Two Gerbich-related antibodies have suggested some association between Gerbich and Rh [68,69]. In addition to being non-reactive with most Ge-negative red cells, they also failed to react with Rh_{null}, D––, and cells with some other variant Rh phenotypes, and one of them also failed to react with D–, k–, and Kp(b–) cells. GPC, the Kell glycoprotein, and the Rh proteins may belong to the same 'junctional' protein complex in the membrane [14]. A possible association between Gerbich and the Vel antigen is described in Section 30.2.2, so Vel may also be part of that complex.

18.4 Other Gerbich antigens

18.4.1 Wb (GE5)

The original anti-Wb (Webb) was found in ABO grouping serum [70]; no further example was found in 2000 Australian sera, but three examples were detected in 7544 British sera [43,71] (Table 18.5). Wb is sensitive to trypsin and sialidase treatment, but resistant to chymotrypsin treatment [64,78,79].

Wb results from Asn8Ser in GPC (*GE*01.05*) [51,80] (Table 18.1). Asn8 in GPC is *N*-glycosylated and loss of this *N*-glycosylation results in an abnormal GPC (GPC.Wb), with an apparent MW 3 kDa lower than that of GPC [78,79]. Treatment of Wb– red cells with endoglycosidase F, which cleaves *N*-linked oligosaccharide chains, results in a reduction in the apparent MW of GPC to that of GPC.Wb; treatment of Wb+ cells with endoglycosidase F has no effect on GPC.Wb. Whether Ser8 of GPC.Wb is *O*-glycosylated has not been determined.

18.4.2 Ls^a (GE6)

Ls^a (Lewis II) [43] is the same as Rl^a [73,81] and is polymorphic in Finns and people of African origin, but rare in other populations (Table 18.5). The original anti-Ls^a was found in an anti-B reagent; other examples were found in sera containing multiple antibodies to low frequency antigens [73] and during pre-transfusion cross-matching [82]. Anti-Ls^a was associated with HDFN necessitating exchange transfusion, but not proven to be the cause [83]. One anti-Ls^a was found by screening over 4000 normal sera from Europeans [43,72] and 19 examples were found in 44 000 Japanese donor sera [74]. Ls^a is trypsin-sensitive, but resistant to ficin and sialidase [84].

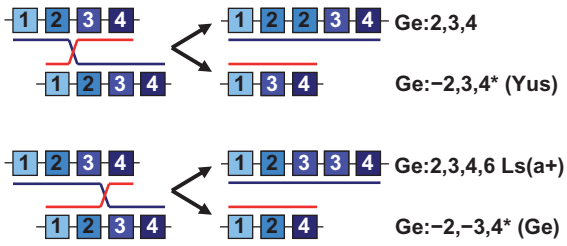


Figure 18.4 Diagram showing two intragenic unequal crossing-over events resulting in four abnormal *GYPC* genes, two with exon deletions and two with exon duplications. A duplication of exon 3 is associated with Ls^a antigen. *The Gerbich-negative phenotypes only occur when there is homozygosity for the aberrant gene (or heterozygosity for two aberrant genes).

Ls(a+) red cells have, in addition to normal GPC and GPD, abnormal GPC and GPD molecules, each with an apparent MW 5.5 kDa greater than its normal counterpart [84]. Both abnormal structures immunostain very strongly with anti-Ge3. GPC.Ls^a also binds monoclonal anti-Ge4 and GPD.Ls^a binds anti-Ge2. *GYPC* from Ls(a+) individuals has a duplication of exon 3 (*GE*01.06.01*) (Figure 18.3) [10,85]. The presence of an extra exon 3 explains why GPC.Ls^a and GPD.Ls^a are of increased size and have strongly expressed Ge3 antigens.

Intragenic crossing-over has been suggested as a mechanism to explain the origin of the *GYPC* exon 2 and exon 3 deletions responsible for the Ge:–2,3,4 and Ge:–2,–3,4 phenotypes, respectively [10,18]. Each unequal crossing-over event would also generate *GYPC* with a duplication of either exon 2 or exon 3 (Figure 18.4). The junction of two contiguous exons 3 would produce a unique amino acid sequence, probably representing the Ls^a antigen. A synthetic peptide (TPTIMDIVVIA-EPDPG) representing the last 11 amino acids encoded by the 3′ end of exon 3 followed by the first five amino acids encoded by the 5′ end of exon 3 inhibited anti-Ls^a [86].

A monoclonal antibody (CBC-96) to an epitope on GPC agglutinated Ls(a+) cells at a significantly higher dilution than that required to agglutinate cells of common Gerbich phenotype, presumably because the epitope is more exposed on the extended molecule [54]. Uchikawa and his colleagues used an appropriate dilution of this antibody to screen red cells of 200 000 Japanese donors. Of the 60 samples strongly agglutinated, eight were Ls(a+) [74]. One of these eight samples had enhanced Ls^a expression and had GPC and GPD molecules with an apparent MW 6 kDa greater than GPC.Ls^a and GPD.Ls^a, resulting from triplication of *GYPC* exon 3 (*GE*01.06.02*) [54] (Figure 18.3). Forty samples (0.02%) had *GYPC* with a duplication of exon 2, the putative result of the unequal crossing-over event responsible for the exon 2 deletion of GPC.Yus (Figure 18.4), and six (0.003%) had a quadruplication of exon 2 [53,54]. As predicted [10], no low frequency antigen associated with GPC and GPD molecules containing multiple products of exon 2 of *GYPC* was found, as this does not create a unique amino acid sequence.

18.4.3 An^a (GE7)

An^a (Ahonen) was found in about 0.06% of Finns and Swedes (Table 18.5) [75]. Anti-An^a has been found in about 1 in 1000 normal sera and does not appear to require red cell stimulation [75]. An^a is destroyed by trypsin, papain, and sialidase [37].

An^a is located on GPD, but not on GPC. It results from an alanine to serine substitution at position 23 of GPC and at position 2 of GPD (*GE*01.07*) [37]. This raises the question of why An^a is only expressed on GPD when the amino acid substitution occurs in both GPC and GPD. An^a does not appear to involve the free amino group at the N-terminus of GPD as acetylation of red cell surface proteins does not affect An^a expression [37]. Consequently, Daniels *et al.* [37] suggested that anti-An^a recognises a conformational difference in the N-terminal region of GPD resulting from the amino acid substitution, which is not apparent when the same substitution occurs within the GPC chain. GPD.An^a in human embryonic kidney cells expressed both An^a and Ge2, as determined by two alloanti-An^a and two alloanti-Ge2 [87].

18.4.4 Dh^a (GE8)

The first anti-Dh^a (Duch) was an IgM 'naturally occurring' antibody found when the serum reacted with the red cells of a Danish blood donor during pre-transfusion compatibility testing [76] (Table 18.5). Identification of anti-Dh^a in sera of two blood donors led to the discovery of an English family with eight Dh(a+) members in three generations [88]. Treatment of intact red cells with trypsin, papain, ficin, pronase, or sialidase destroys Dh^a; chymotrypsin does not [76,88].

Dh^a is located on GPC, but not on GPD, and results from Leu14Phe in GPC (*GE*01.08*) [89,90]. Dh^a is not affected by endoglycosidase F treatment of the cells and so is not dependent on the presence of the *N*-glycan at Asn8 [89]. Dh^a on red cells is sialidase-sensitive, suggesting the necessity for normal *O*-glycosylation of GPC, but anti-Dh^a was specifically inhibited by a synthetic peptide

representing amino acid residues 8–21 of GPC.Dh[a] (NSTAWPFSLEPNPG) [86].

18.4.5 GEIS (GE9)

A very rare antigen initially named Is results from Thr32Asn on GPC and Thr11Asn on GPD (*GE*01.09*) [77] (Table 18.5). GEIS was affected by papain, trypsin, or sialidase treatment of red cells, but not by chymotrypsin. Three examples of anti-GEIS were found in 5447 Japanese.

18.4.6 Four Gerbich antigens of high frequency associated with *GYPC* missense mutations

Antibodies resembling anti-Ge2 or -Ge3 have led to the identification of four antigens of high frequency, absence of which result from missense mutations in *GYPC* [57,91] (Table 18.1).

18.4.6.1 GEPL (GE10)

GEPL– phenotype was associated with a Ge:2,3,4 phenotype with aberrant expression of Ge3 and homozygosity for 134C>T encoding Pro45Leu in GPC and Pro24Leu in GPD (*GE*01.–10*). Immunoblotting revealed apparently normal GPC and GPD, plus additional bands indicating abnormal GPC and GPD molecules 2 kDa lower than GPC and GPD. Anti-GEPL resembled anti-Ge3 [57].

18.4.6.2 GEAT (GE11)

GEAT– phenotype was associated with a Ge:2,3,4 phenotype, with the red cells giving weak reactions with some Gerbich antisera, and with homozygosity for 56A>T encoding Asp19Val in GPC (*GE*01.–11*). Immunoblotting revealed apparently normal GPC and GPD. Anti-GEAT resembled anti-Ge3, but gave variable or weak reactions with Ge:–2,3,4 red cells [57].

18.4.6.3 GETI (GE12)

GETI– phenotype was associated with a Ge:–2,3,4 phenotype, apart from the cells reacting with one autoanti-Ge2, and with homozygosity for 80C>T encoding Thr27Ile in GPC and Thr6Ile in GPD (*GE*01.–12*). Immunoblotting revealed apparently normal GPC and GPD. The proximity of the Thr6Ile substitution in GPD is probably responsible for the loss of Ge2. Anti-GETI resembled anti-Ge2. In addition, a brother and sister with the GETI– phenotype were heterozygous for *GYPC* with the 80C>T mutation and for an intron 1 splice site mutation (IVS2–1g>a), which would be expected to cause splicing out of exon 2, consistent with Ge:–2,3,4 (Section 18.3.3.1) [57].

18.4.6.4 GERW

GERW– phenotype was associated with a Ge:2,3,4 phenotype, except the cells did not react with one alloanti-Ge2, one autoanti-Ge3, and one autoanti-Ge4. Immunoblotting revealed GPC, GPD, and GPC.Ge. The GERW– propositus was heterozygous for (1) *GYPC* encoding Asp58Val in GPC and Asp37Val in GPD, in the region of the insertion of these molecules into the membrane and (2) *GYPC.Ge* (*GYPC* with a deletion of exon 3). The antibody of the propositus did not react with her own cells or with those of four of her eight siblings. It immunostained GPC on blots of normal red cell membranes and appears to react with GPC with Asp58, but not with Val58. It is unclear why this antibody did not react with GPD or with GPC.Yus, both of which have aspartic acid at the position equivalent to amino acid 58 of GPC [91].

18.5 Gerbich antibodies

18.5.1 Alloantibodies

Gerbich antibodies are usually stimulated by pregnancy or transfusion, but some are apparently 'naturally occurring' [27,42,43,47,56]. Eighty-nine (13%) of 664 sera from Gerbich-negative Melanesians had Gerbich antibodies and the frequency of anti-Gerbich was about the same in men as in women [27]. Some Gerbich antibodies may be IgM, but the majority are IgG, mostly IgG1 [92]. The clinical significance of Gerbich antibodies is reviewed in [93].

Gerbich antibodies have not caused a serious HTR and least incompatible red cells can usually be selected for transfusion. A Ge:–2,–3 patient with anti-Ge3 had clinical and laboratory signs of a mild, acute HTR following transfusion of two units of Ge+ red cells [93]. The patient had been transfused uneventfully with Ge+ red cells several years earlier, when the result of a monocyte monolayer assay increased from 2.2 to 79.5% reactivity, predicting the potential to cause an HTR.

Several examples of Gerbich antibodies have caused mild neonatal jaundice, treated by phototherapy (see [94]). Anti-Ge3 has been responsible for late-onset severe anaemia in three babies, two of whom were from the same mother [94,95]. The anaemia was not apparent and did not become severe until 2–4 weeks after delivery. Therefore, pregnancies involving Gerbich antibodies

should be monitored for several weeks. The characteristics of HDFN caused by anti-Ge3 resemble those resulting from Kell antibodies, with only small elevations in reticulocyte and bilirubin levels and unresponsiveness to erythropoietin (EPO), suggesting suppression of erythropoiesis [94,96] (see Section 7.3.5.2). Like the Kell glycoprotein, glycophorin C appears on erythroid progenitors at an early stage of erythropoiesis [97,98], so fetal and neonatal erythroid cells may be phagocytosed by monocytes before they produce haemoglobin. Studies on K562 cells, however, suggest that binding of antibody to GPC causes cytoskeletal rearrangement through protein 4.1R and interference with the EPO signalling cascade, resulting in unresponsiveness to EPO [99]. In addition, ligation of GPC on red cells with murine monoclonal anti-GPC led to exposure of phosphatidylserine (PS) on their surface, a characteristic associated with eryptosis (red cell death) [100].

18.5.2 Autoantibodies

There are four reports of Gerbich autoantibodies causing severe AIHA: three resembled anti-Ge2 (one was IgA, another IgM) [101–103], the other resembled anti-Ge3 [104]. Gerbich-positive red cells of a patient with anti-Ge2 in his serum gave a negative DAT, but anti-Ge2 could be eluted [105]. Immunoblotting with this antibody and with another Ge2-like autoantibody showed that they differed from most alloanti-Ge2 because they bound GPC, but not GPD [105,106]. Both patients had reduced expression of target antigen on their cells. Autoanti-Ge3 behaved like alloanti-Ge3 on immunoblots by binding GPC and GPD [106].

An antibody resembling anti-Ge4 in a Ge:2,3,4 patient with aplastic anaemia and with elliptocytes in her peripheral blood did not cause autoimmune haemolysis [41]. The antibody bound GPC, GPC.Ge, and GPC.Yus, but not GPD. The patient's red cells had a substantially reduced content of GPC, but normal GPD. Two years later, antibody was no longer present in the patient's serum, the GPC content of her red cell membranes had returned to normal, no elliptocytes were present, and the antibody from an earlier sample reacted with her red cells.

18.5.3 Monoclonal antibodies

Many monoclonal antibodies associated with Gerbich define epitopes close to the N-terminus of GPC and behave serologically as anti-Ge4 [5,9,29,30,31,40,53,97, 107–110]. Production of recombinant GPC and its variants in heterologous expression systems has been valuable for analysing epitopes [87,111]. The epitopes of most include Met1 of GPC, but some involve amino acids 16–23 [53,109,110]. One monoclonal anti-Ge4 also reacted with MNS-variant Dantu+ red cells (Section 3.14.1). Several rodent monoclonal anti-Ge3 have been produced [5,39,40]. Rodent monoclonal antibodies behaving serologically as anti-Ge2 bound GPC [60,109] or GPC and GPD [109,112]. An IgM monoclonal anti-Ge2 defined an epitope involving amino acids 15–22 (SLEPDPGM) on GPC, yet did not react with GPC.Ge, possibly because of altered *O*-glycosylation [60]. This antibody cross-reacted with an epitope (amino acids 22–27, EDPDIP) on the cytoplasmic N-terminal domain of band 3. A human monoclonal anti-Ls^a has been produced [110].

18.6 Development and distribution of Gerbich antigens

Ge2 and Ge3 are well developed at birth [22]. Gerbich antigen (type unspecified) was detected in 19 fetuses aged 17.5–28 weeks [43]. During erythropoiesis, GPC and GPD are present on erythroid cells at an early stage of differentiation, though glycosylation may not be complete [97,113]. GPC, detected by a monoclonal antibody (BRIC4) to a glycosylation-dependent epitope, was strongly expressed on 84% of $CD34^+$ cells derived from cord blood [98].

GPC and GPD are not erythroid-specific, though the level of expression and the degree of glycosylation of the proteins may differ in erythroid and non-erythroid cells. Initiation of transcription of *GYPC* probably occurs at different sites in erythroid and non-erythroid cells [19]. GPC has been detected on T-lymphocytes and weakly on B-lymphocytes and platelets; GPC was not detected on granulocytes or platelets [19,97], although GPC was expressed during both erythroid and neutrophil differentiation of $CD34^+$ haemopoietic progenitors [113]. *GYPC* mRNA was detected in human erythroblasts, erythroleukaemic cell lines, and fetal liver, but not in adult liver [7], and in human kidney [8]. Immunostaining with a monoclonal antibody to the cytoplasmic C-terminal domain of GPC and GPD, selected to avoid any effect of differential glycosylation, was apparent on fetal liver (mostly on cells of erythroid lineage), sinusoids of adult liver, kidney glomeruli, and neural cells in the brain [13].

18.7 Functional aspects: association of GPC and GPD with the membrane skeleton

The shape, flexibility, and deformability of red cells is maintained by a submembranous matrix containing the proteins α- and β-spectrin, actin, protein 4.1R, adducin, dematin, tropomyosin, and tropomodulin [114,115]. Transmembrane protein band 3 (anion exchanger) is linked to the cytoskeleton through ankyrin and protein 4.2, representing the major site of attachment of the skeletal network to the membrane (Section 10.7, Figure 10.2). GPC and GPD serve a similar function. Protein 4.1R links GPC and GPD to the spectrin/actin junction, with the phosphoprotein membrane-palmitoylated protein 1 (MMP1, p55) functioning to stabilise the interaction. The Arg-His-Lys tripeptide at positions 86–88 of GPC bind to a sequence within the 30 kDa FERM domain of 4.1R; the tripeptide Tyr-Phe-Ile at the C-terminus of GPC binds to the PDZ domain of MMP1; and the D5 domain of MMP1 binds to 4.1R [116–124]. Protein 4.1R also binds calmodulin and increased levels of Ca^{++} decreases affinity of 4.1R interactions with MMP1 and GPC [124]. Phosphorylation of 4.1R results in dissociation of GPC from the membrane skeleton [125]. Protein 4.1R, therefore, plays an important role in regulating the GPC–4.1R–MMP1 complex. Palmitoylation of MMP1 is essential for membrane organisation and rare individuals who lack the active transferase responsible for MMP1 palmitoylation have an unusual form of haemolytic anaemia [126].

There are about 200 000 molecules of 4.1R per red cell [9], about the same as the total number of GPC and GPD molecules [5]. Patients with hereditary elliptocytosis caused by 4.1R deficiency have a 70–90% reduction of GPC and GPD, are MMP1 deficient, and have complete elliptocytosis [118,127–129]. GPC- and GPD-deficient Ge:–2,–3,–4 red cells have about 25% reduction in 4.1R and about 98% reduction in MMP1 [118]. Red cells of 4.1R knockout ($4.1R^{-/-}$) mice are deficient in GPC and MMP1 [130], and have reduced levels of Rh, Duffy, and Xk proteins as well as possible conformational changes in band 3 and Kell, suggesting the presence of a membrane protein complex linked to the spectrin-actin junction through GPC and 4.1R [14] (Figure 10.2). In $4.1R^{-/-}$ mice GPC sorts to the erythroblast nuclear membrane, rather than the surface membrane, and is lost from reticulocytes during enucleation [131].

The functions of the extracellular domains of GPC and GPD are unknown. Like GPA and GPB, an important function of these heavily sialylated structures could be to contribute to the glycocalyx (see Section 3.23).

18.8 Malaria

The level of invasion of Ge:–2,–3,–4 red cells by the malaria parasite *Plasmodium falciparum* was only 57% of that of Gerbich-positive cells [132]. *P. falciparum* expresses four genes encoding proteins of the Duffy binding-like (DBL) family. These proteins recognise different red cell ligands as a result of mutations in the receptor region of two of the four genes [133]. One of the variants, BAEBL (VSTK) (also known as EBA-140 and PfEBP-2), binds GPC on red cells and could be inhibited from binding to red cells by soluble GPC, but does not bind GPC.Ge, which lacks the product of *GYPC* exon 3 [58,133–136] (although failure to bind Ge:–2,–3 red cells has been disputed [135]). Binding of BAEBL (VSTK) to GPC is dependent on the presence of the single *N*-glycan on GPC, which differs in composition from that on GPC. Ge; neither *N*-deglycosylated GPC nor isolated *N*-glycans inhibited binding of BAEBL (VSTK) to red cells [58]. A selection advantage through the reduced ability of some strains of *P. falciparum* to invade Ge:–2,–3 red cells, could explain the high frequency of this phenotype in parts of PNG, where malaria is endemic, although no evidence for reduced infection of Ge:–2,–3 individuals (as determined by genotyping) was found in a population study [137].

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19 Cromer Blood Group System

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19.1 Introduction

Cromer system antigens are located on the complement regulatory glycoprotein decay-accelerating factor (DAF or CD55), which is attached to the red cell membrane by a glycosylphosphatidylinositol (GPI) anchor. The system includes 18 antigens: 15 of very high frequency; three of low frequency (Table 19.1). All are absent from red cells of the Cromer-null phenotype, the Inab phenotype, an inherited DAF deficiency. Cells lacking the Cromer system antigen Dr^a express the other high frequency Cromer antigens weakly. Complement-sensitive red cells from patients with paroxysmal nocturnal haemoglobinuria (PNH) are deficient in DAF and other GPI-linked proteins and do not express Cromer-system antigens.

CD55, the Cromer gene, is part of the regulator of complement activation cluster on chromosome 1q32, which contains several genes encoding related glycoproteins.

19.2 Decay-accelerating factor (DAF) and the Cromer system

19.2.1 DAF (CD55)

DAF is an intrinsic membrane glycoprotein of red cells, granulocytes, platelets, and lymphocytes, and is widely distributed throughout the body (reviewed in [1,2]). It is also present in soluble form in body fluids including plasma and urine. DAF functions to regulate complement activity (Section 19.5).

DAF is part of a family of glycoproteins anchored to cell membranes by means of the glycophospholipid GPI (reviews in [3,4]). The typical structure of a GPI anchor is shown in Figure 19.1. Other GPI-linked membrane glycoproteins on red cells are acetylcholinesterase (Yt blood group, Chapter 11), ART4 (Dombrock, Chapter 14), CD108 (JMH, Chapter 24), the Emm blood group antigen (Chapter 30), the complement regulatory glycoprotein CD59 (Section 19.5), CD58 (LFA-3), C8-binding protein, and the prion protein, PrP^C. GPI-anchored glycoproteins appear to be preferentially located in lipid rafts, membrane microdomains that float freely in the bilayer and are rich in glycosphingolipids and cholesterol [4,5]. The complement-sensitive red cell population (PNHIII) from patients with the rare, acquired, haemolytic disease paroxysmal nocturnal haemoglobinuria (PNH) are deficient in GPI-linked proteins [6,7] (Section 19.5).

Oligonucleotide probes based on the N-terminal amino acid sequence of DAF were used to isolate cDNA from libraries derived from HeLa epithelial cell line and HL-60 promyelocytic leukaemia cell line [8,9]. The cDNA sequence predicted a 347 amino acid protein preceded by a 34 amino acid N-terminal leader peptide sequence. The DAF polypeptide has four regions of marked homology of about 60 amino acid residues each called complement control protein repeats (CCPs) or short consensus repeats (SCRs). Each CCP domain consists of five β sheets, contains four cysteine residues, and is maintained in a folded conformation by two disulphide bonds [10] (see Figure 20.2). A single *N*-glycan is located between the first two CCPs [11,12]. The rigid stalk linking the four CCP domains to the GPI-anchor of the 70 kDa red cell

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Table 19.1 Antigens of the Cromer system.

Antigen				Molecular basis*		
No.	Name	Frequency	Antithetical antigen	Nucleotides	Exon	Amino acids†
CROM1	Cr^{a}	High		679G (C)	6	Ala227 (Pro)
CROM2	Tc^{a}	High	CROM3, CROM4	155G (T or C)	2	Arg52 (Leu or Pro)
CROM3	Tc^{b}	Low	CROM2, CROM4	155G>T	2	Arg52Leu
CROM4	Tc^{c}	Low	CROM2, CROM3	155G>C	2	Arg52Pro
CROM5	Dr^{a}	High		596C (T)	5	Ser199 (Leu)
CROM6	Es^{a}	High		239T (A)	2	Ile80 (Asn)
CROM7	IFC	High		(Various)		
CROM8	WES^{a}	Low	CROM9	245T>G	2	Leu82Arg
CROM9	WES^{b}	High	CROM8	245T (G)	2	Leu82 (Arg)
CROM10	UMC	High		749C (T)	6	Thr250 (Met)
CROM11	GUTI	High		719G (T)	6	Arg240 (His)
CROM12	SERF	High		647C (T)	5	Pro216 (Lys)
CROM13	ZENA	High		725T (G)	6	His242 (Gln)
CROM14	CROV	High		466G (A)	3	Gln156 (Lys)
CROM15	CRAM	High		740A (G)	6	Gln247 (Arg)
CROM16	CROZ	High		389G (A)	3	Arg130 (His)
CROM17	CRUE	High		639G (A)	5	Leu217 (Trp)
CROM18	CRAG	High		173A (G)	2	Asp58 (Gly)

*Molecular basis of antigen-negative phenotype in parentheses.
†Historically, amino acids were numbered from the first residue of the mature protein, now Asp35.

membrane form of DAF consists of a highly charged, 68 amino acid serine/threonine-rich domain, with 19 predicted *O*-glycosylation sites, and a hydrophobic stretch of 24 amino acids [12] (Figure 19.2).

CD55 (encoding DAF) spans about 40 kb and is organised into 11 exons [13] (Table 19.2). *CD55* belongs to the regulator of complement activation (RCA) cluster of genes, localised to chromosome 1q32, which also contains the genes encoding complement receptor type 1 (CR1, Knops antigen, Chapter 20), CR2, and C4-binding protein [14–17].

19.2.2 Cromer system antigens are located on DAF

In 1987 Spring *et al.* [18] showed that two murine monoclonal antibodies, which are considered Cromer-related because they did not react with Inab phenotype cells and reacted only very weakly with Dr(a–) cells, stained on immunoblots a red cell membrane sialoglycoprotein of apparent MW 70 kDa. This structure was subsequently shown to be DAF [19]. Further confirmation that DAF is the Cromer antigen came from immunoblotting with human alloanti-Cr^{a} and other Cromer system antibodies [18], monoclonal antibody-specific immobilisation of erythrocyte antigens (MAIEA) assays [20,21], tests with recombinant DAF constructs [20–24], and association of mutations in the *CD55* gene with variant Cromer phenotypes (Table 19.1). Red cells of the Cromer-null (Inab) phenotype had only minimal activity with murine monoclonal and rabbit anti-DAF [19,25,26]. PNHIII cells, which are deficient in DAF, did not react with antibodies to high frequency Cromer system antigens [19].

19.3 Inab, the Cromer-null phenotype, and anti-IFC (-CROM7)

The Inab phenotype, a Cromer-null phenotype in which the red cells lack all Cromer system antigens, is very rare. Nine unrelated Inab phenotype individuals have been reported (not including those shown to have a transient Inab phenotype): five Japanese [27–31], two of Moroccan

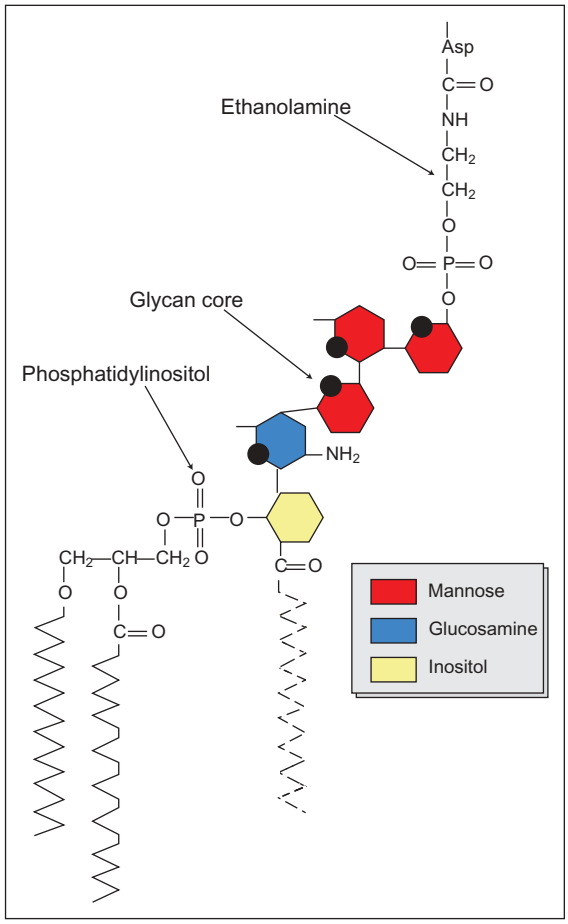


Figure 19.1 Structure of a glycosylphosphatidylinositol anchor. The protein is linked through ethanolamine to a glycan core, attached to phosphatidylinositol, which is embedded in the cell membrane. Three fatty acids are present in red cells. Redrawn from [3], with permission from Taylor & Francis Group.

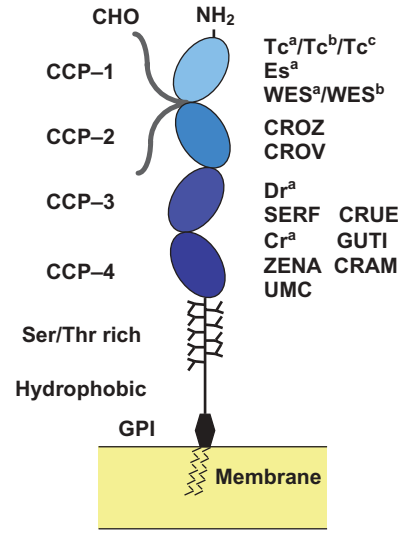


Figure 19.2 Diagrammatic representation of the DAF glycoprotein showing the four complement control protein repeats (CCPs), the *O*-glycosylated serine/threonine-rich region, the hydrophobic region, and the glycosylphosphatidylinositol (GPI) anchor inserted into the cell membrane. Also shown are the locations of the Cromer system antigens on the four CCPs.

descent [32,33], a Jewish American [34], and a white American woman of Italian descent, whose brother also had the Inab phenotype [35]. Homozygosity for four mutations in *CD55* has been reported to be responsible for the Inab phenotype.

1 A nonsense mutation, 261G>A (*CROM*01N.01*), in exon 2 of the original Inab phenotype propositus and in another Japanese propositus: Trp87stop [29,36].

2 A nonsense mutation, 508C>T (*CROM*01N.03*), in exon 4 of another Japanese propositus: Arg170stop [31].

3 A single nucleotide insert, 367A (*CROM*01N.04*) in exon 3, resulting in a reading frameshift and a premature stop codon at Glu128 [32].

4 A 1579C>A change 24 bp upstream of the 3′ end of exon 2 in two Japanese individuals, which creates a novel splice site (TGGT**C**AGA to TGgt**a**aga), giving rise to a 26 bp deletion in the mRNA, resulting a reading frameshift and a translation stop codon immediately downstream of the mutation (*CROM*01N.02*) [28,30].

Sera from eight of the Inab phenotype propositi contained anti-IFC, an antibody reacting with all red cells apart from those of the Inab phenotype [27,29–31, 33–35]. Haemagglutination-inhibition experiments with soluble-recombinant constructs representing different segments of DAF showed that anti-IFC comprises a mixture of antibodies to each of the four CCP domains [29].

There are three reports of patients with a transient Inab phenotype and anti-IFC. On later testing, the antibody had disappeared, or almost disappeared, and DAF expression and Cromer phenotype returned to normal in two patients, and became weakly expressed in the third. One had splenic infarctions [37], one chronic lymphatic leukaemia [38], and one gastrointestinal abnormalities [39]. In one patient, whilst the red cells had the Inab phenotype, lymphocytes, monocytes, granulocytes, and

Table 19.2 Organisation of the *CD55* gene.

Exon	Size (bp)	3′ intron size (kb)	Amino acids*	Region of DAF encoded	Antigens encoded
1		0.5	1–34	5′ untranslated; signal peptide	
2	186	2.3	34–96	CCP-1	$Tc^a/Tc^b/Tc^c$, CRAG, Es^a, WES^a/WES^b
3	192	0.9	96–160	CCP-2	CROZ, CROV
4	100	1.0	160–193	$CCP\text{-}3_A$	
5	86	4.3	193–222	$CCP\text{-}3_B$	Dr^a, SERF, CRUE
6	189	5.4	222–285	CCP-4	Cr^a, GUTI, ZENA, CRAM, UMC
7	126	0.6	285–327	$Ser/Thr\ rich_A$	
8	81	1.9	327–354	$Ser/Thr\ rich_B$	
9	21	1.2	354–361	$Ser/Thr\ rich_C$	
10	118	19.8	(361–400)	*Alu* (alternatively spliced)	
11	956		361–381	Hydrophobic; 3′ untranslated	

Table 19.3 Some population studies on high frequency Cromer antigens.

Antigen	Population	No. tested	No. negative	References
Cr^a	African Americans	>4000	0	[42]
	African Americans	8858	2	[43]
Tc^a	African Americans	950	1	[44]
	White Americans	5000	0	[44]
	Japanese	5000	0	[44]
Es^a	American donors	3400	0	[45]
UMC	Japanese	45 610	0	[46]
GUTI	American donors	>1000	0	[47]
SERF	Thai donors*	1041	1	[48]

*Tested by PCR-RFLP.

platelets expressed CD55, albeit at a lower level than cells of common phenotype [37].

Four of the nine propositi with non-transient Inab phenotype, plus an African American boy who probably had the Inab phenotype [29], had intestinal disorders including protein-losing enteropathy [27], Crohn's disease [34], blood capillary angioma of the small intestine [29], and a chronic progressive gastrointestinal disorder resulting from Budd-Chiari syndrome, a blockage of the hepatic vein [33]. One of the patients with transient Inab phenotype, an 18-month-old boy, had significant gastro-oesophageal reflux and milk and soy intolerance [39]. DAF is present on the epithelial surface of intestinal mucosa [40], but any suggestion of an association between DAF-deficiency and intestinal disease is offset by the absence of any such disorder in two of the Japanese propositi [28,31] and in an 86-year-old Inab phenotype woman and her 70-year-old brother [35]. Mice deficient in the major form of DAF, resulting from a *Daf1* knockout, are more susceptible than control mice to colitis induced by dextran sulphate sodium [41]. Any conclusive evidence for an association between DAF deficiency and inflammatory gastrointestinal disease in humans remains elusive.

19.4 Cromer system antigens and antibodies

19.4.1 Cr^a (CROM1)

In 1965 McCormick *et al.* [42] described an antibody in the serum of an African American antenatal patient, Mrs Cromer, which reacted with red cells of more than 4000 African American donors (Table 19.3), but not with her

own red cells or with those of two of her siblings. Stroup and McCreary [49] recognised a possible serological association between anti-Cr^a and the antibody now called anti-Tc^a. Many more examples have been found in people of African origin, and one in a Spanish American [50].

DAF cDNA deletion-mutants lacking the regions encoding each of the four CCPs were used to transfect Chinese hamster ovary cells [51]. Anti-Cr^a reacted on immunoblots with lysates from these transfected cells with the single exception of those transfected with the cDNA lacking the region encoding CCP-4. Sequencing of genomic DNA from three Cr(a–) individuals revealed a 679G>C change encoding an Ala227Pro in CCP-4 (*CROM*–01*) [22].

19.4.2 Tc^a (CROM2), Tc^b (CROM3), Tc^c (CROM4), and Tc^{a}Tc^b

Two antibodies of identical specificity, shown to be related to anti-Cr^a through common absence from Inab phenotype cells [27], were named anti-Tc^a when a third example was described [44]. One Tc(a–) individual was found as a result of testing red cells from 950 African American donors with anti-Tc^a (Table 19.3).

Anti-Tc^b reacted with red cells of about 6% of African Americans and family studies showed that *Tc^a* and *Tc^b* are alleles. From the results of testing 350 African American donors with anti-Tc^b the following gene and genotype frequencies were calculated: *Tc^a* 0.97, *Tc^b* 0.03; *Tc^a/Tc^a* 0.941, *Tc^a/Tc^b* 0.058, *Tc^b/Tc^b* 0.001 [52].

Red cells of a Tc(a–b–) white woman and her sister, neither of whom had the Inab phenotype, were found to have a low frequency antigen, which was subsequently named Tc^c [53]. Both parents and three of four other siblings were Tc(a+b–c+). Six months after the delivery of a Tc(a+) child, the serum of the Tc(a–b–c+) propositus contained an antibody that represents inseparable anti-Tc^{a}Tc^b; it reacted with neither her own cells nor with those of her Tc(a–b–c+) sister, but did react with Tc(a–b+c–) and Tc(a+b–c–) cells [53]. A second example of anti-Tc^{a}Tc^b has been found in a Tc(a–b–) white woman [54]. Another anti-Tc^c was identified in a Tc(a+c–) pregnant woman with a Tc(a+c+) husband [55].

Arg52Leu and Arg52Pro substitutions in CCP-1, are responsible for Tc^b and Tc^c expression, respectively (*CROM*01.03* and *CROM*01.04*) [22,56,57] (Table 19.1). The *Tc^b* mutation creates a *Stu*I restriction site and both *Tc^b* and *Tc^c* mutations destroy an *Rsa*I site.

19.4.3 Dr^a (CROM5)

Most reported examples of anti-Dr^a have been found in Jews of Uzbekistani origin [58–62], although other examples were found in a Russian woman [36,63] and a Japanese blood donor [29].

In addition to lacking Dr^a, Dr(a–) red cells have weak expression of all other high frequency Cromer system antigens as they have only 40% of normal expression of cell surface DAF [64]. Immunoblotting revealed no gross alteration in Dr(a–) DAF, but did confirm the quantitative difference [64,65].

Dr(a–) phenotype results from homozygosity for 596C>T in exon 5 of *CD55* encoding Ser199Leu within CCP-3 (*CROM*01.–05*) [36,64] (Table 19.1) This mutation results in the loss of a *Taq*I restriction site. Sequencing of cDNA derived from Dr(a–) individuals revealed two *DAF* transcripts: a minor one encoding full-length DAF containing the Ser165Leu substitution and a more abundant form having a 44 nucleotide deletion, which introduces a reading frameshift and the generation of a premature stop codon six codons downstream from the deletion. Any polypeptide produced by the major transcript would consist of an N-terminal leader sequence plus 165 amino acid residues, but lacking the remainder of the molecule, including the GPI anchor [36]. Only DAF encoded by the minor transcript is present at the cell surface, explaining the low levels of DAF and weak expression of the Cromer antigens. The C>T mutation responsible for the loss of Dr^a creates a cryptic splice site 44 nucleotides upstream of intron 4 so that 44 nucleotides of exon 3 are spliced-out of the majority of the mRNA molecules together with intron 4. Site-directed mutagenesis experiments confirmed that the 596C>T change, without the 44 nucleotide deletion, was responsible for loss of Dr^a antigen expression [64].

19.4.4 WES^a (CROM8) and WES^b (CROM 9)

WES^a was detected on the red cells of 61 of 10 982 (0.56%) Finns [66], two of 1610 (0.12%) white Americans [67], seven of 1460 (0.48%) African Americans [67], and five of 245 (2.04%) black North Londoners [68].

Only two examples of antibodies to the high frequency antigen WES^b are known, both found in the sera of black women with WES(a+) red cells [68,69]. The only other known *WES^a* homozygote was a Finnish woman with six WES(a+) and no WES(a–) children [68].

The WES^b/WES^a polymorphism results from Leu82Arg in CCP-1 of DAF [57] (Table 19.1). The *WES^a* allele (*CROM*01.08*) lacks an *Afl*II restriction site. In a MAIEA

assay, two murine monoclonal antibodies to epitopes on CCP-1 blocked binding of anti-Tc^a, whereas a third did not. The opposite result was obtained with the same monoclonal antibodies and anti-WES^b, suggesting that Tc^a and WES^b are on opposing faces of CCP-1 [21]. These results support the molecular model of Kuttner-Kondo *et al.* [10].

19.4.5 Other high frequency Cromer antigens

Eight other Cromer antigens have been described, all of very high frequency. Antigen-negative phenotypes all result from homozygosity for single nucleotide changes encoding amino acid substitutions in DAF [32,47,57,70, 71] (Table 19.1 and Figure 19.2). Some frequency studies are listed in Table 19.3.

19.4.5.1 Es^a (CROM6)

Three examples of anti-Es^a and three Es(a–) propositi are known: a woman of Mexican descent with two Es(a–) siblings [45], a Jewish woman of Tunisian origin with an Es(a–) brother [72], and an African American man [73]. Anti-WES^b reacts slightly less strongly with Es(a–) cells than with Es(a+) cells, whereas two examples of WES(a+b–) cells reacted only very weakly with anti-Es^a, requiring adsorption/elution tests for detection [68]. The close proximity between the amino acid substitutions associated with Es(a–) and that responsible for the WES^b/WES^a polymorphism (positions 80 and 82) explains the serological interaction between Es^a and WES^b.

19.4.5.2 UMC (CROM10)

The only known UMC– propositus is a Japanese blood donor detected during screening for donor antibodies in northern Japan [46]. One of her three siblings was also UMC–.

19.4.5.3 GUTI (CROM11)

Anti-GUTI was found in a previously transfused Chilean man, with a GUTI– sister [47]. Application of a *Mae*II PCR-RFLP assay revealed six heterozygotes for the GUTI mutation among 114 Chilean Mapuche Indians (allele frequency 2.6%).

19.4.5.4 SERF (CROM12)

Anti-SERF was identified in a pregnant Thai woman [70]. A *Bst*N1 PCR-RFLP assay revealed 21 heterozygotes and one homozygote for the SERF mutation among 1041 Thai donors (allele frequency 1.1%) [48].

19.4.5.5 ZENA (CROM13)

Anti-ZENA was identified in a Syrian Turkish antenatal patient [71]. A *Bsr*1 PCR-RFLP assay revealed no alleles with the ZENA mutation among 150 Israeli donors.

19.4.5.6 CROV (CROM14)

Anti-CROV was identified in a Croatian woman who had been pregnant three times [71]. A *Taq*1 PCR-RFLP assay revealed no alleles with the CROV mutation among 100 Croatians.

19.4.5.7 CRAM (CROM15)

Anti-CRAM was identified in a Somali woman in her third pregnancy [71]. CRAM– red cells, with an amino acid substitution at position 247, have weakened expression of GUTI (amino acid 240), but normal expression of the other antigens on CCP-4: ZENA (242), Cr^a (227), and UMC (250). GUTI– cells have weakened expression of CRAM.

19.4.5.8 CROZ (CROM16)

Anti-CROZ was identified in a 78-year-old Australian woman [32]. CROZ– is associated with Arg130His. Arg130 plays an important role in DAF functional activity: Arg130Ala and Arg130Leu substitutions synthesised by site-directed mutagenesis, abolished and significantly reduced C3 convertase activity, respectively [74].

19.4.5.9 CRUE (CROM17)

Anti-CRUE was found in a Thai woman heterozygous for two rare alleles with mutations in exon 5:

1 650T>G, Leu217Trp, presumably responsible for the CRUE– phenotype; and

2 639G>A, Trp213stop, an inactivating mutation that would be predicted to produce no DAF which would be present in the membrane [75].

19.4.5.10 CRAG (CROM18)

Anti-CRAG was found in a woman of Greek ancestry with Leu82Pro in CCP-1 [76]. She was transfused with three incompatible units of blood from random donors, with no adverse consequences.

19.4.6 Other serological characteristics of Cromer system antigens

Cromer system antigens are readily destroyed by treatment of the red cells with α-chymotrypsin, but not by trypsin, papain, ficin, or sialidase, and in this way are easily distinguished from virtually all other blood group antigens. Treatment of intact red cells with the disulphide

bond reducing agents AET and DTT results in only slight weakening of the Cromer antigens. This is surprising, considering that each CCP domain is maintained in its folded configuration by two disulphide bonds (Section 19.2.1).

Haemagglutination-inhibition has demonstrated that Cromer system antigens are present in serum and urine of individuals with the corresponding antigen on their red cells [35,44,46,59,66,68,77]. Anti-Cr^a can be readily removed from sera by adsorption with platelet concentrates [78,79].

19.4.7 Clinical significance of Cromer system antibodies

Cromer system antibodies are mostly IgG, though IgM anti-Cr^a is reported [80]. IgG1 generally predominates, but Cromer system antibodies of all four subclasses have been reported [60–62,66,73,80–83].

Cromer system antibodies are not usually considered clinically significant and there are many reports of successful transfusion of incompatible red cells to patients with anti-Cr^a and -Tc^a [50,84–86]. Anti-Cr^a [85] and -Tc^a [87], however, have been blamed for clinical transfusion reactions, with the anti-Tc^a destroying six units of Tc(a+) red cells, three of them within a day of transfusion. Conclusions from *in vivo* red cell survival studies and *in vitro* functional assays with Cromer system antibodies have varied, some suggesting that the antibodies are of no clinical importance [50,60,61,78,80,83–85,88,89] and others predicting reduced survival of transfused incompatible red cells [34,44,61,73,79,82,83,85,87,90–92]. Only 38% of radiolabelled IFC+ red cells survived 24 hours after injection into an Inab phenotype man with anti-IFC [34] and another example of anti-IFC removed all IFC+ cells within 15 minutes of injection [90]. Anti-Tc^a comprising IgG1, IgG2, and IgG4 gave results in the monocyte monolayer assay (MMA) suggestive of clinical significance; two years later the serum contained only IgG2 and IgG4, and the MMA and *in vivo* red cell survival tests suggested that incompatible transfusion would be well tolerated [83]. In most cases, least incompatible red cells may be selected for transfusion to patients with Cromer system antibodies, although, if possible, antigen-negative red cells should be selected for strong examples of the antibody. Transplantation of a Dr(a+) kidney into a Dr(a–) patient with IgG2 plus IgG4 anti-Dr^a was successful, with good graft function and no increase in titre of the antibody [60].

Despite the indications that Cromer system antibodies often have the potential to be haemolytic, these antibodies are never responsible for clinical signs of HDFN. DAF is present on placental trophoblast epithelial cells derived from the fetus [93]. It is common for strongly reactive Cromer antibodies (anti-Cr^a, -Dr^a, -WES^b, -CRAM) to become weakly reactive or undetectable in maternal plasma during the second and third trimesters of pregnancy, only to reappear shortly after parturition [42,61,69,71,78]. In one case anti-Cr^a could be eluted from the placenta [94]. It is likely, therefore, that maternal Cromer system antibodies become absorbed by the placenta, protecting the fetus from the antibodies.

19.4.8 Monoclonal antibodies

Numerous rodent monoclonal antibodies to DAF have been produced, defining epitopes on each of the four CCPs [13,24,51,95]. They generally behave like anti-IFC as they do not react with red cells of the Inab phenotype and react only very weakly with Dr(a–) cells. Human monoclonal anti-IFC was produced from lymphocytes of an individual with Inab phenotype [30].

19.5 Functional aspects DAF and CD59: GPI-linked complement-regulatory proteins

DAF protects cells from complement-mediated damage by inhibiting the amplification stage of complement activation. DAF inhibits association and accelerates dissociation of C4b2a and C3bBb, the C3 convertases of the classical and alternative pathways, respectively. Classical pathway C3 convertase regulatory function resides within CCP-2 and -3, whereas alternative pathway regulatory function resides within CCP-2, -3, and -4 [96]. DAF has a wide distribution in the body. It is present on granulocytes, monocytes, and lymphocytes [1], and on many epithelial cells, including placental trophoblast epithelium, where it plays a role in protecting the fetus from maternal complement-mediated attack [93].

CD59, also known as the membrane inhibitor of reactive lysis (MIRL), is a complement-regulatory glycoprotein of the Ly-6 superfamily. It inhibits complement-mediated haemolysis by binding to C8 and C9 and preventing assembly of the membrane-attack complex. CD59 is present on red cells, but does not have blood group activity. Like DAF, CD59 is attached to the red cell membrane by a GPI anchor. For reviews on CD59 see [3,97,98].

Paroxysmal nocturnal haemoglobinuria (PNH), a disease characterised by intravascular haemolysis, venous

thrombosis, and blood cytopenias, is caused by somatic mutations in *PIGA*, an X-linked gene that encodes a subunit of an enzyme essential for the biosynthesis of the GPI anchor. Over 100 different mutations, most of which are small insertions or deletions, have been identified, and occur within haemopoietic stem cells [6]. Somatic cells contain only one active X-chromosome, and consequently only one active *PIGA* gene. For the disease to manifest, the affected clone must expand at the expense of normal cells. Healthy people have low numbers of *PIGA* mutated cells, but in PNH an autoimmune process develops in which cytotoxic T cells of the patient target GPI-linked proteins on normal blood cells and selectively destroy the normal blood cell population, leaving the mutated clone, which lack GPI-linked proteins, to proliferate [7,99].

The affected red cells in PNH patients (PNHIII cells) are deficient in all GPI-linked proteins, including DAF and CD59, and can be lysed, *in vitro*, by acidified human serum, a process that involves the activation of the alternative complement pathway. Despite DAF deficiency, red cells of the Inab phenotype show no evidence of haemolysis and Inab phenotype is not associated with any symptoms of haematological disease [25,26,28,29]. Unlike PNH cells, Inab phenotype cells are not lysed by acidified serum or by cobra venom and they are only slightly more susceptible to lysis than normal cells in standard complement-mediated lysis tests, such as lysis in the presence of cold antibody or sucrose. Direct antiglobulin tests on Inab phenotype cells with antibodies to human complement components demonstrated that there is no accumulation of C3 fragments on Inab phenotype cells, as might have been expected in the absence of a C3 convertase inhibitor [25,26]. When CD59 has been inactivated by the addition of monoclonal anti-CD59, however, Inab phenotype red cells are haemolysed by acidified human serum [29,100]. DAF and CD59 share the role of protecting red cells from the activity of autologous complement. CD59 appears more effective than DAF in this respect: a patient with red cell CD59 deficiency resulting from homozygosity for single base deletion within the CD59 gene, but with normal levels of DAF and other GPI-linked glycoproteins, had a mild PNH-like haemolytic anaemia [101,102]. In a complement-mediated lysis sensitivity (CLS) test, the following scores (in CLS units) were obtained: DAF-deficient (Inab) red cells, 4.6; CD59-deficient red cells, 11.7; DAF- and CD59-deficient (PNHIII) red cells, 47.6 [103].

Decreased levels of CD55 and, possibly, CD59 may be a cause of anaemia in children infected with the malarial parasite, *Plasmodium falciparum* [104]

The first CCP domain of DAF, which is not involved in complement regulation, is a ligand for CD97, present on monocytes and granulocytes, and up-regulated on the activation of T and B cells [105]. The function of this interaction is not known.

19.6 DAF as a receptor for pathogenic microorganisms

Like globoside, the P antigen (Section 4.10.1), DAF is exploited as an attachment site on epithelial cells for strains of *Escherichia coli* associated with urinary tract infection, cystitis, and protracted diarrhoea [106]. Fimbriae from 075X-positive *E. coli* agglutinated red cells *in vitro*, with the exception of those with the Inab and Dr(a–) phenotypes [107]. The 075X and other fimbria-like adhesins that bind to Dr^a are referred to as Dr adhesins. *E. coli* and purified Dr adhesins bound Chinese hamster ovary (CHO) cells transfected with normal *DAF* cDNA, but not untransfected cells or cells transfected with *DAF* cDNA encoding the Ser199Leu substitution associated with the Dr(a–) phenotype [108]. In addition to Ser199, Ser189 and, to a lesser extent, Tyr194 and Leu196, are important in binding Dr adhesin, inferring that one loop in CCP-3 is involved in anchorage and subsequent internalisation of Dr-fimbriated *E. coli* by epithelial cells [109,110].

DAF is a ligand for many picornaviruses, including echoviruses and coxsackieviruses, which cause a range of symptoms including diarrhoea, aseptic meningitis, and severe respiratory disease in neonates [2,105]. Some echoviruses are capable of agglutinating red cells [111]. Each of the four CCP domains of DAF are exploited by different viruses [105].

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20 Knops Blood Group System and the Cost Antigens

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20.1 Introduction

The Knops system consists of nine antigens, including three pairs of antithetical antigens (Table 20.1). These antigens are defined by clinically insignificant antibodies that are notoriously difficult to identify. They are all located on complement receptor 1 (CR1, CD35), a member of the complement control protein superfamily, and associated with nucleotide changes in *CR1*. The Helgeson phenotype appears, by conventional serological methods, to be a Knops-null phenotype, though very low levels of CR1 are present on the red cells.

CR1 is part of the regulators of complement activity gene cluster on chromosome 1q32.

Cs^a, an antigen of moderately high frequency, is related serologically to Yk^a, but does not appear to be on CR1. Cs^a and Cs^b comprise the Cost collection (Collection 205, Section 20.8).

20.2 Complement receptor 1 (CR1) and the Knops system

20.2.1 CR1 (CD35)

CR1 is a glycoprotein of about 200 kDa present on red cells, granulocytes, monocytes, B lymphocytes, a subset of T cells, glomerular podocytes, and follicular-dendritic cells in lymph nodes (reviews on CR1 in [1–4]). A soluble form of CR1 (sCR1) is present in plasma. The primary structure of the CR1 polypeptide has been elucidated from the cDNA sequence [5–7]. The most common allotype of CR1 (CR1*1) consists of 2039 amino acids, including a 41 amino acid N-terminal signal peptide (cleaved from the mature protein), a 1930 amino acid extracellular domain, a 25 amino acid transmembrane region, and a 43 amino acid cytoplasmic domain. Like some other complement regulatory proteins, including decay-accelerating factor (Chapter 19), the extracellular domain is organised into regions of amino acid sequence homology, each comprising about 60 residues, called complement control protein repeats (CCPs) or short consensus repeats (SCRs). The extracellular domain of the CR1*1 allotype consists of 30 CCPs (Figure 20.1). Each CCP domain contains four cysteine residues and is maintained in a folded conformation by two disulphide bonds (Figure 20.2). Further homology divides the N-terminal 28 CCPs into four regions called long homologous repeats (LHRs), each comprising seven CCPs.

Four allotypes of CR1 of different molecular weight have been identified: the common CR1*1 allotype (190 kDa under non-reducing conditions), the less common CR1*2 allotype (220 kDa), and the rare CR1*3 (160 kDa) and CR1*4 (250 kDa) allotypes [1–4,8]. These allotypes differ by the numbers of LHRs making up the extracellular domain and may have arisen as a result of intragenic unequal crossing-over [9]. The number of CR1 molecules per red cell differs considerably from person to person,

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Table 20.1 Antigens of the Knops system.

Antigen				Molecular basis*		
No.	Name	Frequency	Antithetical antigen	Nucleotides	Exon	Amino acids
KN1	Kn^a	High	KN2	4681G (A)	29	Val1561 (Met)
KN2	Kn^b	Low	KN1	4681G>A	29	Val1561Met
KN3	McC^a	High	KN6	4768A (G)	29	Lys1590 (Glu)
KN4	Sl1	High	KN7	4801A (G)	29	Arg1601 (Gly)
KN5	Yk^a	High		4223C (T)	26	Thr1408 (Met)
KN6	McC^b	Low	KN3	4768A>G	29	Lys1590Glu
KN7	Sl2	Low	KN4	4801A>G	29	Arg1601Gly
KN8	Sl3	High		4828T (A)	29	Ser1610 (Thr)†
KN9	KCAM	§		4843A (G)	29	Ile1615 (Val)

*Molecular basis of antigen-negative phenotype in parentheses.
†Arg1601 also required for KN8 (Sl3) expression.
§High in Caucasians, relatively low in Africans.

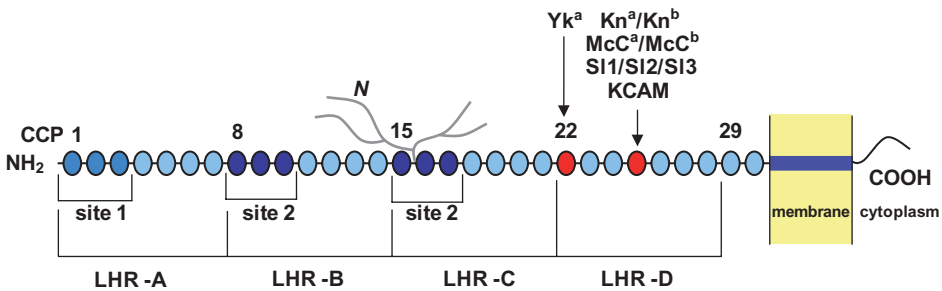


Figure 20.1 Diagrammatic representation of the most common allotype of CR1 (*CR1*1*), showing the 30 complement control protein repeats (CCP), the four long homologous repeats (LHR), one of the 6–8 *N*-linked oligosaccharides (*N*), the transmembrane region, and the cytoplasmic domain, the active sites (site 1 and the duplicated site 2), the position of the Knops polymorphisms in CCP-22 and CCP-25.

varying from 20 to over 800 [10]. This quantitative polymorphism is independent of the size polymorphism and is associated with several SNPs in *CR1*, including one responsible for a *Hind*III RFLP in white people, but not in African Americans (for reviews on CR1 polymorphisms see [4,11]).

There are 25 potential sites for *N*-glycosylation [6], but only 6–8 *N*-glycans per molecule [1]. CR1 is not *O*-glycosylated [12].

The 133–160 kb *CR1* gene is located on chromosome 1q32, within the regulator of complement activity (RCA) cluster (see Section 19.2.1). *CR1* is organised into 39 exons (*CR1*1* allele) or 47 exons (*CR1*2*). Each LHR is represented by 8 exons. In each LHR, CCPs 1, 5, and 7 are encoded by one exon each; CCPs 2 and 6 by two exons each; and CCPs 3 and 4 by a single exon [9,13] (Table 20.2). The major transcription start site is 111 bp upstream of the translation-initiating ATG codon [9].

20.2.2 Knops system antigens are located on CR1

In 1991, Rao *et al.* [14] and Moulds *et al.* [15] independently demonstrated that Knops system antigens are situated on CR1. Immunoprecipitation of radiolabelled red cell membrane proteins with anti-Kn^a, -McC^a, -Sl1 (Sl^a then), and -Yk^a produced bands on SDS polyacrylamide

gels identical to those produced by precipitation with monoclonal anti-CR1. Furthermore, after immunoprecipitation with monoclonal anti-CR1, affinity purified CR1 could be detected on immunoblots with human anti-Kn/McC serum [14]. When both CR1*1 and CR1*2 allotypes were present, two bands were detected with the monoclonal and human antibodies [14,15]. The location of Kn^a, McC^a, Sl1, and Yk^a on CR1 was confirmed by neutralisation of the corresponding antibodies with soluble, recombinant CR1 [16] and by the MAIEA assay with monoclonal anti-CR1 [17]. All Knops polymorphisms have now been shown to be associated with single nucleotide polymorphisms in *CR1* (Section 20.4 and Table 20.1). In contrast, the immunochemical methods described above gave negative results with anti-Cs^a, suggesting that Cs^a is not on CR1 [15–17].

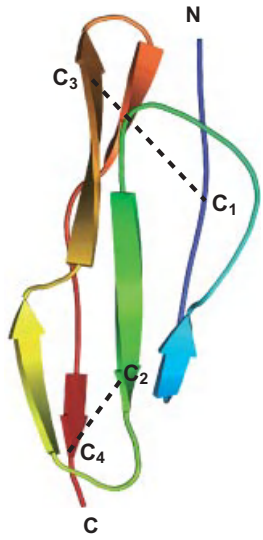


Figure 20.2 Model of a CCP domain, showing the five β sheets and four conserved cysteine residues (C) forming two disulphide bonds (dashed lines) (Provided by N. Burton.)

20.3 Helgeson, a mod phenotype in the Knops system

The major serological characteristic that led to the Knops, McCoy, and Sl antigens being ranked together has been their apparent absence from the red cells of one of the discoverers of Kn^a, Margaret Helgeson (M.H.), and from other red cells of the same phenotype [18–22]. Apparent absence of Yk^a from these cells was demonstrated later [10]. In fact, Helgeson phenotype cells do not represent a true Knops-null phenotype because they express very low levels of Knops antigens [10,14,15], and may even be agglutinated in antiglobulin tests by the most potent examples of Knops antibodies [10]. Consequently, Helgeson phenotype is not associated with an antibody to a generic Knops system antigen. The Helgeson phenotype has an incidence of about 1% in Caucasians and African Americans [21,22].

Red cells of the Helgeson phenotype have a very low number of CR1 molecules per red cell, approximating

Table 20.2 Domains of CR1 encoded by the 39 exons of *CR1*1*.

LHR-A		LHR-B		LHR-C		LHR-D			
Exon	CCP	Exon	CCP	Exon	CCP	Exon	CCP	Exon	Domain
2	1	10	8	18	15	26	22	34	29
3	2a	11	9a	19	16a	27	23a	35	30
4	2b	12	9b	20	16b	28	23b	36	TMa
5	3,4	13	10,11	21	17,18	29	24,25	37	TMb
6	5	14	12	22	19	30	26	38	cyto
7	6a	15	13a	23	20a	31	27a	39	3′ UT
8	6b	16	13b	24	20b	32	27b		
9	7	17	14	25	21	33	28		

Exon 1 encodes the leader peptide.
LHR, long homologous repeat; CCP, complement control protein domain; TM, transmembrane domain; cyto, cytoplasmic domain; UT, untranslated.

Table 20.3 Frequencies of Knops antigens.

Antigen	Population	No. tested	No. positive	Antigen frequency	References
Kn^a	Caucasians	4553	4498	98.8%	[18,21]
	African Americans	894	883	98.8%	[21]
Kn^b	Caucasians	229	10	4.4%	[24]
McC^a	Caucasians	3860	3802	98.5%	[19,21]
	African Americans	1539	1461	94.9%	[19,21]
McC^b	African Americans	371	168	45.3%	[25]
Sl1	Caucasians	833	815	97.8%	[20,22]
	African Americans	480	257	53.5%	[20,22]
Yk^a	Caucasians	2889	2598	89.9%	[21]
	African Americans	1117	1098	98.3%	[21]

10% of normal [10,14,15]. Knops antigens could be detected on CR1 of Helgeson phenotype cells by immunoprecipitation, flow cytometry, or MAIEA [14,15,17]. Levels of CR1 were often normal on red cells of individuals who lacked only one of the Knops system antigens and who had made the corresponding antibody [15]. Expression of Knops antigens, as detected by an antiglobulin test, correlates strongly with the number of CR1 molecules per red cell. Cells with between 20 and 100 CR1 molecules are negative with Knops antibodies by the antiglobulin test (Helgeson phenotype), cells with 100–150 molecules are weak or negative depending on the antibody used, and cells with more than 200 molecules are generally positive with all antibodies tested [10]. Helgeson phenotype appears to result from inherited low copy number of CR1, whereas absence of single antigens in individuals who may make the corresponding antibody results from mutations within the *CR1* gene. There is some, but not complete, correlation between the Helgeson phenotype and the polymorphic genotypes associated with low CR1 copy number, including that responsible for the *Hind*III restriction site [23]. Helgeson phenotype red cells have significantly lower CR1 copy number than the more common 'low copy number' phenotypes.

20.4 Antigens of the Knops system

Knops antigens and their molecular backgrounds are listed in Table 20.1 and some antigen and allele frequencies in Tables 20.3 and 20.4. All the Knops polymorphisms are associated with SNPs in exon 29 of *CR1*, encoding amino acid changes in CCP-25, except Yk^a, with a SNP in exon 26, encoding an amino acid change in CCP-22.

20.4.1 Kn^a and Kn^b (KN1 and KN2)

Kn^a, the original Knops antigen first reported in 1970 [18], has an incidence of about 98–99%, although any antigen frequency study in the Knops system will be compromised by the presence of Helgeson alleles. An antibody in a serum containing anti-Kp^a, which reacted with red cells of 4.2% of Kp(a–) Australian blood donors, was considered to be anti-Kn^b because it reacted with virtually all Kn(a–) McC(a+) red cell samples, but with no Kn(a–) McC(a–) samples [24]. No other examples of anti-Kn^b are reported.

The Kn^a/Kn^b (*KN*A/KN*B*) substitution represents Val1561Met in CR1 [26].

20.4.2 McC^a and McC^b (KN3 and KN6)

McC^a (McCoy) was identified and shown to be associated with Kn^a by Molthan and Moulds [19]. Although Kn^a and McC^a have frequencies well in excess of 90%, 53% of McC(a–) individuals were also Kn(a–). The frequency of Mc(a–) is 1–2% in white Americans, but varies between 90 and 97% in different surveys of African Americans and West Africans [19,21,32]. Anti-McC^b is antithetical to anti-McC^a in black people [25,29]: 45.3% of black donors are McC(b+), which includes all Kn(a+) McC(a–), but no Kn(a–) McC(a–) individuals.

The McC^a/McC^b polymorphism is associated with Lys1590Glu [29]. Discrepancies between phenotype and genotype in about 6% of samples were accounted for mainly by genes encoding low CR1 copy number.

Table 20.4 Frequencies of some Knops alleles.

Population	Alleles										Refs
	Kn^a	*Kn*^{b}	*McC*^{a}	*McC*^{b}	*Sl1*	*Sl2*	*Yk*^{a}	*Yk*^{b*}	*KCAM*$^+$	*KCAM*$^-$	
Caucasians	0.99	0.01	0.99	<0.01	0.99	<0.01	0.71	0.29			[26,27]
European Brazilians	0.97	0.03	0.98	0.02	0.97	0.03			0.76	0.24	[28]
Malians	0.90	0.10	0.69	0.31	0.31	0.69					[26]
West Africans			0.69	0.31	0.21	0.79					[29]
Africans									0.1	0.9	[30]
African Americans	0.98	0.02	0.78	0.22	0.48	0.52					[26]
African Brazilians	0.99	0.01	0.76	0.24	0.42	0.58			0.32	0.68	[28]
Chinese Han	1.00	0	1.00	0	1.00	0			0.82	0.18	[31]
Asian Brazilians	1.00	0	1.00	0	1.00	0			0.79	0.21	[28]

*Yk^b represents absence of Yk^a as anti-Yk^b has not been found.

Recombinant soluble CR1 (sCR1) containing Lys1590 inhibited anti-McC^a, but not anti-McC^b, whereas sCR1 containing Glu1590 inhibited anti-McC^b, but not anti-McC^a.

20.4.3 Sl1, Sl2, and Sl3 (KN4, KN7, and KN8)

Sl1 (previously named Sl^a, Swain-Langley, and McC^c) is an antigen of high incidence in white people, but of distinctly lower frequency in black people [20,22]. McC(a–) black people are Sl1–, whether they are Kn(a–) or Kn(a+), but 45% of Kn(a+) McC(a+) African Americans are also Sl1–. Kn(a–) McC(a–) white people are also Sl1–, but only 1% of Kn(a+) McC(a+) white people were Sl1– and all four Kn(a+) McC(a–) individuals found in testing 722 white donors were Sl1+. Sl1– has a frequency of around 70% in West Africans [32].

Anti-Sl2 (originally called anti-Vil) appeared to be antithetical to anti-Sl1 when testing red cells from black people, but no Sl2+ white person was found [20].

The Sl1/Sl2 polymorphism is associated with Arg1601Gly in CR1 [29]. Discrepancies between phenotype and genotype in about 12% of samples were mainly accounted for by genes encoding low CR1 copy number. Soluble CR1 containing Arg1601 inhibited anti-Sl1, except when it also contained Glu1590 (McC^b), but not anti-Sl2. The change from a positively charged lysine to a negatively charged glutamic acid at position 1590 probably affects the conformation of the Sl1 epitope around position 1601 [29]. McC(a–) Sl(a+) phenotype is not found in black people [22,25,29]. Soluble CR1 containing Gly1601 inhibited anti-Sl2, but not anti-Sl1.

The specificity previously named anti-Sl^a was subdivided when an Sl(a+) donor was found to have an apparent anti-Sl^a [33]. Her red cells reacted with six anti-Sl^a, renamed anti-Sl1, but not with two anti-Sl^a, renamed anti-Sl3. She was homozygous for 4810A (Arg1601), typical of Sl1+, but also homozygous for 4828T>A, encoding Ser1610Thr. Consequently, it appears that Sl3 is a conformational epitope requiring both Arg1601 and Ser1610. No homozygotes for 4828A, encoding Sl3– phenotype, were found in 89 Caucasians, 41 black Africans, or 86 Asian Americans, but allele frequencies for 4828A of 0.04, 0, and 0.01 were found in those three populations, respectively [33].

20.4.4 Yk^a (KN5)

Yk^a was described as a new antigen related to Cs^a because the original anti-Yk^a (York) failed to react with two Cs(a–) samples, yet the red cells of Mrs York were Cs(a+) [34]. The frequency of the *Yk*^{a} allele, calculated from the data shown in Table 20.3, is 0.6826 in white Americans and 0.8696 in African Americans [21], and by molecular testing is 0.71 in Caucasians [27].

The incidence of the Cs(a–) Yk(a–) phenotype in white Americans is 1.63% [21]. If there were no association between Cs^a and Yk^a, the expected phenotype frequency would be 0.48%, about three times less than that observed. Cs(a–) Yk(a–) has an incidence of 0.35% in African Americans [21], about 17 times that expected

assuming no association. Of McC(a–) white people, 37% were Yk(a–) and 29% Cs(a–); 2.2% of McC(a–) black people were Yk(a–) and 17% were Cs(a–) [19]. These figures differ substantially from those expected if no relationship existed between McC^a, Yk^a, and Cs^a; 100 times more McC(a–) donors were Yk(a–) Cs(a–) Kn(a–) than would be predicted from the frequencies of these antigens. Although the original Helgeson phenotype cells (M.H.) are Yk(a–) Cs(a–), three other examples were Yk(a–) Cs(a+) [10].

Yk(a–) results from homozygosity for 4223C>T in exon 26 of *CR1*, encoding Thr1408Met in CCP-22 [27].

20.4.5 KCAM (KN9)

The original anti-KCAM was initially considered to be anti-McC^a, but recognises the product of a SNP encoding Ile1615 in KCAM+ and Val1615 in KCAM– [30]. Four of nine anti-McC^a and six of 19 anti-'Kn/McC' also appeared to have KCAM specificity. Serological tests do not appear to show reliable concordance between phenotype and genotype [35]. Frequencies of KCAM predicted from molecular genotyping are as follows (Table 20.4): Chinese 97%; Caucasians 94%; African Brazilians 54%; Africans 19% [28,30,31].

20.4.6 Some other serological characteristics of Knops antigens

Knops system antigens are generally resistant to treatment of the red cells with ficin and papain, although this may depend on the antibody and method of enzyme treatment used. Kn^a, McC^a, and Yk^a are destroyed by trypsin and chymotrypsin treatment of the cells, which helps to distinguish them from Cs^a, and also destroyed, or at least weakened, by the disulphide bond reducing agents AET and DTT, also distinguishing them from Cs^a [36].

Knops antigens show a variation in strength between individuals that correlates with red cell CR1 levels [10]. A reduction in expression of Knops antigens has often been observed to occur with red cell storage, but this could not be demonstrated conclusively in controlled tests with Knops antibodies against freshly bled red cells and cells stored for 35 days [37]. CR1 density per red cell decreases with cell aging, possibly due to protease cleavage of the protein near its stalk [2].

Strength of the Knops antigens is also affected by presence of an *In(Lu)* gene (Section 6.8.2) [38]. In(Lu) Lu_{null} cells gave lower mean titration scores with anti-Kn^a, -McC^a, -Yk^a, and -Sl1, and with anti-Cs^a, than did Lu(a–b+) or Lu(a+b+) cells from members of the same family. This effect of *In(Lu)* was not confirmed in a later study, by comparing In(Lu) cells with unrelated donors of common Lutheran phenotype [39].

Knops antigens are generally well expressed on red cells from cord samples. Two babies of McC(a–) mothers with high titre anti-McC^a were also McC(a–) at birth, but became McC(a+) within their first year of life [40]. Maternal anti-McC^a may have been responsible for impaired McC^a antigen expression *in utero*.

20.5 Knops system antibodies

20.5.1 Antibody characteristics

The term 'high-titre low-avidity' (HTLA) was used for many years to describe antibodies to a variety of antigens, including those of the Knops system. Although most of these antibodies react at high dilution despite their low avidity, some examples do not share these characteristics and the HTLA label is of little value.

Knops antibodies are generally troublesome to work with. This partly results from the variation in antigen strength, but also because it is difficult to adsorb the antibodies to completion or to obtain active eluates from weak antigen-positive cells. Consequently, it is almost impossible to distinguish antigen-negative cells from weakly positive cells, especially when stored or 'travelled' red cells are used.

Knops antibodies are generally IgG; they react by an antiglobulin test and do not bind complement [21,41]. There is little information regarding IgG subclass: one Knops system antibody was IgG4 [42]; another contained IgG1, IgG3, and IgG4, as well as IgA [43].

There is only one report of an apparently 'naturally occurring' Knops system antibody; anti-Kn^a in a woman who denied previous pregnancy or transfusion [44]. Of 602 blood donors lacking one or more of the Knops antigens or Cs^a, none had made a corresponding antibody [21]. Most people with one or more Knops antibodies have been transfused, but there are a few examples of anti-Kn^a, -McC^a, and -Yk^a stimulated by pregnancy alone. About 50% of sera with Knops antibodies or anti-Cs^a also contained antibodies to other red cell antigens [21].

20.5.2 Clinical significance

Knops antibodies are clinically benign, apart from the hazard of masking the presence of more dangerous antibodies that are commonly present in the same serum. Knops antibodies should be ignored when selecting

blood for transfusion. There are numerous accounts of patients with one of these antibodies being transfused with no ill effects. Radiolabelled incompatible red cells in patients with Knops antibodies show either normal or only slightly reduced survival [42–45], and *in vitro* phagocytosis assays often give very low scores [42,44]. *In vitro* functional assays involving monocytes may, however, give false positive results with Knops antibodies, because these antibodies can bind red cells to monocytes *via* CR1 rather than the Fc receptor, FcγR1 [46].

There is no report of HDFN caused by a Knops antibody, despite numerous opportunities. CR1 expression on red cells is reduced during pregnancy, reaching its nadir in the third trimester and returning to normal within 48 h post-partum [47].

20.6 Functional aspects of CR1, a complement-regulating protein

Red cell CR1 binds C3b/C4b-coated immune complexes and transports them to the liver and spleen where they are transferred to macrophages for processing. Ligation of CR1 triggers a complex Ca^{++}-dependent signalling cascade that promotes phosphorylation of the cytoskeletal proteins α-adducin and β-spectrin. This correlates with increased membrane deformability, which could play an important role in the immune-adherence clearance process [48].

CR1 has decay-accelerating activity for C3 and C5 convertases of the classical and alternative pathways and acts as a cofactor for the factor I-mediated cleavage of C3b and C4b (reviews in [1,4]). *In vivo* and *in vitro* haemolysis of PNHIII red cells, which have CR1 but are deficient in DAF and CD59 (Chapter 19), suggests that CR1 plays a minor role in protection of red cells from complement-mediated lysis.

CR1 appears to represent a privileged site on red cells for IgG binding, as relatively large quantities of IgG may be bound to CR1 without subsequent lysis or phagocytosis of the red cells [49]. This may explain why Knops system antibodies do not significantly reduce the survival of transfused incompatible red cells (Section 20.5.2).

20.7 CR1 associations with malaria and other pathogens

Red cells infected with selected cultures of the malarial parasite *Plasmodium falciparum* form rosettes with infected and uninfected red cells. Rosetting is associated with severe disease by clogging the microvasculature of vital organs including the brain. Rowe *et al.* [50] showed that infected cells do not form rosettes with uninfected red cells that have very low levels of CR1 (Helgeson phenotype) and that there is substantially reduced rosetting with Sl1– red cells, compared with Sl1+ cells. Furthermore, compared with Sl1+ cells, Helgeson phenotype red cells and other cells with the Sl1– phenotype showed reduced levels of binding to COS-7 cells transfected with the *P. falciparum var* gene expressing PfEMP1, the parasite ligand involved in rosetting. In Kenya, children with the genotype for Sl1– were less likely to have cerebral malaria than children with the genotype for Sl1+ and, in particular, those with Sl1– genotype and heterozygous for McC^a and McC^b were less likely to have cerebral malaria [51]. Sl1– is present in about 70% of West Africans, 40–50% of African Americans, but in only about 2% of white Americans (Section 20.4.3), so it is feasible that the Sl1– phenotype has a selective advantage in areas where *P. falciparum* malaria is endemic. The relationship between CR1 and malaria, however, is far from straightforward [52,53]. In Thailand a low level of red cell CR1 appeared to be a risk factor for developing severe malaria [54] and in the Gambia no association between severe malaria and *CR1* alleles encoding Sl2 or McC^b was observed [55]. Studies with soluble CR1 fragments provided no evidence that either the McC^a/McC^b or Sl1/2 polymorphisms had any effect on red cell *P. falciparum* invasion or rosette disruption [56].

The *P. falciparum* adhesin PfRh4 binds to CR1 on red cells, but only to CCPs 1–3 [57], remote from the site of the Knops polymorphisms in LHR-D (Figure 20.1). In addition to the role played in rosetting, CR1 is involved in control of complement activation and immune complex formation during malaria infection and is also a receptor for parasite invasion [53]. PfRh4 binding to CR1 affected neither C3b nor C4b binding, but it did inhibit decay-accelerating activity [57].

Some pathogenic bacteria, such as *Mycobacterium tuberculosis*, *M. leprae*, and *Legionella pneumophila*, utilise CR1 for invading phagocytes through adherence and phagocytosis [58]. Presence of McC^b and *Sl2* alleles appears to be associated with resistance to *M. tuberculosis* infections in Mali [59] and homozygosity for McC^b to confer protection from leprosy in Malawi [60]. Complement-dependent binding of gram-negative bacteria (*Escherichia coli* and *Neisseria meningitidis*) to red cell CR1 reduces intravascular phagocytosis and oxidative burst by monocytes and neutrophils, with the

Table 20.5 Cs^a frequencies.

Population	No. tested	No. Cs(a+)	Cs^a frequency	References
Northern Europeans	363	354	97.5%	[62]
Black Africans and Americans	53	51	96.2%	[62]
White Americans	2028	1931	95.2%	[21]
African Americans	894	883	98.8%	[21]
Yk(a–) white Americans	96	84	87.5%	[34]
Yk(a–) African Americans	13	12	92.3%	[34]

possible effect of reducing systemic inflammatory responses [61].

20.8 The Cost collection: Cs^a and Cs^b (COST1 and COST2)

When Giles *et al.* [62] described three patients with antibodies reactive with the red cells of 98% of Northern Europeans, they named this antibody anti-Cs^a after two of the original patients, Mrs Co. and Mrs St. Numerous family studies have shown that Cs^a is inherited as a dominant character and that it is not part of the ABO, MNS, Rh, Kell, Duffy, Kidd, Yt, or Scianna systems, is probably not part of P and Lewis, and that there is a possible association between Cs^a and Do^a [62,63]. Table 20.5 shows the results of frequency studies with anti-Cs^a.

Anti-Cs^a share many characteristics with Knops system antibodies (Section 20.5). They are difficult to work with, primarily because of the variability in expression of the Cs^a antigen, and anti-Cs^a are of no significance clinically.

An antibody in a multiply transfused woman with a weak Cs^a antigen was named anti-Cs^b [64]. Fifty-six of 59 Cs(a–) samples were Cs(b+); the remaining three were Cs(a–b–), suggesting the presence of a third allele. Fifty-five (31%) of 175 Cs(a+) samples were Cs(b+).

Despite phenotypic associations with the Knops system antigens, especially Yk^a (see Section 20.4.4), Cs^a and Cs^b are not included in the Knops system for the following reasons: Cs^a could not be shown to be on CR1 [15,17]; Cs^a was easily detected on three of four Helgeson phenotype samples [10]; and Cs^a is resistant to treatment of red cells with trypsin, chymotrypsin, and AET [36]. The nature of the association between Cs^a and the Knops system remains obscure.

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21 Indian Blood Group System and the AnWj Antigen

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21.1 Introduction

In^b/In^a, a polymorphism in people from the Indian subcontinent and in Arabs, results from an Arg46Pro substitution in CD44. In^a and In^b are of low and high incidence, respectively. The Indian system also contains two antigens of very high frequency, IN3 and IN4 (Table 21.1). CD44 is a ubiquitous glycoprotein with a variety of functions, mostly associated with its ability to bind hyaluronan, a component of the extracellular matrix. The gene encoding CD44 is on chromosome 11p13.

The high frequency antigen AnWj (901009) has not been assigned to the Indian system, but is either located on an isoform of CD44 or is closely associated with it.

21.2 CD44 and the Indian antigens

21.2.1 CD44

CD44, which is present on cells of most tissues, is a member of the link module superfamily of proteoglycans (for review see [1]). It is a major red cell membrane component of apparent MW 80 kDa [2,3]. The *CD44* gene spans 50 kb of DNA and consists of 20 exons [4,5]. CD44 exists as multiple isoforms, arising partly from alternative splicing of 10 variant exons (Figure 21.1) and partly from variation in glycosylation [4,5]. CD44s (standard), which contains products of none of the alternatively spliced exons, is the isoform present on haemopoietic cells. Exon 1 encodes the signal sequence. The 248 amino acid N-terminal extracellular domain can be divided into two regions:

1 the *N*-glycosylated link module encoded by exons 2–3, which is maintained in a folded configuration by three Cys-Cys bonds; and

2 an *O*-glycosylated mucin-like domain encoded by exons 4–7, which contains sites for covalent linkage of the glycosaminoglycan chondroitin sulphate.

Exon 8 encodes the 21 amino acid membrane-spanning domain and exon 9 the 72 amino acid C-terminal cytoplasmic tail (Figure 21.2) [6–8]. The cytoplasmic domain interacts with the membrane skeleton [3]: CD44 can bind protein 4.1R and ankyrin [9] of the two band 3-macrocomplexes (see Figure 10.2) and 4.1R-deficient red cells lack CD44 [10], yet protein 4.2 deficiency causes elevated CD44 expression [11]. There are an estimated 6000 to 10 000 copies of CD44 per red cell [12].

CD44 was assigned to chromosome 11 by testing a panel of somatic cell hybrids with the original anti-CD44 [13] and localised to 11p13 [14,15].

21.2.2 Indian antigens are located on CD44

Spring *et al.* [3] showed that In^a and In^b are carried on the CD44 glycoprotein. Immunoblotting of membranes from antigen-positive cells under non-reducing conditions with human anti-In^a and -In^b revealed an 80 kDa component of identical mobility to CD44; no such component was detected in membranes from In(a–b+) and

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Table 21.1 Antigens of the Indian system.

Antigen				Molecular basis*		
No.	Name	Frequency	Antithetical antigen	Nucleotides	Exon	Amino acids
IN1	In^a	Low	IN2	137G>C	2	Arg46Pro
IN2	In^b	High	IN1	137G (C)	2	Arg46 (Pro)
IN3	INFI	High		255C (G)	2	His85 (Gln)
IN4	INJA	High		488C (G)	2	Thr163 (Lys)

*Molecular basis of antigen-negative phenotype in parentheses.

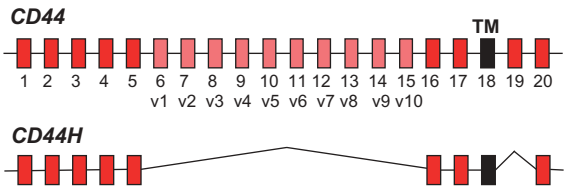


Figure 21.1 Organisation of *CD44* showing the 20 exons, including the 10 alternatively spliced exons, v1–v10, within the region encoding the extracellular domain (exons 1–17) and the exon encoding the transmembrane domain (TM, exon 18). The lower figure (*CD44H*) shows the exons encoding the haemopoietic form of CD44, CD44s.

In(a+b–) cells, respectively. CD44 glycoprotein isolated from human red cell membranes by immunoprecipitation with monoclonal anti-CD44 reacted with anti-In^b on an immunoblot. Anti-In^b was shown, by radioimmunoassay, to bind CD44 glycoprotein isolated from human red cells and leucocytes [3,16] and positive results were obtained in MAIEA assays performed with human alloanti-In^b and mouse monoclonal anti-CD44 [17,18].

21.3 Indian antigens

21.3.1 In^a and In^b (IN1 and IN2)

In 1973, a new antigen present on red cells of about 3% of Indians from Bombay was named In^a by Badakere *et al.* [19,20]. Two years later Giles [21] found that an antibody to a public antigen, Salis, was antithetical to anti-In^a and the antibody became anti-In^b.

The In^b/In^a polymorphism results from Arg46Pro at the N-terminal of an α1 helix within the link module of CD44 [22] (Table 21.1, Figure 21.1). This was confirmed by cDNA transfection and site-directed mutagenesis experiments with Jurkat human leukaemia cell line. Other changes detected in some In(a+b–) individuals, encoding Tyr109Ser and Glu239Gly, do not appear to affect expression of the Indian antigens.

Of 1749 Bombay Indians, 51 (3%) were In(a+) [20]. This gives a frequency for *IN*A* of 0.0147, and the following genotype frequencies can be deduced: *IN*A/A* 0.0002; *IN*A/B* 0.0290; *IN*B/B* 0.9708. A higher frequency of In^a was found in some Arabs: 10.6% of Iranians and 11.8% of Arabs in Bombay were In(a+) [23]. In^a is virtually unknown in other populations. Two of 700 Indian blood donors were In(b–) [24], far in excess of the number expected from the calculated genotype frequencies given above. Of 251 members of the Asian immigrant population of northern England, two were In(a+b–), 8 In(a+b+), and 241 In(a–b+); again an excess of In(a+b–) [25].

21.3.2 IN3 (INFI) and IN4 (INJA)

IN3 and IN4 are two antigens of high frequency, shown to be located on CD44 by immunoblotting and by MAIEA analysis [26]. Anti-IN3 was found in three pregnant Moroccan women. IN:–3,4 phenotype in the three propositi was associated with His85Gln in the β4 strand of the link module of CD44 (*IN*02.–03*). Anti-IN4 was found in two pregnant Pakistani women. IN:3,–4 phenotype in the two propositi and in the father and two siblings of one of the propositi was associated with Thr163Lys in the mucin-like C-terminal extension (*IN*02.–04*) (Table 21.1, Figure 21.1).

IN:–3 and IN:–4 red cells were In(a–b+) but showed weakness of In^b in titration studies [26]. Observation of three-dimensional models of the link module indicates that Arg46 (IN2) and His85 (IN3) may be in close proximity on the folded protein, but that Thr163 (IN4) would

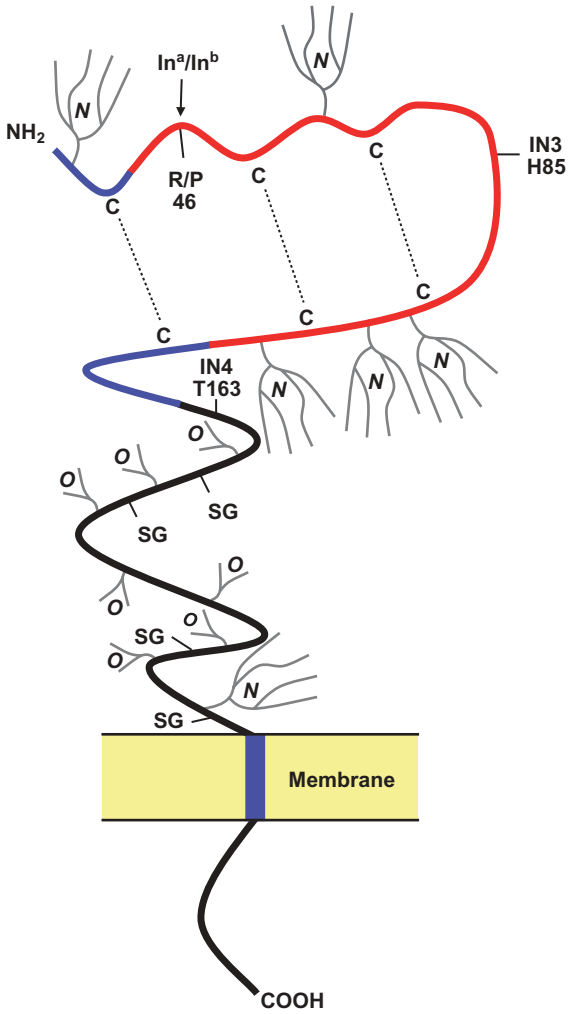


Figure 21.2 Model of CD44s. The extracellular domain consists of two regions: a membrane-proximal region (black) containing one *N*-glycosylation site (*N*), several *O*-glycosylation sites (*O*), and several Ser-Gly chondroitin sulphate linkage sites (SG); and a distal region containing five *N*-glycosylation sites and six cysteine residues (C), which suggests the presence of three disulphide bonds (dotted lines), the link module (red), and flanking extended lobes (blue). The position of the amino acid substitution responsible for the In^a/In^b polymorphism and IN3 and IN4 antigens are shown.

not be [27,28], suggesting that amino acid substitutions responsible for the rare IN:–3 and IN:–4 phenotypes affect the conformation of the link module and reduce binding of anti-In^b. Furthermore, binding of a monoclonal antibody to CD44 appeared to block binding of anti-In^b, -IN3, and -IN4, suggesting either that binding of the monoclonal antibody affects the conformation of the whole link module or that In^b, IN3, and IN4 are in closer proximity than suggested by the three-dimensional models.

21.3.3 Antigen characteristics

Indian antigens are destroyed by the proteases papain, pronase, trypsin, and chymotrypsin, but are resistant to treatment of red cells with sialidase. They are also destroyed by the disulphide bond reducing agents AET and DTT. Higher concentrations of the reducing agents than generally used for treating red cells may be required to destroy antigen expression.

Red cells from cord samples and from pregnant women show reduced expression of In^a, with about 25% of the adult number of In^a antigen sites [29]. Red cells of pregnant women have about 38% of the normal adult number, the number of sites returning to normal 3–6 months after delivery. No weakness of In^b was detected on cord red cell samples by serological titration [25].

CD44 is present in serum [30,31] and In^b can be detected in serum by haemagglutination inhibition.

21.4 Effects of *In(Lu)* on CD44 and Indian antigens

Telen *et al.* [30,32] demonstrated by flow cytometry that a CD44 monoclonal antibody (A3D8) showed markedly reduced levels of binding with red cells of the In(Lu) phenotype. In(Lu) is associated with low expression of Lutheran antigens and some other red cell antigens, and with mutations in *EKLF* (see Section 6.8). One example of In(Lu) cells bound between 25 and 39% of the quantity of anti-CD44 bound by normal cells [32]. Reduced binding was not seen with Lu$_{null}$ cells or with Lu$_{mod}$ cells of the X-linked type (Section 6.8.3), and the Lu$_{null}$ cells may even have had enhanced binding of CD44 antibodies [33]. With some CD44 antibodies the reduced binding to In(Lu) cells can also be detected by conventional serological techniques [3,34]. In inhibition experiments, normal human sera reduced binding of anti-CD44 to red cells by 67% whereas serum from an In(Lu) individual reduced binding by only 33% [30]. Immunoprecipitation and immunoblotting of membranes from In(Lu) cells revealed only a trace of CD44 [2,3,12,34], whereas Lu$_{null}$ and X-linked Lu$_{mod}$ cells had normal, or even slightly enhanced, CD44 expression [33,35].

Anti-In^b has a reduced titre with In(Lu) cells, compared with cells of normal Lutheran type and with Lu$_{null}$ and X-linked Lu$_{mod}$ cells [3,36], and anti-IN3 and -IN4 reacted weakly or not at all with In(Lu) cells [26]. A band of markedly reduced intensity was seen with membranes from In(Lu) cells blotted with anti-In^b [3].

Red cells of a girl with a novel form of congenital dyserythropoietic anaemia associated with heterozygosity for an *EKLF* mutation (Glu325Lys) had a gross deficiency of red cell CD44 and were In(a–b–): they were also IN:–3,–4 (as determined by immunoblotting [26]), Co(a–b–), AnWj–, and weak for LW^{ab} [37–39] (see Section 15.4 for further information).

21.5 Indian antibodies

Indian antigens appear to be good immunogens. Thirty of 39 In(a–) donors immunised for anti-D production with D+ In(a+) red cells made anti-In^a [40]. One anti-In^b and one anti-IN4 were produced in untransfused women during their first pregnancies [26,36]. Anti-In^a and -In^b often agglutinate antigen-positive red cells directly, although the strength of reaction is generally enhanced by antiglobulin [21,25,40].

Radiolabelled In(a+) red cells were eliminated from the circulation of two individuals with anti-In^a within 20 minutes, suggesting potential for a transfusion reaction [40]. There is one case of anti-In^b causing an immediate HTR after infusion of 50 ml of incompatible blood [24]. Reduced *in vivo* survival of In(b+) cells in a patient with anti-In^b was observed at 24 h [36]. Antigen-negative red cells for transfusion to patients with anti-In^b, -IN3, or -IN4, may be very difficult to obtain, though In(Lu) cells would probably be suitable for transfusion. No Indian antibody has been implicated in HDFN and it has been suggested that binding of anti-In^b to CD44 on fetal monocytes and macrophages could have a blocking effect on FcγR1 [41].

Numerous murine monoclonal antibodies to non-polymorphic epitopes on CD44 have been produced, one of which had In^b specificity [42].

21.6 Functional aspects of CD44

CD44 proteoglycan is a ubiquitous structure: very few tissues or cells lack CD44. It is present on circulating red cells, B and T lymphocytes, granulocytes, and monocytes, but not platelets [3,30,34], and is present on thymus, central nervous system white matter, epidermis, skeletal muscle, and epithelium from stomach, intestine, liver, bladder, lung, and breast [12,43].

Many roles have been attributed to CD44, the functions being regulated by the inclusion of the products of the various alternatively spliced exons. Functions of CD44 include the following: adhesion of leucocytes to endothelial cells, stromal cells, and the extracellular matrix (ECM); participation in T and B cell activation in response to immunological stimuli; lymphocyte–endothelial cell interactions involved in the localisation of lymphocytes to the site of inflammation; modelling of the ECM during wound healing and embryonic development. CD44 has also been implicated in tumour metastasis (reviews in [44,45]). Most of these interactions involve binding of CD44 to hyaluronan, a high molecular weight glycoaminoglycan that is a major component of the ECM and is also present on cell surfaces. CD44 also binds collagen, fibronectin, and laminin; proteins of the ECM that fill the spaces between cells [1]. CD44 appears to play a regulatory role in normal haemopoiesis (review in [46]). Hyaluronan binding by CD44 may be involved in adhesion of haemopoietic progenitors, including burst-forming units-erythroid (BFU-E), to the bone marrow stroma [46,47]. Surface expression of CD44 in mice progressively decreased 30-fold in late-stage erythroblasts [48]. Consequently, CD44 on circulating red cells may be vestigial, its functions being completed during erythropoiesis.

CD44 contains three copies of a hyaluronan binding motif, BX_7B (B = arginine or lysine; X_7 = seven non-acidic amino acids), present in proteins that bind hyaluronan [49]. Experiments involving site-directed mutagenesis identified several amino acid residues that were considered important in the binding of CD44 to hyaluronan, one of which was Arg46 (the second B of a BX_7B hyaluronan-binding motif). Mutating Arg46 to Gly in CD44 constructs abolished hyaluronan binding [49]. In(a+b–) phenotype results from homozygosity for *CD44* alleles encoding Pro46 (Section 21.3.1), yet the In^a Arg-46Pro substitution does not reduce hyaluronan binding to intact CD44s, *in vitro* [22]. A combined modelling and mutagenesis study has subsequently suggested that Arg41, Tyr42, Arg78, and Tyr79 are critical for hyaluronan binding, and Lys68, Asn100, Asn 101, and Tyr105 support binding [50]. All of these residues, except Lys68, come together in a three-dimensional module, as a contiguous linear patch [27]. Glycosylation of Asn25 and Asn120, which are outside the link module, also play a key role in hyaluronan binding.

21.7 AnWj (901009)

Anti-Anton [51] and anti-Wj [52] were names given to alloantibodies and autoantibodies that failed to react with In(Lu) phenotype cells and with cord cells, but did react with Lu_{null} cells. It subsequently became clear that anti-Anton and -Wj had the same specificity [53,54] and the name AnWj was given to the antigen they define.

21.7.1 Inheritance and frequency

The rare AnWj– phenotype is usually acquired and may be transient, but one family demonstrated that it could also be inherited [55]. Two of seven siblings of an AnWj– Arab woman with anti-AnWj were AnWj–. The consanguineous parents and the six children of the propositus were AnWj+, suggesting that the AnWj– phenotype in this family results from homozygosity of a rare recessive gene. The family study demonstrated that AnWj is not controlled by *LU*, or by *ABO*, *MNS*, *RH*, *KEL*, *FY*, *JK*, *XG*, or *XK*.

Anti-AnWj screening of red cells from 2400 American donors revealed three In(Lu) samples, but no AnWj– red cells with normal Lutheran antigens [56].

21.7.2 AnWj and the In(Lu) phenotype

Like In^b and several other red cell antigens outside the Lutheran system, AnWj is expressed only very weakly on red cells of individuals with the dominant gene *In(Lu)* (see Section 6.8). Usually AnWj cannot be detected on these Lu_{mod} cells by direct testing, but anti-AnWj can be adsorbed and eluted from them [55]. Lu_{null} and X-linked Lu_{mod} cells have normal AnWj expression [51,57].

AnWj differs from Lutheran and Indian system antigens in being resistant to trypsin, chymotrypsin, and the disulphide bond reducing agent AET.

21.7.3 Development of AnWj

Analysis of red cells from 36 infants revealed that the age at which conversion from AnWj– to AnWj+ takes place varies from infant to infant, but occurs between the ages of three days and 46 days and requires less than one day to complete [58]. This rapid, 'all or nothing' phenomenon is unexpected as the red cells in the circulation are not all produced at the same time and no evidence could be found for a conversion factor in the serum. Whatever causes the change from AnWj– to AnWj+ in infants might be reversible on rare occasions, as the adult AnWj– phenotype with concurrent presence of anti-AnWj has been found to be transient in several patients and in a healthy individual [54,58–60].

21.7.4 AnWj as a receptor for *Haemophilus influenzae*

H. influenzae is a commensal bacterium of the throat of most healthy people, but it may also cause respiratory tract infections and, more seriously, is a major cause of bacterial meningitis in young children. Some strains of *H. influenzae* express fimbriae (short, thread-like processes attached to the cell walls), which are probably involved in adherence to nasopharyngeal epithelial cells.

Fimbriae-bearing strains of *H. influenzae* isolated from patients with invasive disease and respiratory tract infections agglutinated most red cell samples from adults, including Lu_{null} cells, but did not agglutinate cord cells, In(Lu) cells, or AnWj– red cells of both acquired and inherited types [61,62]. Anti-AnWj inhibited agglutination of AnWj+ red cells by the bacteria.

H. influenzae bound to buccal epithelial cells, including those from one individual with transient AnWj– red cells, two with *In(Lu)* genes, and several neonates, but did not bind to buccal epithelial cells from three AnWj– members of the Arab family with inherited AnWj– phenotype [62,63]. Adherence of *H. influenzae* to epithelial cells was not inhibited by anti-AnWj [63]. Although the receptors for *H. influenzae* adherence on red cells and epithelial cells may not be identical, they appear to have a common genetic basis.

21.7.5 Anti-AnWj

Anti-AnWj may be autoantibodies or alloantibodies, or apparent alloantibodies in patients, usually with lymphoid tumours, with an acquired AnWj– phenotype. In the only family with more than one AnWj– member, the propositus and her AnWj– sister both had anti-AnWj and both had been pregnant, but not transfused [55]. It is a little surprising that anti-AnWj can be stimulated by pregnancy considering the very low level of AnWj antigen on neonatal red cells.

Anti-AnWj has been incriminated in severe HTRs [60,64–68], although there are several cases where AnWj+ cells have been transfused uneventfully to patients with anti-AnWj. The haemolytic potential of anti-AnWj has been supported by *in vivo* red cell survival studies [64,69] and monocyte monolayer analysis [67,70]. In a patient with autoanti-AnWj and depressed red cell AnWj expression, radiolabelled autologous red cells survived normally, but AnWj+ allogeneic cells had reduced survival

[71]. If available, In(Lu) cells are most suitable for transfusing patients with anti-AnWj.

There was no indication of HDFN in any of the children of a mother with alloanti-AnWj [55].

Two monoclonal antibodies with AnWj specificity were produced from mice immunised with human T-cell lines derived from patients with acute lymphocytic leukaemia [72].

21.7.6 AnWj may be located on CD44 glycoprotein

In(a–b–) red cells of the patient with a novel form of CDA and a gross deficiency of red cell CD44 were AnWj– [37–39]. Positive results were obtained in MAIEA analyses performed with either anti-CD44 or -AnWj murine monoclonal antibodies and anti-In^b or -AnWj human antibodies [17]. The CD44-negative human leukaemia cell line, Jurkat, transfected with *CD44* cDNA reacted with human and mouse monoclonal anti-AnWj [73]. Immunoblotting with anti-AnWj revealed an 80 kDa component in normal red cells and in Chinese hamster ovary cells (CHO) and murine erythroleukaemia (MEL) cells transfected with *CD44* cDNA, but not in non-transfected CHO or MEL cells, or in In(Lu) red cells. The anti-AnWj also bound a 200 kDa structure in red cells and transfected CHO cells. Telen *et al.* [73] suggest that AnWj antigen is located on a trypsin-resistant region of an isoform of CD44 that is not present on red cells of the newborn. AnWj might also be on the 200 kDa chondroitinated isoform of CD44.

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22 Ok Blood Group System

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22.1 Introduction

The three antigens of the Ok system, Ok^a (OK1), OK2, and OK3, are of very high frequency and located on the immunoglobulin superfamily (IgSF) molecule basigin, a receptor for *Plasmodium falciparum* invasion. The rare antigen-negative phenotypes result from single amino acid substitutions on basigin (Table 22.1).

22.2 Basigin, the Ok glycoprotein

Immunoblotting of membranes from Ok(a+) red cells with murine monoclonal anti-Ok^a or human alloanti-Ok^a showed that Ok^a is situated on a glycoprotein ranging in apparent MW 35–68 kDa [1]. The N-terminal 30 amino acids of purified Ok glycoprotein [2] were found to be identical to those of basigin, also known as CD147 or EMMPRIN [3,4]. Mouse NS-0 cells transfected with basigin cDNA expressed Ok^a, except when the cDNA was from an Ok(a–) individual [2].

Human basigin cDNA was isolated both by screening a human cDNA library with a fragment of mouse basigin cDNA [5] and by expression cloning of cDNA derived from human T cell and B cell libraries [6]. Basigin is a single chain transmembrane molecule with a signal peptide of 18 amino acids, an N-terminal extracellular domain of 187 amino acids, a 24 amino acid membrane-spanning domain, which contains a single charged glutamic acid residue and a leucine zipper, and a 40-residue cytoplasmic domain. It is a member of the immunoglobulin superfamily of adhesion molecules and receptors (see Chapters 6 and 16) and the extracellular domain is organised into one C2 set and one V set IgSF domains (Figure 22.1) [6,7]. Three *N*-glycans, one on the C2 set domain and two on the V set domain, comprise about 50% of the mass of the glycoprotein.

BSG, the gene encoding basigin, consists of 10.8 kb organised into eight exons (Figure 22.1) [8]. Approximately 95 kb of the 5′ flanking sequence contained three Sp1 and two AP2 sites, but no TATA or CAAT box. *BSG* was mapped to 19p13.3 by fluorescence *in situ* hybridisation [9].

22.3 OK antigens and antibodies

22.3.1 Ok^a (OK1)

The producer of the original anti-Ok^a (S.Ko.G) came from a small Japanese island. Her parents were consanguineous and two of her three siblings were also Ok(a–) [10]. No other Ok(a–) individual was found as a result of testing red cells from 400 people from S.Ko.G's home island, 870 donors from other parts of Japan, 3976 American blood donors of 'Oriental appearance', 9053 white Americans, 1570 African Americans, and 1378 Mexican Americans.

Basigin cDNA from three Ok(a–) individuals contained a mutation encoding Glu92Lys in the N-terminal C2-set IgSF domain [2] (Table 22.1). One other Japanese and one Korean Ok(a–) individuals were also homozygous for the Glu92Lys mutation. Another Ok(a–)Japanese was heterozygous for the Glu92Lys mutation; no mutation was detected in the other allele [11]. No Ok(a–) allele (274A) was detected in 907 Chinese and Tibetans [12].

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Table 22.1 Antigens of the Ok system.

Antigen			Molecular basis*		
No.	Name	Frequency	Nucleotides	Exon	Amino acids
OK1	Ok^a	High	274G (A)	4	Glu92 (Lys)
OK2	OKGV	High	176G (T)	2	Gly59 (Val)
OK3	OKVM	High	178G (T)	2	Val60 (Met)

*Molecular basis of antigen-negative phenotype in parentheses.

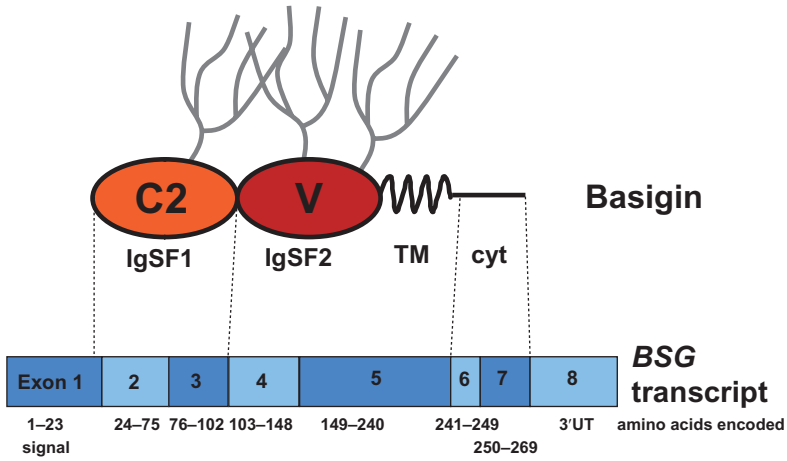


Figure 22.1 Diagrammatic representation of basigin showing the C2 and V set IgSF domains with one and two *N*-glycans, respectively, the transmembrane domain (TM), and the cytoplasmic tail (cyt). Also shown is the relationship of the protein structure to the eight exons of *BSG*, including a 23 amino acid signal peptide.

Ok^a is not affected by treatment of intact cells with trypsin, chymotrypsin, papain, pronase, or sialidase, or with the disulphide bond reducing agent AET.

22.3.2 Anti-Ok^a

S.Ko.G had not been pregnant, but had been transfused at least once. Her Ok(a–) sister had five children, at least two of whom were Ok(a+), but had not made anti-Ok^a. Three hours after injection of radiolabelled Ok(a+) red cells, only 10% remained in the circulation of S.Ko.G. A mononuclear phagocyte assay with anti-Ok^a gave similar values to those obtained with antibodies known to have caused significant shortening of red cell survival [10]. Ideally antigen-negative red cells would be selected for transfusion to a patient with anti-Ok^a, but, owing to their extreme rarity, least incompatible red cells should be used with extreme caution.

IgG, IgM, and IgA monoclonal anti-Ok^a have been produced from spleen cells of mice immunised with human teratocarcinoma or embryonic kidney cell lines [1,13].

22.3.3 OK2 (OKGV) and OK3 (OKVM)

Two alloantibodies resembled anti-Ok^a as they did not react with Ok(a–) red cells, but were made by patients whose red cells reacted with alloanti-Ok^a and at least some monoclonal anti-Ok^a. The patients were homozygous for different mutations encoding single amino acid substitutions in basigin (Table 22.1). Anti-OK2 was produced by a previously transfused woman of Iranian origin with a history of recurrent miscarriages [11] and anti-OK3 by a prenatal patient with no known history of transfusion or previous pregnancy [14].

22.4 Tissue distribution and function of basigin

Basigin is present on all leucocytes and human leukaemic cell lines and has been detected on all human cells examined, although some tissues show differentiation-related expression [1,2,6]. Early haemopoietic progenitors express Ok^a strongly, but the level of expression decreases during erythroid development [15].

Basigin is a pleiotropic molecule with multiple functions (reviewed in [4,16]). It has also been named extracellular matrix metalloproteinase inducer (EMMPRIN) because on tumour cells it induces production of collagenase and other extracellular matrix metalloproteinases (MMP) and basigin may be involved in tumour invasion and metastasis. In healthy tissue, basigin may function in embryonic development or wound healing by causing dermal fibroblasts to increase their MMP production, thus facilitating tissue remodelling [7]. Basigin is a leucocyte activation-associated glycoprotein and is highly expressed on activated T and B lymphocytes, monocytes, and macrophages [6]. Basigin interacts with integrins in the cell membrane and probably regulates binding of cells to laminin in basement membranes [4]. In breast carcinoma cells, interactions between hyaluronan, CD44 (Chapter 21), and basigin contribute to the localisation and function of the monocarboxylate transporters MCT1 and MCT4 in the plasma membrane [17]. Basigin-null mice are blind owing to deficiency of MCTs in the retina. Fetal basigin-null mice have severely impaired implantation and males are sterile [4].

On red cells, basigin also appears to interact specifically with MCT1 and MCT4, which transport monocarboxylates, such as lactate and pyruvate, across the plasma membrane [18]. Basigin functions as a chaperone for translocation of the MCTs to the plasma membrane, where its continued presence and correct conformation are critical for transporter function [19]. In mice, masking of basigin by $F(ab')_2$ fragments of monoclonal anti-basigin disrupts the migration of red cells out of the spleen, inducing splenomegaly, anaemia and, consequently, erythropoietin-mediated erythropoiesis [20]. Basigin may, therefore, play an important role in the recirculation of mature red cells from the spleen into the general circulation.

22.5 Basigin and malaria

Glycophorins A, B, and C, and CR1 are red cell surface proteins with blood group activity that have been recognised as receptors for some strains of the most virulent human malaria parasite, *Plasmodium falciparum* (Chapters 3, 18, and 20). None, however, is required for red cell entry of all strains of the parasite. PfRh5 is a member of the *P. falciparum* reticulocyte-binding protein homologue family that appears to play an essential role in red cell selection and invasion by merozoites of all strains of *P. falciparum* [21]. In 2011, Crosnier *et al.* [22] screened a library of 40 red cell surface proteins with PfRh5 and found that the parasite ligand bound basigin. Red cell invasion by *P. falciparum* was inhibited by soluble basigin, blocked by two basigin (anti-Ok^a) antibodies, and substantially reduced in red cells from two unrelated Ok(a–) individuals and in cultured red cells in which basigin expression was knocked-down by inhibitory RNA. Ok(a–) red cells, which have an altered form of basigin, had reduced affinity for PfRh5. The requirement for basigin in red cell entry applied to 15 strains of *P. falciparum* implying a universal entry pathway and anticipation of the development of an effective malaria vaccine.

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23 Raph Blood Group System

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23.1 Introduction

The Raph system contains just one antigen, MER2 (RAPH1), located on the tetraspanin CD151. The true MER2– phenotype, associated with the presence of anti-MER2, is very rare and results from mutations in *CD151*, but there is a quantitative red cell polymorphism in which red cells of about 8% of Caucasians are serologically MER2–.

23.2 CD151 and the tetraspanin superfamily

The four-transmembrane domain superfamily (TM4SF) of integral proteins (tetraspanins) comprises 33 members in humans (for reviews see [1,2,3,4]). Tetraspanins are widely distributed on animal cells and several are known to be expressed on human haemopoietic cells including CD9, CD37, CD53, CD63, CD81, CD82, CD151, and CD231, though only CD151 and CD82 have been detected on mature red cells [5,6]. In addition to the characteristic four membrane-spanning domains, tetraspanins have short, cytosolic N- and C-termini and one small (EC1) and one large (EC2) extracellular loop. EC2 comprises 78–150 amino acids and contains four conserved cysteine residues, two of which are in a CCG motif. EC2 of most tetraspanins also contains a PxxCC motif, one or two disulphide bonds, and at least one *N*-glycan (Figure 23.1). Tetraspanins in the cell membrane aggregate with each other and with a variety of other transmembrane proteins, especially integrins, to form clusters known as tetraspanin-enriched microdomains. Integrins are heterodimeric, transmembrane receptors, consisting of non-covalently linked α and β subunits (see Section 16.8). They are two-way signalling molecules that dynamically link the extracellular matrix (ECM) with the cytoskeleton [4].

CD151 (TSPAN24) was originally identified as a platelet and endothelial cell marker [8]. Isolation of CD151 cDNA from megakaryoblastic and T-cell leukaemia cell lines revealed that CD151 is a 253 amino acid protein with a single *N*-glycosylation site and six cysteine residues in EC2 [9,10].

CD151 was located on chromosome 11p15.5 by *in situ* hybridisation and radiation-hybrid mapping [11,12]. It comprises 8 exons: exon 2 encodes the N-terminal domain; exon 3, the first transmembrane domain (TM1), EC1, TM2, and the cytoplasmic loop; exon 4, TM3; exons 5 and 6, EC2; exon 7, TM4; and exon 8, the C-terminal domain [12] (Figure 23.1).

23.3 CD151 is the Raph glycoprotein

The gene encoding MER2 was assigned to chromosome 11p15 by somatic cell hybridisation in 1987 [13]. In 2004 Karamatic Crew *et al.* [5] noticed that the genes for MER2 and CD151 were on the same region of chromosome 11. They confirmed that CD151 expresses the MER2 antigen by showing that (i) a monoclonal anti-CD151 reacted with red cells and recognised the MER2 polymorphism, (ii) incubation of MER2+ red cells with human alloanti-MER2 blocked binding of murine anti-CD151 and murine anti-MER2, and (iii) MER2– individuals who

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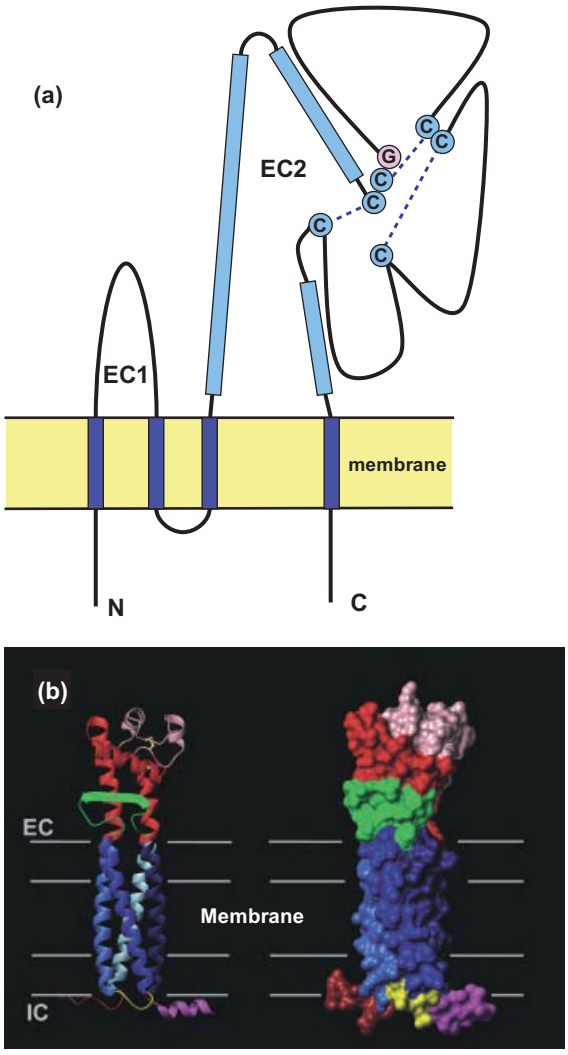


Figure 23.1 (a) Diagram of CD151, a typical tetraspanin, showing the four membrane-spanning domains, the cytosolic N- and –termini, the small extracellular loop (EC1) and large extracellular loop (EC2), comprising three α-helices (blue boxes), six Cys residues (C), one of which is part of the Cys-Cys-Gly (CCG) motif, and three disulphide bonds (dotted lines). (b) Ribbon and space-fill models of a tetraspanin, showing EC1 in green, the three α-helices of EC2 in red, and the remainder of EC2 in brown (reproduced from Seigneuret [7], with permission from Elsevier). EC, extracellular; IC, intracellular.

had made anti-MER2 were homozygous for mutations in *CD151*.

23.4 MER2 (RAPH1) antigen and anti-MER2

23.4.1 Effects of enzymes and reducing agents

MER2 is resistant to papain and sialidase treatment of red cells, but is destroyed by trypsin, chymotrypsin, pronase, and the disulphide bond reducing agent AET.

23.4.2 MER2– phenotype and alloanti-MER2

True MER2– phenotype is very rare and has only been found in individuals with alloanti-MER2. Human anti-MER2 was found in three individuals living in Israel, but originating from a Jewish community in India [14]. Two were siblings and the third was unrelated. All three were homozygous for a single nucleotide insertion (G383) in exon 5 of *CD151*, causing a frameshift and premature stop signal at codon 140 (*RAPH*–01N.01*) [5]. This would result in a translated protein lacking most of EC2 and TM4. It is unlikely that this truncated protein would reach the plasma membrane or that the severely truncated EC2 region would fold to a functional protein domain, and so these individuals could be considered to have a Raph-null phenotype. All had renal failure requiring dialysis and regular blood transfusion (Section 23.5). In two cases the antibodies were detected before commencement of dialysis and before the patients had been transfused [14]. All three antibodies react by an antiglobulin test with anti-IgG.

Alloanti-MER2 has been identified in four women, all of whom had been pregnant and are homozygous for *CD151* exon 6 mutations encoding amino acid substitutions in EC2: 511C>T, Arg171Cys (*RAPH*–01.01*) in women of Pakistani and Turkish origin [15]; 533G>A, Arg178His (*RAPH*–01.02*) in a Turkish woman [5]; 494G>A, Arg165Gln in a Caucasian woman [16].

23.4.3 The MER2 quantitative polymorphism and the effect of *In(Lu)*

The strength of MER2 varies considerably between red cell samples [13]. Of 1016 English blood donors, 8% were MER2– by an antiglobulin test. Analysis of 103 Northern European families with a total of 294 children confirmed that this polymorphism is inherited. The serological

MER2– phenotype, with no production of anti-MER2 and no detected mutations in *CD151*, probably represents the low end of a spectrum of red cell expression, with a site density below the threshold for detection by indirect agglutination techniques. Expression of MER2 on erythroid cells decreases throughout *ex vivo* erythropoiesis and MER2 is detectable on erythroid progenitors of apparent MER2– individuals [5,17]. In addition, MER2 is abundant on platelets of individuals with both MER2+ and MER2– red cell phenotypes, though there may be some degree of correlation between strength of antigen expression on red cells and platelets [5]. The reason for the individual variation in the expression of MER2 on red cells and platelets is unknown.

Titration scores with anti-MER2 and red cells of members of a large Sardinian family demonstrated that the dominant inhibitor gene *In(Lu)* (representing mutations in *EKLF*) exerted a slight depressing effect on MER2 expression (see Section 6.8.2.5). Scores for nine family members with *In(Lu)* (Lu_{mod}) varied from 0–15 with a mean of 6, whereas scores for 12 members without *In(Lu)* [Lu(a–b+)] varied from 12–21 with a mean of 16.

23.4.4 Clinical significance of anti-MER2

The two Israeli siblings with anti-MER2 and hereditary nephritis have received numerous transfusions of cross-match incompatible blood over a number of years with apparently no ill effects or indications of reduced red cell survival [14]. An 81-year-old woman with anti-MER2 showed indications of an HTR following transfusion of a third unit of red cells and results of a monocytes monolayer assay suggested that the antibody had the potential to be clinically significant [15]. Least incompatible red cells should be selected for transfusion to patients with anti-MER2.

23.5 Disease associations and functional aspects

The functions of tetraspanins are multifarious. Together with their partner molecules in the tetraspanin-enriched microdomains, they are involved in cell adhesion, signalling, and intracellular trafficking. They appear to function as molecular facilitators and transmembrane linkers, by recruiting signalling enzymes and tethering them to integrins, and by engaging other transmembrane proteins in specific lateral associations to form a network referred to as the tetraspanin web. Expression of certain tetraspanins and their partners is deregulated in some human malignancies [4,18]. Enhanced CD151 expression correlates with increased risk of metastasis or reduced survival in colon, lung, and prostate cancers. Metastasis is reduced in *Cd151* knockout mice, demonstrating that CD151 expression on host cells, as well as on tumour cells, plays a role in tumour progression [19]. CD151 is present on nearly all epithelial, endothelial, and fibroblastic cells, and associates tightly in cell membranes with the laminin-binding integrins α3β1, α6β1, α6β4, and α7β1. For reviews see [1–4].

MER2 demonstrates well how the study of an obscure blood group of little clinical importance in blood transfusion can provide important information on the functions of proteins in a variety of tissues. All three Israeli patients with CD151 deficiency had end-stage renal failure and the siblings also had severe blistering of the shins, neurosensory deafness, bilateral lachrymal duct stenosis, nail dystrophy, and β-thalassaemia minor [5,20]. CD151 binds integrin α3β1 on glomerular podocytes, enhancing adhesion strength between the podocytes and laminin of the glomerular basement membrane, important in the maintenance of a functional filtration barrier [21]. CD151 is also involved in dermal–epidermal adherence in the skin. Deafness in the CD151-deficient patients suggests that CD151 plays a similar role in the inner ear.

Severe anaemia observed in the CD151-deficient patients can be attributed, in part at least, to the occurrence of β-thalassaemia minor, but these patients require much higher doses of recombinant erythropoietin to maintain their haemoglobin levels between 80 and 100 g/L than other patients with β-thalassaemia trait undergoing chronic haemodialysis [22]. This suggests that their marrow response to erythropoietin is impaired, which may result from defects in the membrane assembly of integrins in erythroid progenitors that impact on signalling pathways also utilised by the erythropoietin receptor [5].

Modelling of CD151 EC2 suggested that both Arg178His and Arg171Cys, present in other MER2– patients with anti-MER2, are consistent with a protein that has lost the MER2 epitope, but retained integrin binding functionality. This explains why these patients showed none of the symptoms seen in the CD151-deficient Israelis [5,15].

CD151-deficiency in some strains of mice leads to similar kidney defects to those in CD151-deficient humans, but to no skin lesions or hearing impairment [21,23,24].

23.6 Tetraspanin CD82 is also present on red cells

The only other tetraspanin to be detected on mature red cells is CD82 (TSPAN27) and, like CD151, CD82 displays a quantitative polymorphism. About 86% of 115 blood donors had CD82-negative red cells, as determined by an antiglobulin agglutination test, yet CD82 was detected on erythroid progenitor cells of an individual with CD82-negative red cells [6].

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24 JMH Blood Group System

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24.1 Introduction

The original John Milton Hagen (JMH1) antigen represents determinants on the signalling protein semaphorin 7A (Sema7A, CD108). JMH:–1 is usually an acquired phenotype most often found in people over 50 years old. Inherited variants of JMH lacking antigens JMH2 to JMH6 represent amino acid substitutions in Sema7A resulting from homozygosity for missense mutations in *SEMA7A*.

24.2 The JMH glycoprotein is semaphorin 7A (CD108)

Semaphorins are glycoproteins found throughout the animal kingdom; at least 20 have been found in man. Semaphorins exist as secreted, membrane-spanning, and GPI-linked forms and consist of a sema domain, a highly conserved form of a seven-blade β-propeller fold, linked through a cysteine-rich region to an immunoglobulin domain [1–3].

SEMA7A cDNA was cloned by screening human cDNA libraries with sequences derived from human expressed sequence tags (EST) identified by comparison with a herpes viral semaphorin [4] and independently from a partial amino acid sequence of a glycoprotein isolated by immunoprecipitation with a CD108 antibody [5]. Nascent Sema7A is a 666 amino acid polypeptide, which includes a 44 amino acid signal peptide and an 18 amino acid GPI anchor motif. The mature protein consists of a 438 amino acid sema domain, containing four *N*-glycosylation and six myristoylation sites, plus an 86 amino acid immunoglobulin domain of the C2 set, containing one *N*-glycosylation site (Figure 24.1) [3–6].

SEMA7A consists of 14 exons and was localised to 15q22.3-q23 by radiation hybrid mapping and fluorescence *in situ* hybridisation [4,6].

Immunoblotting and immunoprecipitation with human anti-JMH and with a monoclonal JMH-related antibody (H8) showed that JMH is located on a structure of apparent MW 76 kDa in JMH+ red cells, but not in JMH– cells [7]. This GPI-linked protein, which is cleaved from the red cell by phosphatidylinositol-specific phospholipase C and is not present on the complement-sensitive population of red cells (PNHIII) of patients with paroxysmal nocturnal haemoglobinuria [7,8] (see Chapter 19), was subsequently shown to be Sema7A [9]. Sema7A contains 19 cysteine residues, so some disulphide bonding is likely; JMH expression is destroyed by disulphide bond reducing agents. JMH variants have been shown to be associated with mutations in *SEMA7A* (Section 24.3). JMH– red cells have normal expression of the GPI-linked proteins CD55 and CD59, so their Sema7A deficiency does not result from defective biosynthesis of the GPI anchor [6].

24.3 JMH (JMH1)

Sabo *et al.* [10] coined the term anti-JMH for a collection of antibodies identified in many reference laboratories and found predominantly, but not exclusively, in elderly patients. In many cases JMH– is probably an acquired phenotype. In some patients with anti-JMH, JMH may

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not have been totally lost as the red cells give a weakly positive DAT, and in some cases anti-JMH can be eluted [6,11]. JMH– phenotype may be transient, but in some cases the JMH– phenotype remained stable for decades [6]. JMH is usually expressed only very weakly on the red cells of neonates, achieving full strength during the first few years of life. JMH is destroyed by proteases (papain, trypsin, chymotrypsin) and by the disulphide bond reducing agent AET, but is not affected by sialidase.

In a family in which the JMH– phenotype was shown to be inherited, JMH– appeared in three generations, suggesting autosomal dominant inheritance [12]. None of the JMH– members of the family had anti-JMH, and their red cells did not give a positive DAT.

No nucleotide changes encoding amino acid substitutions were detected in the coding or promoter regions of *SEMA7A* of any JMH– individuals. Reticulocytes from JMH– individuals expressed full-length *SEMA7A* transcripts, suggesting that their Sema7A deficiency results from a post-transcriptional mechanism, and their lymphocytes could be stimulated to express normal levels of Sema7A [6].

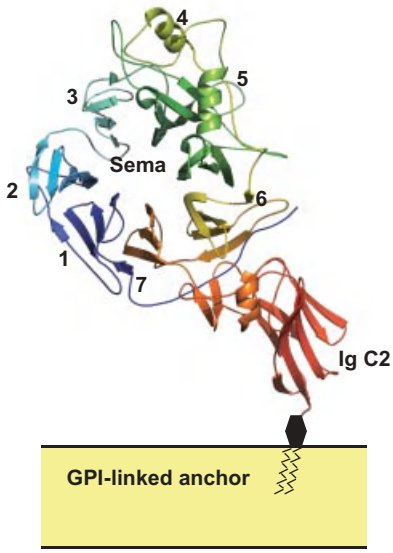


Figure 24.1 Three-dimensional model of Sema7A showing the seven propellers of the Sema domain, the loops of the Ig-like domain, and the location of the GPI-linked anchor in the red cell membrane. (3-D model provided by Nicholas Burton.)

24.4 JMH variants

Rare phenotypes have been recognised in JMH+ individuals with alloantibodies to high frequency antigens that are non-reactive with JMH– cells. These phenotypes appear to be inherited in an autosomal recessive manner. The JMH-like antibodies differ from anti-JMH because they do not react with the antibody makers' own JMH+ cells or with the JMH+ cells of some of their siblings [6,13–16]. Five of these variant phenotypes result from homozygosity for mutations in *SEMA7A* encoding single amino acid substitutions [6,16], and the associated antigens of high frequency are numbered JMH2 to JMH6 (Tables 24.1 and 24.2). Normal levels of Sema7A are expressed on red cells lacking variant JMH antigens [6].

Table 24.1 Antigens of the JMH system.

Antigen			Molecular basis*		
No.	Name	Frequency	Nucleotides	Exon	Amino acids
JMH1	JMH	High	Not known		Protein deficiency
JMH2	JMHK	High	619C (T)	6	Arg207 (Trp)
JMH3	JMHL	High	620G (A)	6	Arg207 (Gln)
JMH4	JMHG	High	1379G (A)	11	Arg460 (His)
JMH5	JMHM	High	1381C (T)	11	Arg461 (Cys)
JMH6	JMHQ	High	1040G (T)	9	Arg347 (Leu)

*Molecular basis of antigen-negative phenotype in parentheses.

Table 24.2 Reactions of anti-JMH and JMH-related alloantibodies with JMH– and JMH variant red cells [6,16].

Phenotype	Antibodies to:						Origin
	JMH1	JMH2	JMH3	JMH4	JMH5	JMH6	
JMH:–1	–	–	–	–	–	–	
JMH:–2	+	–	–	+	+	nr	Japan
JMH:–3	+	–	–	+	+	nr	Canada and Germany
JMH:–4	+	+	+	–	+	nr	USA
JMH:–5	+	+	+	–	–	nr	Poland
JMH:–6	+	nr	nr	nr	nr	–	Native Canadian

nr, not reported.

24.5 Anti-JMH

24.5.1 Human antibodies

JMH– patients with anti-JMH often have no history of transfusion or pregnancy. Of seven anti-JMH, five were IgG4 and two were IgG1 [17], although an IgG3 anti-JMH has been described [18].

There are numerous cases where patients with anti-JMH have been transfused with JMH+ blood with no adverse effects [10,11,19,20]. One such patient received 20 units of JMH+ blood in 10 months, with the expected haemoglobin rise [20]. Radiolabelled JMH+ red cells often survive normally in patients with anti-JMH [10,11,20], but there are reports of slightly accelerated clearance of JMH+ cells [14,17,21] and of JMH antibodies giving positive results in monocyte functional assays [14,17,18,21]. One JMH antibody is reported to have caused an acute intravascular HTR, but evidence that the JMH antibody was responsible for the reaction is limited [22]. Least incompatible red cells should be selected for transfusion to patients with anti-JMH.

There are no reports of JMH antibodies causing HDFN, unsurprising considering JMH antigens are expressed very weakly on cord red cells.

24.5.2 Monoclonal antibodies

Monoclonal antibodies of the CD108 cluster of differentiation may also be considered anti-JMH [9]. A monoclonal antibody (H8), produced from a mouse immunised with a human lymphoid cell line derived from a patient with acute lymphocytic leukaemia, appeared to have the same specificity as anti-JMH5 [23,24].

24.6 Functional aspects

Sema7A is widely expressed. In addition to red cells it has been detected on neurons of the brain and spinal cord, activated lymphocytes, monocytes and macrophages, and fibroblasts, and in thymus, spleen, bone, gonads, gut, heart, kidney, and placenta [1].

Most semaphorins bind directly to plexins, a family of proteins that also have a sema domain. Sema7A functions as both a neural and immune semaphorin through PlexinC1, with the β propeller structures of dimers of Sema7A and PlexinC1 interacting in an edge-on orientation [3].

Sema7A has both immune and neurological functions, although its function on red cells is not known. Sema7A interacts with integrins. The sema domain of Sema7A contains a conserved integrin-binding motif Arg-Gly-Asp (267–269), although analysis of the Sema7A crystal structure suggests that the motif is buried and unlikely to be recognised by integrins [3]. Sema7A on activated lymphocytes may assist in initiating inflammatory cascades through the α1β1 integrin by stimulating macrophages to produce proinflammatory cytokines [25]. Sema7A also promotes central and peripheral axon outgrowth through β1 integrin-dependent regulation of mitogen-activated protein kinase (MAPK) pathways [26].

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25 I and i Antigens, and Cold Agglutination

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25.1 Introduction

The subject of cold agglutination, as defined by Roelcke [1], involves, 'the occurrence and reaction of autoantibodies, reacting optimally in the cold (0°C) with red blood cells'. These autoantibodies are termed cold agglutinins. Low titre cold agglutinins are present in the sera of all adults. The most prevalent of these autoantibodies is a heterogeneous assembly of specificities called anti-I; antibodies that react with the red cells of almost all adults, but do not react, or at best react only weakly, with the red cells of neonates. Anti-I are generally weak, but potent examples may be found as autoantibodies in patients with cold agglutinin disease (CAD) or following *Mycoplasma pneumoniae* infection. In a rare inherited phenotype called adult i the red cells express very little I antigen. Alloanti-I is generally present in sera of i adults.

The i antigen has a reciprocal relationship with I. It is expressed only very weakly on the cells of most adults, but strongly on fetal, neonatal, and adult i red cells. Anti-i cold agglutinins may be haemolytic and are often present in sera of patients with infectious mononucleosis.

I and i determinants are carbohydrate structures carried on glycolipids and glycoproteins. They are internal structures of ABH-active oligosaccharides. The i-active structure is a linear chain of repeating *N*-acetyllactosamine units and is the precursor of the branched I-active structures. In normal development, i antigen is converted to I antigen by the branching of linear oligosaccharide chains. This conversion is catalysed by a β1,6-*N*-acetylglucosaminyltransferase, the product of *GCNT2*. Adult i results from *GCNT2* mutations.

I and i are not the product of alleles. Before *GCNT2* was recognised as being responsible for I biosynthesis, I and i comprised the Ii collection, but subsequently I (I1) formed the I blood group system and i (207002) remained in the Ii collection.

Although most cold agglutinins are Ii antibodies, many other specificities are known that detect determinants on carbohydrate structures of membrane glycoproteins and glycolipids (Section 25.9).

25.2 I (I1) and i antigens

Wiener *et al.* [2] were the first to give the name I to an antigen of variable strength detected by an autoantibody of very high titre. Reports of more anti-I soon followed and most reacted only very weakly with cord red cell samples [3,4]. I antigen strength varies from person to person and titration scores follow a normal distribution curve [5]. Treatment of red cells with proteases or with sialidase generally enhances expression of I and i antigens. The number of I antigen sites per red cell has been estimated at between 32 000 and 500 000 [6–8].

When Marsh and Jenkins [9,10] found two cold agglutinating antibodies that reacted strongly with cord cells

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and adult i cells, but only very weakly with normal adult cells, the opposite of anti-I, they named these antibodies anti-i. Some potent anti-i react quite strongly with adult cells, but their titre may be 1000 times higher with cord cells. Adult i cells generally have higher expression of i than cord cells. The number of i antigen sites has been estimated to be between 20 000 and 65 000 on cord cells and between 30 000 and 70 000 on adult i cells [8].

Expression of i is depressed on red cells of adults with an *In(Lu)* gene, a dominant inhibitor of various red cell antigens resulting from *EKLF* mutations (Section 6.8).

25.3 Biochemistry and molecular genetics

25.3.1 Structure of I and i

Ii antigens are carbohydrates and present on the interior structures of the complex oligosaccharides that carry ABH and Lewis antigens (Sections 2.2 and 2.3). Like the ABH-active structures, Ii determinants on red cell membranes are detected on three major classes of macromolecules:

1 *N*-linked oligosaccharides of glycoproteins;
2 simple glycolipids; and
3 complex glycolipids (polyglycosylceramides).

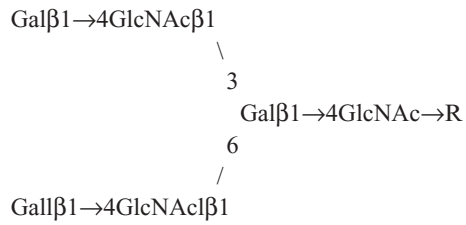
Ii determinants are either accessible or present as partially or totally masked antigens. They are not only detected on red cells, but on many other cell types and in various body secretions (Section 25.5). Recommended reviews on Ii biochemistry are found in [1,11–14] and some important papers on I and i biochemistry are [15–23].

I and i antigens are based entirely on Type 2, Galβ1→4GlcNAc (*N*-acetyllactosamine), chains. They may be concealed in ABH-active oligosaccharides and revealed by the stepwise removal of terminal monosaccharides by chemical degradation [15,16]. Anti-i detect linear structures, prevalent on fetal cells. The basic i structure is an unbranched polylactosamine comprising at least two *N*-acetyllactosamine units:

$$\text{Gal}\beta1 \rightarrow 4\text{GlcNAc}\beta1 \rightarrow 3\text{Gal}\beta1 \rightarrow 4\text{GlcNAc} \rightarrow \text{R}.$$

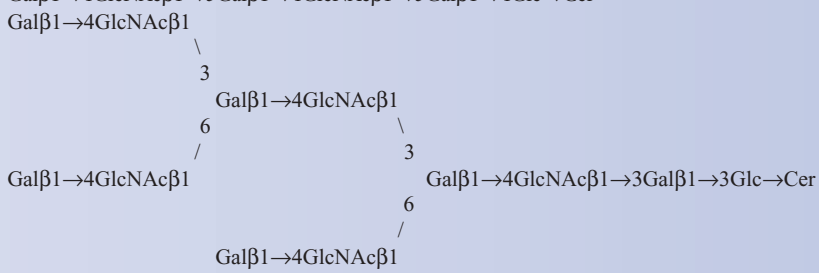
Paragloboside, which has a single lactosamine unit, is not i active; hexasaccharides of three lactosamine units are generally better inhibitors of anti-i than the tetrasaccharide shown above [20,23].

I antigen activity is associated with the branched structure



There are relatively few of these branched structures present on the glycolipids and glycoproteins of fetal cells because most branching occurs after birth [17]. Highly branched polyglycosylceramide molecules, not present in the membranes of fetal or neonatal cells, have a high level of I activity. Ii-active oligosaccharide chains on glycoproteins are generally more complex and linked through GlcNAc to asparagine. Examples of i- and I-active glycolipids are shown in Table 25.1.

Table 25.1 Examples of i- and I-active glycolipids.

i	Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glc→Cer
I	

Much of the chemistry of Ii antigens has been worked out with the aid of numerous monoclonal autoantibodies that differ in their fine specificities. Although no two anti-I appear to be identical they can be subdivided into three general categories [21]:

1 those recognising the Galβ1→4GlcNAcβ1→6 branch (typified by the antibodies Ma. and Woj.);

2 those recognising the Galβ1→4GlcNAcβ1→3 sequence with branching (Step.);

3 those requiring both branches for activity (Phi.).

The basic i and I structures are usually further glycosylated. Ii antibodies vary in their ability to combine with determinants that have additional glycosylation. Terminal Gal residues may be fucosylated by H-transferase to produce an H-active structure, which, in turn, may act as an acceptor substrate for A- and B-transferases to catalyse the addition of GalNAc and Gal respectively (Section 2.3). In red cells of the rare O_h (Bombay) phenotype, in which terminal fucosylation of Ii-active oligosaccharides does not occur because of the absence of H-fucosyltransferase, I antigen expression is enhanced (Section 2.12.6). Destruction of H antigen by treatment of red cells with α1,2-fucosidase from *Aspergillus niger* elevates I expression [24]. Alternatively, the terminal Gal of Ii-active oligosaccharides may be sialylated. This prevents fucosylation and subsequent conversion to A- and B-active structures. These sialylated structures have moderate I or i activity, which is enhanced by sialidase treatment [11,22,25].

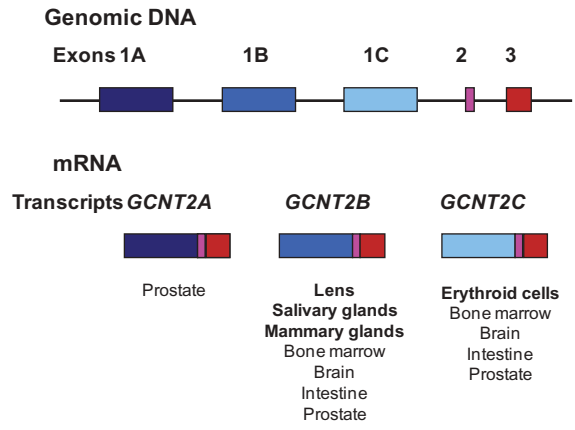


Figure 25.1 Organisation of *GCNT2*, showing the three exons 1 and exons 2 and 3. Below are the three transcripts and some examples of cells and tissues where they are expressed.

25.3.2 Biosynthesis of I

The biosynthesis of i requires the sequential action of β1,3-*N*-acetylglucosaminyltransferase and β1,4-galactosyltransferase. The i antigen is transformed into an I-active structure by the I branching enzyme, a β1,6-*N*-acetylglucosaminyltransferase [11,22,25].

Chinese hamster ovary (CHO) cells usually express i, but no I. Bierhuizen *et al.* [26] used a gene transfer procedure (similar to that described in Section 2.3.1.1 for isolation of an α1,2-fucosyltransferase gene) in order to clone cDNA encoding a β1,6-*N*-acetylglucosaminyltransferase from a cDNA expression library derived from human teratocarcinoma cells, which express large quantities of I-active branched structures. Following primary and secondary transfection, CHO cells expressing I antigen were isolated by panning with anti-I (Ma). The cloned cDNA contained an open reading frame and hydropathy analysis predicted a type II transmembrane protein with topology characteristic of a glycosyltransferase (Section 2.3). I activity of CHO cells transfected with the cloned cDNA appeared to result from branching of i-active *N*-acetyllactosamine chains at GlcNAcβ1→6Gal linkages, suggesting that the cloned gene, *GCNT2*, encodes the I-branching enzyme, a β1,6-*N*-acetylglucosaminyltranferase (IGnT). This was confirmed by the identification of mutations in *GCNT2* responsible for the adult i phenotype [27] (Section 25.4.3).

The coding sequence of *GCNT2* is divided over three exons, but there are three alternative versions of exon1 – exons 1A, 1B, and 1C – encoding 402, 400, and 402 amino acids, respectively [28,29] (Figure 25.1). Transcripts, active in different tissues, contain one of the three first exons plus exons 2 and 3. The transcript active in erythroid precursors contains exons 1C, 2, and 3 (*GCNT2C*). Expression of *GCNT2C* is almost restricted to bone marrow [28,29]. During *ex vivo* erythropoiesis there was a dramatic increase in expression of the *GCNT2C* transcript, with concomitant decrease in *GCNT2B* transcript [29]. The conversion of i to I during early infancy suggests activation of *GCNT2C* during that period. *GCNT2C* is regulated by the transcription factor CCAAT/enhancer binding protein α (C/EPBα) [30]. Dephosphorylation of Ser21 of C/EPBα stimulates transcription of *GCNT2C* and, ultimately, poly-*N*-acetyllactosamine branching [31].

Mapping of 39 expressed sequence tags (ESTs), one of which was identical to *GCNT2*, located *GCNT2* on chromosome 6p24-p23 [32].

Table 25.2 Frequency of adult i phenotype in various populations, determined by testing red cells with anti-I.

Population	No. tested	Adult i		References
		No.	Frequency (%)	
England, London	17 000	0		[3]
France	10 090	1	0.01%	[33]
USA, New York	22 000	5†	0.02%	[2]
African Americans, Detroit	8552	1	0.01%	[34]
Japan	1017	0		[35]
Taiwan	562	0		[36]

†Four black, one white.

25.4 Adult i and other rare phenotypes

25.4.1 Serology, inheritance, and frequency of adult i

Red cells of the rare adult i phenotype are rich in i antigen and but have very low levels of I. Anti-I is usually present. Black people with adult i (i_2) usually have less i and more I than white people (i_1) [3,4,9,10]. Anti-I from i_1 individuals can be adsorbed by i_2, but not i_1, cells.

Numerous family studies have shown that adult i is inherited as a recessive character. In some families with adult i members, red cells of obligate heterozygotes have reduced I and enhanced i compared with cells from unrelated controls [10].

Table 25.2 shows results of several population studies carried out by screening with anti-I. Molecular testing of 51 white donors revealed one heterozygote for an allele responsible for adult i (505A) [28] (see Section 25.4.3), which predicts an incidence of adult i phenotype of about 1 in 10 000.

Only two i adults, one white and one black, were found by screening 2.5 million serum samples from American donors for the presence of anti-I [37]. In another study, eight i adults were found as a result of testing sera of 22 700 pregnant women (0.035%) for anti-I, but only four were found among 135 100 non-pregnant patients (0.003%) [38]. These figures suggest that I depression may be a transient phenotype in pregnancy.

25.4.2 Association between adult i and congenital cataracts

In 1972, Yamaguchi *et al.* [39] reported four adult i propositi with a total of four i and seven I siblings. All eight adult i individuals had congenital cataracts (inherited lens opacity); all seven I individuals had normal vision. In Japan, adult i phenotype is almost invariably accompanied by cataracts [40,41]. Of 31 Japanese adult i donors and patients, 29 suffered impaired vision due to cataracts, and in the 11 families studied there was no recombination between Ii phenotype and cataracts [41]. Two of 92 Chinese in Taiwan with congenital cataracts had the adult i phenotype [36]. In people of European origin, adult i phenotype is not generally accompanied by congenital cataract [42], but the association has been reported in two Caucasian families [43,37], in four Arab and one Persian Jewish families from Israel [44,45], and in two Pakistani families [46].

25.4.3 Molecular basis of adult i and its association with congenital cataracts

Adult i results from homozygosity (or compound heterozygosity) for mutations in *GCNT2*, that include missense mutations, nonsense mutations, and a deletion of most of the coding region of the gene (Table 25.3). All prevent poly-*N*-acetyllactosamine branching in red cells and, therefore, conversion of i to I. Heterozygosity for a pair of *cis* mutations in *GCNT2* (1054G>A, Gly352Arg; 1184C>Tm Ala395Val) was associated with weak I and no i red cell expression [49], a phenotype previously reported as I_{int} [10].

Poly-*N*-acetyllactosamine branching appears to be important in maintaining transparency of the lens. Whereas only *GCNT2C* is expressed in erythroid cells, only *GCNT2B* is expressed in lens epithelial cells [28]. Mutations in exon 1C only inactivate the product of *GCNT2C* and cause adult i red cell phenotype, but leave

Table 25.3 Some *GCNT2* mutations responsible for adult i phenotype.

Mutation†	ISBT *GCNT2**	Exon	Amino acid change†	Cataracts	Ethnicity	References
243T>A	*01W.01*	1C	Asn81Lys	No	East Asian	[47]
505G>A	*01W.02*	1C	Ala169Thr	No	Caucasian	[28]
651delA	*N.07*	1C	Val244stop	No	East Asian	[48]
683G>A	*01W.03*	1C	Arg228Gln	No	Caucasian	[28]
816G>C, 1006G>A	*N.04*	2, 3	Glu272Asp, Gly336Arg	Yes	East Asian	[29]
935G>A		2	Gly312Asp	Yes	Persian Jewish	[45]
978G>A	*N.05*	2	Trp328stop	Yes	Arab	[44]
1043G>A	*N.01*	3	Gly348Glu	Yes	East Asian	[27,29]
1148G>A	*N.02*	3	Arg383His	Yes	East Asian	[27,29]
del exon 1B,1C,2,3	*N.06*	1B,1C,2,3	No protein	Yes	East Asian, Pakistani	[27,28,46]

†Nucleotides and amino acids counted for *GCNT2B* and its product [26]; *GCNT2C* has six more nucleotides and two more amino acids [28,29].

GCNT2B and, therefore, the lens unaffected. Mutations in exon 2 or 3, however, affect all three *GCNT2* transcripts, resulting in adult i and congenital cataracts [28,29].

The equivalent gene in mice is organised in a similar fashion to human *GCNT2*, but mice deficient in the common exon did not develop cataracts earlier than wild-type mice [50].

25.4.4 Other rare phenotypes

Red cells of seven of 5864 healthy blood donors in Mumbai reacted very weakly with anti-I, but did not react with anti-i [51]. The I antigen on these red cells was either weaker or of about the same strength as that on cord cells. Analysis of two large three-generation families showed the rare phenotype to be inherited, although the mode of inheritance appeared to be complex, depending partly on ABO group as all individuals with the unusual phenotype were group A_1 or A_1B [51,52]. Similar phenotypes with depressed expression of both I and i have been found outside India in A_1, B and O individuals [53,54].

25.5 Distribution of Ii antigens

Clausen and Hakomori [13] refer to I and i as histo-blood group antigens because, like ABH, they are not restricted to red blood cells, but are found on the surface of most human cells and on soluble glycoproteins in body fluids.

25.5.1 Body fluids

I antigen can be detected by haemagglutination-inhibition in milk with most anti-I [55–57] and in saliva with rare examples of anti-I [56,58,59]. A high-titred autoanti-I was inhibited to a varying extent by all 181 salivas tested, including saliva from neonates, with no correlation between degree of inhibition and the presence of ABH or Lewis substances [59]. With anti-I Sti., however, the concentration of I substance in saliva was a function of ABH secretor status, non-secretors having much greater quantities of salivary I substance than secretors [60,61]. This result is not surprising as non-secretor salivas lack the H-transferase responsible for fucosylation of the I-active structures. I antigen is generally difficult to detect in plasma by haemagglutination-inhibition [58,59], but one anti-I serum was inhibited by all 39 plasmas tested [62]. Unlike saliva, there is no relationship between I concentration in plasma and ABH secretor status [60–62].The average level of I antigen in plasma from neonates was 25% of that in plasma from adults [62]. Milk from four I women contained I substance and anti-I (or anti-HI) [57].

Saliva, milk, and plasma from i adults contained normal quantities of soluble I antigen, and milk from one i adult inhibited her own anti-I [55,59,61]. It is likely that these studies were carried out with body fluids from i adults of the European type, without cataracts and with mutations in exon 1C of *GCNT2*. The transcripts active in salivary and mammary glands are predominantly *GCNT2B* (Figure 25.1), explaining the presence of

secreted I in these i adults. It would be expected that body fluids from i adults with cataracts, and with mutations affecting exons 2 or 3, would be deficient in I.

Plasma from an individual with I– i– red cells had a reduced quantity of plasma I antigen [61].

There appears to be some i antigen present in saliva and milk, which can be detected by a minority of anti-i [55,58,63]. Haemagglutination by anti-i is inhibited by serum or plasma from most adults and from cord blood samples [58,62–64]. An i-active glycoprotein, with no detectable I, A, B, or H activity, was isolated from serum on an anti-i affinity column [64].

I and i antigens have been detected in amniotic fluid, urine, and ovarian cyst fluid [58,65,66].

25.5.2 Other blood cells

I and i are present on lymphocytes from cord and adult blood [67,68]. Anti-I and -i are potent cold lymphocytotoxins, effective at killing B and T lymphocytes [67,69,70]. Monoclonal anti-i were specific for subsets of B lymphocytes and for most pre-B cells in adult bone marrow [71]. Anti-I and -i are also cytotoxic for peripheral blood monocytes and macrophages, and about 25% of granulocytes; they are equally effective at killing granulocytes obtained from either maternal or cord blood samples [72,73]. Platelets tested with anti-I by flow cytometry produced a broad distribution curve, with the majority of platelets having a low density of I antigens compared with A and B antigens [74].

25.5.3 Other tissues

The gastrointestinal mucosae and the mucins they secrete have been studied in detail for Ii antigens, especially with anti-I (Ma), which detects Galβ1→4GlcNAcβ1→6. This I (Ma) structure is detected in gastrointestinal glycoproteins from ABH non-secretors, but not those from secretors, where it is concealed by the ABH immunodominant monosaccharides [75,76]. The expression of the three *GCNT2* transcripts in various cells and tissues are shown in Figure 25.1 and in references [28,29].

The i antigen is a characteristic of dividing cells present on a variety of cell types including lymphoblasts, fibroblasts, erythroblasts, and thymocytes [77].

25.5.4 Other animal species

Wiener *et al.* [78] tested red cells from over 160 different animal species and found that the distribution of Ii antigens cuts across taxonomic lines. Red cells of most adult primates, including chimpanzees and various monkeys, resemble human neonatal and adult i cells by lacking I and expressing i [78–80]. In tests on red cells of various non-primate species (cat, dog, guinea pig), there was no evidence of a developmental change of i to I [80].

25.6 Ontogenesis and oncogenesis

Ii antigens represent developmental antigens on red cells and in many other tissues. In red cells, and probably most other tissues, conversion begins around the time of birth. Fetal and neonatal red cells have very little I antigen expression and very few branched chains. The increase in I strength and concomitant reduction in i antigen expression reaches adult levels at the age of between 6 and 18 months [10,81] and coincides with the branching process of oligosaccharide chains [17]. High expression of i antigen is also a characteristic of immature and less differentiated adult cells [13]. Conversion from i to I is part of a continual process of erythroid differentiation; circulating 'young' red cells have higher i expression than 'old' cells [82]. The expression of the *GCNT2C* transcript increases dramatically during differentiation of erythroid progenitors from $CD34^+$ cells [29]. The i antigen is detected in the germinating layer of squamous epithelium, such as that from the intestine, whereas branching of the oligosaccharides occurs in the more differentiated cell layers and i is no longer present [83].

I and i often demonstrate altered expression on neoplastic cells and may be considered onco-developmental antigens (reviews in [12,84,85]). *GCNT2* expression, regulated by transforming growth factor (TGF) β, is closely related to metastasis in breast cancer. Enzyme activity enhances cell detachment, migration, and invasion [86].

25.7 I and i antibodies

25.7.1 Anti-I

25.7.1.1 Autoanti-I

The original anti-I was an autoantibody that caused AIHA [2] as were most subsequent examples of high titred anti-I agglutinins [5,87,88]. Potent cold reactive antibodies responsible for cold agglutinin disease (CAD) are usually of I specificity. CAD is reviewed in [1,68,89,90]. These antibodies are generally monoclonal, accounting for the heterogeneity of their specificity. They are usually IgMκ, but IgMλ and IgG autoanti-I occur [1].

They directly agglutinate I-positive red cells at 4°C with varying thermal amplitude, but are generally inactive above 30°C. One autoanti-I active at 30°C caused an acute HTR in a small child when two units of blood were transfused immediately after their removal from the refrigerator [91].

Transient, polyclonal, or oligoclonal autoanti-I may arise from infection, most typically by *Mycoplasma pneumoniae* [1,89]. At least 50% of patients with pneumonia induced by *M. pneumoniae* produce high-titred cold agglutinins during the three weeks after the onset of respiratory symptoms. It is likely that the pathogen modifies a sialylated I-active receptor making it immunogenic and that antibody is then produced to the modified structure [92–94]. Sialylated I determinants are recognised by anti-Sia-lb2 (anti-Gd) and anti-Sia-b1 (anti-Fl) cold agglutinins (Section 25.9) and these antibodies occur together with anti-I in the majority of cases of *M. pneumoniae*-induced cold agglutinin production [95,96]. Most people have weak, cold-reactive autoanti-I in their serum [97]. Analysis by adsorption and elution of 22 sera containing cold agglutinins that could not be clearly defined as anti-I or -i revealed that all contained a separable mixture of both antibodies [98].

25.7.1.2 Alloanti-I

Anti-I of fairly high titre is usually present in the sera of i adults. Although adult i red cells are not totally devoid of I, the anti-I can be referred to as alloanti-I as it does not react with autologous cells. These antibodies are almost invariably IgM and usually only active at low temperatures.

Rare examples may be haemolytic and have a thermal range up to 37°C [55] and some anti-I with a thermal range below 37°C can cause shortened survival of transfused I+ red cells [99]. Less than 1% of radiolabelled I+ red cells survived 15 minutes after injection into an i adult with anti-I, a clear indication that his antibody had the potential to provoke a dangerous transfusion reaction [100]. One anti-I became potentially clinically significant after transfusion of 6 units of I+ blood [101]. Judd [102] has described in detail how he would manage cold agglutinins in a transfusion setting.

25.7.1.3 Anti-I lectin

Lectin prepared from the gonads of *Aplysia depilans*, a marine mollusc (sea slug), behaved serologically as anti-I [103]. All other lectins resembling anti-I have specificities dependent on the presence of ABH or P antigens [103] (Section 25.7.6).

25.7.2 Anti-i

Alloanti-i has not been recognised. The first three examples of autoanti-i were from patients with reticulosis, one of whom died with AIHA [3,4]; a fourth example was in a patient with myeloid leukaemia [88]. Anti-i is a rare alternative to anti-I in CAD [89]. Anti-i are heterogeneous in specificity [8,16,77,104].

Anti-i is often found in the serum of patients with infectious mononucleosis and occasionally causes haemolysis. Estimates of the proportion of infectious mononucleosis patients with anti-i vary between 8 and 90%, but only very few develop haemolytic complications [79,105–107]. These antibodies may be IgM or, in some cases, may be IgG anti-i combined with IgM anti-IgG [79,106–111]. One of the IgM anti-i behaved like a Donath-Landsteiner antibody (haemolysis in the presence of complement following incubation of red cells in serum at 4°C and subsequent warming to 37°C) [110], as did an IgG anti-i detected in a patient with chronic paroxysmal cold haemoglobinuria [112].

The presence of anti-i is associated with immunodeficiency. Autoanti-i activity was detected in 50% of patients with Wiskott–Aldrich syndrome, a rare X-linked recessive immunodeficiency [71], and in 64% of patients with HIV/AIDS [113]. Infection with Epstein-Barr virus, the pathogen associated with infectious mononucleosis, is endemic in HIV/AIDS.

Maternal IgG autoanti-i can cross the placenta and has resulted in positive DATs with cord cells and mild neonatal jaundice [114,115]. Acute intravascular haemolysis in a patient with anti-i followed infusion of two units of blood deemed compatible by an immediate spin crossmatch technique [116].

Monoclonal anti-i have been produced by heterohybridomas of mouse myeloma and human lymphoid cells [71,83].

25.7.3 Structure of I and i antibodies

A rat monoclonal antibody, 9G4, recognises a cross-reacting idiotypic determinant present on virtually all pathogenic anti-I and -i cold agglutinins and specifically inhibits haemagglutination by these antibodies [117,118]; it does not generally react with cold agglutinins of other specificities (anti-Pr, etc.) [119]. The epitope recognised by 9G4 is on an IgM heavy chain variable region derived from a single, highly conserved common gene segment V4-34 (previously called V_H4-21) [71,120–122]. Although all non-pathogenic monoclonal, naturally occurring anti-i also appear to be V4-34 encoded antibodies [123], this is not the case for non-pathogenic anti-I where V_H3

genes are often involved [124]. Site-directed mutagenesis analysis demonstrated that both Trp7 and an Ala23-Val-Tyr motif of framework region 1 (FR1) of V4-34 encoded immunoglobulin are required for I binding [125]. Based on the three-dimensional structure of an anti-I cold agglutinin [126], those amino acids essential for I antigen binding appear to form an extensive hydrophobic patch in FR1 [125].

A majority of IgM Rh antibodies also have the V4-34 encoded heavy chain segment. In addition to their Rh specificity, these antibodies have cold agglutinin activity directed against I/i antigens, although patient-derived cold agglutinins never detect Rh antigens [127,128]. The same amino acid residues in FR1 were found to be essential for both anti-D and anti-i reactivity of V4-34-encoded IgM anti-D [129].

25.7.4 Anti-I^T

Anti-I^T defines an antigen expressed strongly on cord cells, weakly on most adult cells, and very weakly on adult i cells [130]. I^T is expressed very weakly on red cells of Melanesians with South-East Asian ovalocytosis [131,132] (Section 10.9). Cold agglutinins were detected in 76% of Melanesians from Papua New Guinea; of the six analysed in detail, one was anti-I and five were anti-I^T [130]. Anti-I^T was found in 84% of Yanomama Indians of Venezuela [133]. Outside of those populations, rare examples of anti-I^T are often IgG and found in patients with Hodgkin's lymphoma or other lymphoproliferative diseases [134–136]. These antibodies may be active at 37°C and responsible for AIHA [134,136]. Six examples of IgG autoanti-I^T were not apparently clinically significant as judged by response to incompatible transfusion and *in vitro* survival studies [137,138]. IgM anti-I^T can also cause AIHA [139,140].

25.7.5 Anti-j

Cold agglutinins in two patients, which agglutinated adult and cord red cells, behaved as anti-Ii and were named anti-j [141]. These antibodies reacted with protease- and sialidase-treated red cells, but not with cells treated with endo-β-galactosidase, which cleaves Type 2 oligosaccharide chains. The antibodies were inhibited by linear (i) and branched (I) Type 2 structures. The two anti-j were unusual cold agglutinins as they were IgMλ molecules; they resembled most pathogenic anti-I and -i by expressing the 9G4 idiotype, a characteristic of antibodies encoded by a gene utilising a V4-34 sequence.

25.7.6 Ii antibodies and the H, ABO, and P groups

Considering the heterogeneity of Ii antibodies and the close biochemical association between Ii and the H, A, and B antigens, it is of no surprise that some antibodies appear to show a preference for I determinants with attached H, A, or B immunodominant monosaccharides. The most abundant of these antibodies is anti-HI, which does not react, or at least reacts only very weakly, with I-positive cells of the rare H-deficient phenotypes (described in Section 2.14.8). Some anti-I resemble anti-HI by giving stronger reactions with O or A_2 cells than with A_1 red cells [3,88,142]. Anti-Hi has also been reported [143]. Some anti-I react more strongly with A, B, or AB cells than with O cells. These antibodies have been called anti-AI (or -A_1I) [4,142,144,145], -BI [144,146–148], and -(A+B)I [149]). Anti-HILe^b (or -ILe^{b}H) agglutinated only O or A_2, I-positive, Le(a–b+) cells [150,151].

P1 antibodies that do not agglutinate P1+ cord or P1+ adult i red cells are called anti-IP1 [152]. An antibody reacting only weakly with cord and adult i cells, and not at all with p cells, was named anti-IP [153]. Anti-I^TP was responsible for fatal AIHA [154].

25.8 I and i antigens and disease

Red cells of patients with dyserythropoietic conditions often have elevated expression of i [73,155–158]. These conditions include thalassaemia, sickle cell disease, congenital dyserythropoietic anaemia II, Diamond–Blackfan syndrome, myeloblastic erythropoiesis, sideroblastic erythropoiesis, refractory anaemia, paroxysmal nocturnal haemoglobinuria, and acute leukaemias. Red cells with increased i antigen also appear in the circulation of people subjected to repeated phlebotomy [159]. I expression is not generally decreased in these conditions [156]. When red cell production is inadequate to meet demands, the proliferative stress on the erythroid precursors results in shortened maturation time before the red cell precursors appear in the peripheral blood. This could account, at least in part, for enhanced i expression.

The i antigen is detected on lymphocytes from adults and cord samples [67]. In chronic lymphocytic leukaemia (CLL) there is a reduction in lymphocyte i expression, as detected by some anti-i [160]. In acute lymphoid leukaemia, blast cells have as much i antigen as normal lymphocytes, whereas in acute myeloid leukaemia, blast cells have much less i antigen [161]. Lymphoblasts can be

distinguished from myeloblasts by their i expression in undifferentiated acute leukaemia and in chronic myeloid leukaemia in blast-cell crisis [162].

The late activation of *GCNT2* may have evolved as a mechanism to prevent ABO HDFN [31]. Despite the common occurrence of maternal-fetal ABO incompatibility, and the prevalence of IgG ABO antibodies, particularly in group O individuals, severe HDFN caused by ABO antibodies is rare (Section 2.14.3). This results, in part, from the linear structure of A- and B-active oligosaccharides on fetal and neonatal red cells, which prevent monogamous bivalency (the binding of both Fab arms) of IgG, and weakens the interaction between antibody and antigen.

25.9 Other cold agglutinins

In addition to anti-I and -i, cold agglutinins of numerous other specificities have been defined, mostly by Roelcke and his colleagues (for reviews see [1,163]). These antibodies and some characteristics of the determinants they define are listed in Table 25.4. All comply with the definition of cold agglutinins: autoantibodies to red cell antigens that react optimally in the cold (0°C) [1]. Apart from anti-j, they are usually IgMκ monoclonal antibodies.

The most abundant cold agglutinins, after anti-I and -i, are the Pr antibodies. These detect protease-labile determinants on the *O*-linked sialotetrasaccharides and sialotrisaccharides found predominantly on glycophorins A and B. These glycoproteins also carry blood group M, N, S, and s antigens and so Pr is described in Section 3.6.4. Pr antibodies are heterogeneous and have been subdivided. Anti-Pr_1, -Pr_2, and -Pr_3 are distinguished from each other by the effects of certain chemical modifications of their determinants [1]. Anti-Sa, like anti-Pr_2, detects an antigen present on glycophorin A and also on some gangliosides [179,180]. All of 15 anti-Pr contained Vκ light chains, with Vκ IV subgroup predominating [181]. A few anti-Pr and -Sa are IgAκ [165,182]. Pr antibodies may be associated with rubella infection [183] and Pr autoantibodies may cause severe AIHA (Section 3.6.4).

Anti-Sia-lb (anti-sialo-linear-branched, formerly anti-Gd) represents a heterogeneous collection of antibodies detecting protease-resistant, sialidase-sensitive antigens

Table 25.4 Antigens defined by cold agglutinins, showing reactions of the antibodies with adult (Ad), cord (Cd), adult i (i ad), papain- (Pap) and sialidase-treated (Sial) red cells.

Antigen	Ad	Cd	i ad	Pap	Sial	Comments	References
I	+	w	w	+	+	Branched; glycoproteins and glycolipids	See text
i	w	+	+	+	+	Linear; glycoproteins and glycolipids	See text
I^T	w	+	w	+	+		See text
j	+	+	+	+	+	Linear and branched; glycoproteins and glycolipids	[141]
$Pr_{1–3}$	+	+	+	0	0	*O*-glycans of glycophorins	*
Pr_a	+	+	+	0	+		*
Sa	+	+	+	w	0	*O*-glycans of glycophorins and gangliosides	[164,165] *
Sia-lb1 (Gd_1)	+	+	+	+	0	Sialylated linear and branched; glycolipids	[166,167]
Sia-lb2 (Gd_2)	+	+	+	+	0	Sialylated linear and branched; glycolipids	[166,167]
Sia-b1 (Fl)	+	w	w	+	0	Sialylated branched; glycolipids	[168,169]
Sia-l1 (Vo)	w	+	+	+	w	Sialylated linear; glycolipids	[169,170]
Li	w	+	+	+	0	Sialylated linear; glycolipids	[169,171]
Lud	+	w	+	w	0		[172]
Me	+	+		+	+	Enhanced by human milk	[173]
Om	+	+		+	+	Not enhanced by human milk	[174]
Ju	+	+		w	w		[175]
IgM^{WOO}	0	0	0	0	+	Type 1 chain	[176]
Rx	+	w	+	+	+	pH optimum 6.5; previously Sd^x	[177,178]

*See Section 3.5.4.

+, strong agglutination; w, relatively weak agglutination compared with +; 0, no agglutination.

located on gangliosides and created by the α2,3-sialylation of I- and i-active structures (branched and linear) on glycolipids [1,166,169]. Anti-Sia-lb1 require only a terminal sialic acid residue for activity (NeuNAcα2,3–); anti-Sia-lb2 also require subterminal Gal (NeuAcα2→3Galβ1–) [167]. Sia-lb antibodies also bind sialyl-Le^a (sLe^a) and sialyl-Le^x (sLe^x) structures (see Table 2.3) expressed on nucleated cells and in soluble cancer-related mucins [184].

Sia-b1 (Fl) is located on glycolipids with sialylated branched structures, whereas Sia-l1 (Vo) and Li are probably α2,3-sialylated linear structures on glycolipids [94,168–171,184].

Very little is known about the biochemistry of the Lud, Om, Me, and Ju antigens. Anti-Lud recognises α2,3-sialylated Type 1 chain sequences [1,185]. Activity of anti-Me (which is not related to anti-M^e of the MNS system), is enhanced by preheated human milk, but not by individual milk sugars [173]. Anti-Om activity is slightly reduced by human milk [174], which distinguishes it from anti-Me. The cold agglutinin IgM^{WOO}, which agglutinates sialidase-treated cells, but not untreated cells, recognises the Type 1 chain Galβ1→3GlcNAcβ1→3Galβ1→4Glc/GlcNAc [176]. Anti-Rx was originally named anti-Sd^x because it appeared to be inhibited by Sd(a+) but not Sd(a–) urine [177,186]. This was shown to be a non-specific effect, probably resulting from the extreme pH dependency of the antibody [178].

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26 Gill Blood Group System

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26.1 Introduction

GIL, the only antigen of the Gill system, is an antigen of very high frequency located on the water and glycerol channel aquaporin-3 (AQP3). The GIL– phenotype results from homozygosity for a splice site mutation in *AQP3*.

26.2 GIL (GIL1) and anti-GIL

Five examples of anti-GIL have been identified, all in white women who had been pregnant at least twice [1]. No GIL – individual was found by screening 23 251 white Americans or 2841 African Americans with anti-GIL. Red cells of two of the babies of mothers with anti-GIL gave a positive DAT, but there were no clinical symptoms of HDFN. Anti-GIL may have been responsible for an HTR and results of monocyte monolayer assays with two anti-GIL suggested a potential to cause accelerated destruction of transfused GIL+ red cells.

26.3 Aquaporin-3 and GIL

Aquaporin-3 (AQP3) is a member of the aquaporin family of water channels described in Chapter 15 (for reviews see Chapter 15 and [2]). Aquaporins, including AQP3, have the characteristic 'hourglass' structure, spanning the membrane six times, shown in Figure 15.1.

AQP3 cDNA was isolated from a rat kidney cDNA library by screening with a PCR product with a sequence similar to that of other aquaporins [3]. Rat *AQP3* cDNA was used to screen a human kidney cDNA library [4] and the isolated human *AQP3* cDNA used to screen a human placental genomic library [5]. *AQP3* comprises six exons that encode the following amino acid residues: exon 1, 1–36; exon 2, 37–78; exon 3, 79–125; exon 4, 126–165; exon 5, 166–237; exon 6, 238–292. The 5′-flanking region has a TATA box, two Sp1 sequences, and some consensus sequences including AP2 sites [5]. *AQP3* was shown, by fluorescence *in situ* hybridisation, to be located on chromosome 9q13 (erratum to [4]). The predicted protein consists of 292 amino acids, with the typical aquaporin six membrane-spanning topology, *N*-glycosylated at Asn141 on loop C (Figure 15.1). Unlike AQP1, which forms tetramers in the membrane, AQP3 appears to exist in red cell membranes in multiple oligomeric forms (dimers, trimers, tetramers) composed of weakly associated monomers [6].

In 1998, Roudier *et al.* [7] showed that AQP3 is present in red cells and then, in 2002, that AQP3 is the GIL blood group antigen [8]. They found by immunoblotting with anti-rat AQP3, which cross-reacts with human AQP3, that red cells of two individuals with the GIL– phenotype were deficient in AQP3. Both GIL– individuals, one from the USA and one from France, were homozygous for G>A in the invariant 5′ donor splice site of intron 5 (IVS5+1g>a), resulting in a transcript lacking exon 5 and introducing a reading frameshift and premature termination of translation, predicting a truncated protein lacking amino acids 165–237. The sister and 10 children of one of the propositi were heterozygous for the mutation and expressed approximately a half-dose of AQP3 on their red cells. COS-7 cells became GIL+ following transfection with *AQP3* cDNA.

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26.4 Functional aspects

Based on their permeability characteristics, there are two types of aquaporins: those permeated only by water, which includes AQP1 (Chapter 15), and the aquaglyceroporins, which are permeated by water and by small solutes, especially glycerol, and includes AQP3. AQP3 is permeable to glycerol and water, plus urea and hydrogen peroxide [2,9].

In addition to red cells, AQP3 is present in a variety of tissues, including kidney, skin, lung, eye, and colon, where it is located in the basolateral plasma membrane. GIL–, AQP3-deficient individuals are apparently healthy, possibly because the absence of AQP3 can be compensated by other aquaporins [1,8]. *AQP3* knockout mice showed a urinary concentrating defect, manifesting as nephrogenic diabetes insipidus, and skin defect arising from failure to maintain skin hydration [2]. Mice do not have AQP3 on their red cells [6]. The function of AQP3 in red cells is not known, although it could make them less susceptible to osmotic stress during exposure to high glycerol concentration [7]. GIL– red cells exhibited a drastic reduction of glycerol permeability, but water and urea transports were normal [8].

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27 Junior and Langereis Blood Group Systems

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27.1 Introduction

Jr^a (JR1) and Lan (LAN1) are antigens of very high frequency in most populations. In 2012 they were shown to be located on the ATP-binding cassette transporters ABCG2 and ABCB6, respectively, and so became the sole representatives of the Junior and Langereis blood group systems.

27.2 ATP-binding cassette (ABC) transporters

ABC transporters form one of the largest and most diverse protein superfamilies and are present in all living cells and organisms. Forty-eight ABC transporters are present in humans and are located within external and internal membranes of cells. They are classified into seven subfamilies, ABCA to ABCG, based on their gene and amino acid sequences and domain organisation. A typical active ABC transporter comprises two transmembrane (TM) domains, consisting of between six and eleven membrane-spanning α-helices, and two nucleotide-binding domains (NBDs) (Figure 27.1). Half-transporters have only one TMD and one NBD, and are functionally dependent homodimer or heterodimer formation. Full transporters are usually found in the external membrane, whereas half-transporters are generally located in subcellular organelles. The NBDs, which bind and hydrolyse ATP to fuel transport activity, contain three characteristic motifs: a signature motif (LSGGQ) unique to this superfamily, flanked by Walker A and Walker B motifs.

ABC transporters translocate multifarious hydrophobic substrates across biological membranes. They have a wide variety of functions and genetic defects lead to various diseases, including cystic fibrosis. They have also been implicated in multidrug resistance in cancer. For reviews on ABC transporters, see [1–3].

27.3 Junior system, Jr^a antigen, and ABCG2

The first five examples of anti-Jr^a were described briefly in 1970 by Stroup and MacIlroy [4], who were able to test the families of four of the propositi and found a total of seven Jr(a–) siblings, none of whom had made anti-Jr^a.

27.3.1 Frequency and ethnic distribution

Jr(a–) is much less rare in Japanese than in most other populations (Table 27.1). Frequencies vary greatly in different regions of Japan with an incidence of Jr(a–) of around one in 60 in the Niigata region of northwest Japan. Jr(a–) has been found in people of Northern European extraction [9–12], particularly in Gypsy populations [12,13], in Bedouin Arabs [14], and in a Vietnamese [15].

27.3.2 Anti-Jr^a

Anti-Jr^a may be stimulated by transfusion or by pregnancy and has been detected in untransfused Jr(a–)

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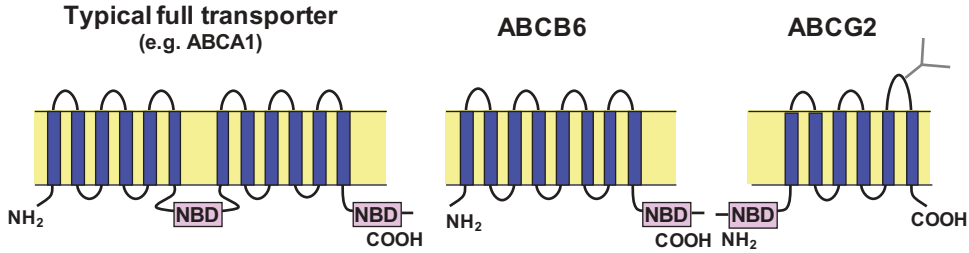


Figure 27.1 Diagram of three ABC proteins in a membrane: a typical full transporter and two half-transporters, ABCB6 (Langereis) and ABCG2 (Junior). NBD, nucleotide binding domain.

Table 27.1 Frequency of Jr^a.

Population	No. tested	No. negative	Antigen frequency	References
Japanese	19 298	5	0.9997	[5]
Japanese	28 744	19	0.9993	[6]
Japanese, Osaka	994	2	0.9980	[7]
Japanese, Niigata	460	8	0.9826	[7]
American	9545	0		[8,9]

women during their first pregnancy [16,17]. IgM anti-Jr^a was found in the sera of two Jr(a–) brothers who had not been transfused. Most anti-Jr^a are IgG and those that have been subclassed were predominantly IgG1, sometimes together with IgG3 [14,16–19]. Anti-Jr^a may fix complement [5,15,20–22].

Many transfusions of Jr(a+) red cells to patients have resulted in no signs of haemolysis and no adverse effects, although incompatible transfusion may cause a sharp rise in the titre of the anti-Jr^a, resulting in signs of an acute HTR in subsequent transfusions [21]. A patient with anti-Jr^a developed rigors after transfusion of 150 ml of crossmatch-incompatible blood [23]; another signs of a mild delayed HTR after transfusion of multiple units of Jr(a+) red cells [15]. Injection of radiolabelled Jr(a+) red cells into a patient with anti-Jr^a resulted in moderate destruction of the cells with no Jr(a+) cells remaining after 24 hours [10]. Five of 14 anti-Jr^a gave results suggesting potential clinical significance on monocyte monolayer assays [24], as did assays on some other patients [15,21]. Least incompatible red cells may be suitable for transfusion to most patients with anti-Jr^a, but Jr(a–) red cells should be selected in cases where the anti-Jr^a is of high titre.

Anti-Jr^a is a dangerous antibody in pregnancy and has been implicated in severe and fatal HDFN [11,12, 19,22], although in other pregnancies with maternal anti-Jr^a indications of HDFN have been no more than a positive DAT on cord cells or mild neonatal jaundice [5,16,17,20,25].

An IgG3 human monoclonal anti-Jr^a was produced from lymphocytes of a blood donor with anti-Jr^a [6].

27.3.3 ABCG2 and the molecular basis for Jr(a–)

In 2012 Zelinski *et al.* [26] and Saison *et al.* [27] reported, in the same issue of *Nature Genetics*, identification by very different methods that the gene encoding Jr^a is *ABCG2*. One group used SNP analysis on genomic DNA from six Jr(a–) individuals to locate a homozygous region at chromosome 4q22.1, and then identified *ABCG2* as only one of the four genes in the region expressed on red cells [26]. The other group used monoclonal anti-Jr^a to isolate a protein from cat red cells, which express the antigen strongly. The protein was then identified as an orthologue of human ABCG2 by mass spectrometry [27]. Homozygosity or compound heterozygosity for 13 different mutations in *ABCG2* were found in Jr(a–) individuals (Table 27.2). All are inactivating mutations, with the exception of a missense mutation encoding Val12Met in the N-terminal sequence before the NBD. This mutation was found in a Jr(a–) individual with anti-Jr^a, so probably prevents protein expression in the red cell membrane.

ABCG2 is located at chromosome 4q22.1, spans over 66 kb, and contains 16 exons, with the translation start site in exon 2. The promoter region has a CCAAT box, but no TATA box, and several Sp1 sites plus AP1 and AP2 sites downstream from a putative CpG island [29]. ABCG2 is a 72 kDa protein consisting of 665 amino acids.

Table 27.2 *ABCG2* mutations associated with Jr(a–) phenotype.

Nucleotide change	Location	Amino acid change	Population	References
34G>A	Exon 2	Val12Met	Asian, Caucasian	[26,28]
187–197delATATTATCGAA	Exon 2	Ile63Tyr fs 54stop	European	[27]
337C>T	Exon 4	Arg113stop	Caucasian	[28]
376C>T	Exon 4	Gln126stop	Asian, Caucasian	[26–28]
542–543insA	Exon 6	Phe182Val fs14 stop	European	[27]
706C>T	Exon 7	Arg236stop	Asian, European, European Gypsy, N. African	[26–28]
730C>T	Exon 7	Gln244stop	European	[27]
736C>T	Exon 7	Arg246stop	Caucasian	[26,28]
784G>T	Exon 7	Gly262stop	Caucasian	[28]
791–792delTT	Exon 7	leu264His fs 14stop	European Gypsy, Caribbean	[27]
875–878dupACTT	Exon 8	Phe293Leu fs 8stop	Caribbean	[27]
1111–1112delAC	Exon 9	Thr37Leu fs 20stop	Pakistani	[27]
1591C>T	Exon 13	Gln531stop	Caucasian	[28]

Jr(a–) phenotype arises from either homozygosity or compound heterozygosity.

It has a single TMD, with six membrane-spanning α-helices, an N-terminal NBD, and is glycosylated at Asn596 in the third extracellular loop (Figure 27.1). As a half-transporter, ABCG2 forms dimers or possibly oligomers in order to function (for review see [30,31]).

ABCG2 is present in many different human cells and may have multiple functions. It was first identified as a multidrug resistance protein [32]. It also functions as a high-capacity uric acid transporter, and Gln126stop, which is a cause of Jr(a–) phenotype, is considered a major cause of primary gout in Japan [33]. Val12Met, however, also responsible for Jr(a–), was not found to confer any risk. A genome-wide association study identified an association between *ABCG2* and serum uric acid levels and risk of gout in people of European and African origin [34]. Significant elevation of serum urate levels were not detected, however, in the absence of ABCG2 in pregnant Jr(a–) women [27]. Porphyrin levels were very low in the plasma and elevated in the red cells of Jr(a–) individuals, suggesting that ABCG2 may share the function of red cell porphyrin transporter with ABCB6 [27] (Section 27.4.3).

27.4 Langereis system, Lan (LAN1) antigen, and ABCB6

A severe HTR resulted in the identification of a new public antigen, Lan [35]. The patient, Mr Lan, had a Lan– brother. Two other public antigens, Gn^a and So, were later shown to be the same as Lan [36–38].

Table 27.3 Frequency of Lan in various populations.

Population	No. tested	No. negative	Antigen frequency	References
American	6653	1	0.9998	[36,38–41]
British	28 992	0	>0.9999	[42,43]
Japanese	713 384	14	>0.9999	[44]
Black South African*	6000	4	0.9993	[45]

*Including donors of mixed ethnic origin.

27.4.1 Lan antigen: frequency and variants

Screening of red cells from almost 40 000 blood donors from America, Britain, and Holland (mostly Caucasian) with alloanti-Lan revealed only two Lan-negatives (1 in 20 000) and with monoclonal anti-Lan of red cells from 713 384 Japanese donors revealed 14 Lan-negatives (1 in 50 000) (Table 27.3). Anti-Lan has been reported in two African Americans [46,47].

A quantitative variant, in which Lan is expressed very weakly, has also been shown to be inherited [48]. Red cells with this Lan-weak phenotype can easily be mistaken for

Table 27.4 *ABCB6* mutations associated with Lan– phenotype [44,56].

Nucleotide change	Location	Amino acid change	Population
197–198inG	Exon 1	Ala66Gly fs 96stop	European
574C>T	Exon 2	Arg192Trp	N. African
717G>A	Exon 3	Gln239stop	European
953–956delGTGG	Exon 4	Gly318Ala fs 8stop	European
1533–1543dupCGGCTCCCTGC	Exon 9	Leu515Pro fs 17stop	European
1690–1691delAT	Exon 11	Met564Val fs 2stop	Japanese
1709–1710delAG	Exon 11	Glu570Gly fs 21stop	European
1867delinsAACAGGTGA	Exon 14	Gly623Asn fs 3stop	European
1942C>T	Exon 14	Arg648stop	European, N. African
1985–1986delTC	Exon 15	Leu662Pro fs 15stop	European
2256 + 2t > g	Intron 16	Splicing defect	European

Lan– phenotype arises from either homozygosity or compound heterozygosity.

Lan–. Five of 15 apparent Lan– samples were subsequently shown to have weak Lan on testing with more potent reagents [49].

Cord red cells had higher Lan activity than adult cells, as determined by a monoclonal antibody [44].

27.4.2 Anti-Lan

Anti-Lan may be stimulated by transfusion or pregnancy [35,36,38,39,50,51]. There is no report of 'naturally occurring' alloanti-Lan; none of the Lan– siblings of Lan– propositi has anti-Lan. Lan alloantibodies are mostly IgG1 and IgG3, although IgG2 and IgG4 may also be present [18,24,50,52]. Some anti-Lan fix complement [35,43,50,53], others do not [39,50].

The original anti-Lan was responsible for an immediate HTR characterised by fever and chills [35]. The potential of other examples of anti-Lan to cause red cell destruction has been demonstrated by *in vivo* red cell survival studies and *in vitro* functional assays [24,53–55]. Ideally Lan– red cells should be selected for transfusion to patients with anti-Lan, although least incompatible red cells may be suitable for patients with weak examples of the antibody.

Anti-Lan has not been implicated in serious HDFN. Two babies of mothers with anti-Lan and whose cord red cells gave a positive DAT received phototherapy and one of them (whose red cells were also coated with anti-c and -Jk^a) was transfused with Lan+ red cells [41,51].

The only reported autoanti-Lan was in a patient with mild AIHA [54]. Her red cells appeared to have depressed Lan expression and gave a weakly positive DAT. Monoclonal anti-Lan was produced from lymphocytes of a donor with anti-Lan [44].

27.4.3 ABCB6 and the molecular basis of Lan–

Helias *et al.* [44] used monoclonal anti-Lan to purify the Lan antigen from red cells: an 80 kDa membrane protein identified by mass spectrometry as ABCB6. Ten inactivating mutations were identified in 11 unrelated Lan– individuals: nine were homozygous and two were compound heterozygotes (Table 27.4). Homozygosity for a missense mutation, Arg192Trp, was also responsible for a true Lan– phenotype associated with anti-Lan production [56]. Heterozygosity for three other *ABCB6* mutations (826C>T, Arg276Trp; 85–87delTTC, Phe29del; 1762G>A, Gly588Ser) appeared to cause a reduced level of Lan expression, suggesting that they are also null alleles [56].

Human *ABCB6* gene was identified by screening a human liver cDNA library with an expressed sequence tag (EST) revealed by searching a human EST database for an orthologue of a mouse ABC gene [57]. The cDNA predicted an 842 amino acid ABC half-transporter with a TMD containing eight membrane-spanning α-helices (Figure 27.1). *ABCB6* is located on chromosome 2q36, spans 11 kb, and contains 19 exons. ABCB6 exists in two forms of MW 104 kDa and 79 kDa in mitochondrial and plasma membranes [58]. During haem biosynthesis in erythroid cells, ABCB6 appears to function by importing porphyrin into mitochondria. In mature red cells, which have no mitochondria, ABCB6 may export porphyrins from the cells to prevent their accumulation [58,59].

ABCB6 may also have a general role of regulating haem biosynthesis in non-erythroid cells. Another porphyrin transporter, possibly ABCG2 (Jr^a protein, Section 27.3.3), could compensate for the absence of ABCB6 in the Lan– phenotype [44].

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28 Er Antigens

28.1 Introduction, 493
28.2 Er antigens, 493
28.3 Antibodies, 494

28.1 Introduction

Er^a and Er^b, the products of alleles, are high and low frequency antigens, respectively; Er3 is defined by an antibody produced by an Er(a–b–) individual. These three antigens constitute collection 208 of the ISBT terminology, the Er collection: Er^a is ER1 (208001); Er^b is ER2 (208002); and Er3 is ER3 (208003).

28.2 Er antigens

28.2.1 Er^a and Er^b (ER1 and ER2): inheritance and frequencies

Families of two of the original three Er(a–) propositi described by Daniels *et al.* [1] in 1982 showed Er(a–) to be an inherited character. In 1988, Hamilton *et al.* [2] described an antibody to a low frequency antigen, named Er^b, that reacted with five of six Er(a–) red cell samples. In one family the presence of Er(a–) in two generations resulted from an Er(a–b+) × Er(a+b+) mating [2]. Er^a and Er^b, therefore, appear to be inherited regularly as co-dominant alleles. Family studies have shown that Er is not part of the ABO, MNS, P1PK, Duffy, Kidd, or Dombrock systems [1–3].

Er(a–) phenotype has only been found in people of European origin ([1,4–6] and several other unpublished examples), including a Mexican family [7], although an abnormal Er(a–) phenotype was identified in a Japanese family [3] (see below). No Er(a–) individual was found in tests on red cells from 63 762 mostly white [1,4,8] and 13 521 Japanese [3] blood donors.

Four of 605 random white donors were Er(b+) and the frequency of the Er^b allele is calculated as 0.0033 [2]. If the existence of a third allele is disregarded, the Er^a allele has a frequency of 0.9967 and Er(a–) would only be expected in about 1 in 100 000 white people.

Red cells of a Japanese woman and two of her siblings were negative with five anti-Er^a (including the original), but reacted with three others [3]. Positive and negative results were confirmed by adsorption techniques. The serum of the propositus, who had been transfused twice and pregnant three times, contained an antibody that resembled anti-Er^a: it reacted with all cells except Er(a–b+) cells and those of the propositus and two of her siblings.

28.2.2 Er3

Er(a–b–) phenotype has been identified in two unrelated individuals and an Er(a+b–) daughter had an Er(a–b+) mother, suggesting the presence of a third allele [2,9]. One of the Er(a–b–) individuals, a Caucasian man with consanguineous parents, had an antibody, named anti-Er3, that reacted with all red cells tested, including Er(a–) cells [9]. Anti-Er3 reacted with the red cells of the other Er(a–b–) person, suggesting that the man with anti-Er3 may have an Er-null phenotype, whereas the other Er(a–b–) individual could have another active allele that produces neither Er^a nor Er^b.

28.2.3 Antigen characteristics

Er^a is fully expressed on cord cells and is not sensitive to the treatment of red cells with proteases (trypsin, chymotrypsin, papain, ficin, pronase), sialidase, or the

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disulphide bond reducing agent AET. Incubation of red cells in low pH EDTA/glycine buffers, often used in antibody-elution tests, resulted in loss of Er^a and Er3 [9,10]. There was total loss of Er^a at pH 2.0, partial loss at pH 2.5, and no apparent loss at pH 3.0 [10].

Er^b is resistant to treatment of red cells with ficin, papain, or DTT [2].

28.3 Antibodies

All the recorded producers of anti-Er^a have been transfused and/or pregnant [1,3–7,11]. The antibodies are IgG and do not fix complement [1,3,4,7]. In two patients with anti-Er^a, Er(a+) red cells gave a positive DAT after transfusion, but there were no signs of haemolysis [1,4]. The patient with anti-Er3 showed signs suggesting mild haemolysis following transfusion of one unit of incompatible red cells [9]. Monocyte phagocytosis assays and *in vivo* red cell survival studies provided additional evidence that Er^a antibodies are not clinically significant [1,4,7], but that the anti-Er3 was potentially significant [9]. Red cells of three babies born to women with anti-Er^a gave positive DATs, but none had HDFN [1,6,11].

The producers of the only two known anti-Er^b had been pregnant, but not transfused; both had Er(a+b+) husbands [2,12]. Er(b+) red cells from babies of both of the women with anti-Er^b gave strongly positive DATs, but there were no other indications of HDFN.

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29 Low Frequency Antigens

29.1 Antigens, 495
29.2 Antibodies, 495
29.3 Additional information on some of the antigens and antibodies, 496

29.1 Antigens

Many red cell antigens occur only very rarely in most populations and have not been shown to belong to any of the existing blood group systems or collections. Some have only been found in a solitary family. In the numerical notation, low frequency antigens (LFAs) make up the 700 series. The criteria for joining this series of antigens are as follows:

1 the antigen must have a frequency of less than 1%;
2 it must be an inherited character;
3 it must not be part of an existing blood group system or be related closely enough to another antigen to merit collection status;
4 it must have been shown to be serologically distinct from all other antigens of low frequency;
5 antibody and red cells carrying the antigen must be available, so that further examples can be identified.

LFAs of the 700 series are listed in Table 29.1. Many numbers have become obsolete, either because the corresponding antigens have been elevated to blood group systems or collections, or because they have become extinct because antibody or antigen-positive red cells are unavailable. Frequencies of LFAs are shown in Table 29.2.

When recombination is demonstrated between the gene controlling an LFA and that for a blood group system, the LFA is considered not to be part of that system. None of the 700 series antigens has been shown to be independent of all blood group systems and none of the genes encoding those antigens has been identified.

29.2 Antibodies

Frequency of occurrence and some characteristics of antibodies to LFAs are shown in Table 29.3. Like most other blood group antibodies, antibodies to some LFAs arise from immunisation caused by pregnancy or transfusion; a few have been responsible for HDFN (Section 29.3.4). In most cases, however, the antibodies arise as a result of no known stimulus and are often found together with other antibodies to LFAs. Some sera contain numerous antibodies to LFAs. Serum samples from the same donor taken at different times may contain different specificities and the antibodies often react by different methods. Occasionally, sera containing an 'immune' antibody to an LFA also contain apparently 'naturally occurring' antibodies to other LFAs. As an example, the serum of a healthy blood donor, Mrs Tillett, contained the following antibodies to red cell antigens of low frequency: anti-Pt^a, -M^g (MNS11), -Vw (MNS12), -Ri^a (MNS16), -Hut (MNS19), -Dantu (MNS25), -Or (MNS31), -Go^a (RH30), -Rh32, -Evans (RH37), -Wr^a (DI3), -Wd^a (DI5), -Rb^a (DI6), -ELO (DI8), -Bp^a (DI10), -Mo^a (DI11), -Vg^a (DI13), -BOW (DI15), -NFLD (DI16), -Jn^a (DI17), -Tr^a (DI19), -Ls^a (GE6), and eight unpublished specificities.

Antibodies to LFAs usually come to light for one of the following reasons:

1 the antibody causes HDFN;
2 a single red cell sample reacts with a patient's serum during compatibility testing;
3 a serum blood grouping reagent contains a contaminating antibody to an LFA that gives an unexpected reaction during red cell phenotyping;

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Table 29.1 Low frequency antigens: the 700 series.

Number	Name	Symbol	References
700002	Batty	By	[1]
700003	Christiansen	Chr^a	[2]
700005	Biles	Bi	[3]
700006	Box	Bx^a	[4,5]
700017	Torkildsen	To^a	[6]
700018	Peters	Pt^a	[7]
700019	Reid	Re^a	[8]
700021	Jensen	Je^a	[9]
700028	Livesay	Li^a	[10]
700039	Milne		[11]
700040	Rasmussen	RASM	[12]
700044		JFV	[13]
700045	Katagiri	Kg	[14]
700047	Jones	JONES	[15]
700049		HJK	[16]
700050		HOFM	[17]
700052		SARA	[18]
700054		REIT	[19]
		SHIN	[20]

4 an antibody is detected when sera are screened with antigen-positive red cells;
5 an additional specificity is found in a serum known to contain one or more antibodies to LFAs when it is tested against cells of rare phenotype.

When red cells react with a serum known to contain a certain antibody to an LFA, the assumption cannot be made that those red cells carry that LFA, because the serum may contain more antibodies than those previously known to be present. Consequently, cross-adsorption/elution tests must be carried out for confirmation. The cross-adsorption method is not infallible, however, as antibodies of related, but different, specificities might be removed together from a serum by adsorption with red cells apparently expressing only one of the antigens.

29.3 Additional information on some of the antigens and antibodies

29.3.1 Pt^a (700018)

Many examples of anti-Pt^a have been found, all in sera containing other 'naturally occurring' antibodies to LFAs

Table 29.2 Frequencies of low frequency antigens.

Antigen 700		Population	No. tested	No. positive	Antigen frequency	References
002	By	English	31 522	2	0.0001	[21]
003	Chr^a	Danish	500	1	0.0020	[2]
005	Bi	American	1110	0		[3]
006	Bx^a	English	24 106	2	<0.0001	[4,5,22]
017	To^a	Norwegian	6461	1	0.0002	[6]
018	Pt^a	New Zealand	14 500	0	<0.0001	[7]
		Norwegian	21 825	0	<0.0001	[7,23]
		English	10 200	1	0.0001	[24]
019	Re^a	Canadian	>10 000	0	<0.0001	[8]
		English	6635	1	0.0002	[25]
		Welsh	4770	0		[25]
021	Je^a	Danish	>1000	0		[9]
039	Milne	New Zealand	2643	0		[11]
040	RASM	North American	9541	0		[12]
044	JFV	German	1014	0		[13]
045	Kg	Japanese	68 395	131	0.0019	[26]
047	JONES	Caucasian	16 746	1	<0.0001	[15]
050	HOFM	Dutch	926	0		[17]
054	REIT	Canadian	4086	0		[19]
	SHIN	Japanese	3000	1	0.0003	[20]

Table 29.3 Frequencies and characteristics of antibodies to low frequency antigens.

Antibody Anti-700	Antibody frequency[1]	Immune	Present in multispecific sera[2]	References
002 By	1/7987 0/2000	Yes[3]	Yes	[1,22,27]
003 Chr[a]			No	[2]
005 Bi		Yes[3]	No	[3]
006 Bx[a]	0/8000 1/23 081	[4]	Yes	[4,5,22]
017 To[a]	66/5704 ~48/300		No	[6,28,29]
018 Pt[a]			Yes	[7,24,30]
019 Re[a]	0/2358	Yes[3]	No	[8,25]
021 Je[a]	1/>100 000		No	[9]
028 Li[a]		Yes	No	[10]
039 Milne	58/1242	[5]	Yes	[11]
040 RASM	0/543	Yes[3]	No	[12]
044 JFV	0/534	Yes[3]	No	[13]
045 Kg	0/57 147	Yes[3] [6]	No	[14,26]
047 JONES	0/2000	Yes[3]	Yes	[15]
049 HJK		Yes[3]	No	[16]
050 HOFM		Yes[3]	No	[17]
052 SARA	1/3150	Yes[3]	Yes	[18,31]
054 REIT		Yes[3]		[19]
SHIN	0/19 380[7]	[8]	Yes	[20]

[1]The number of sera found to contain the antibody in the total number of sera from random blood donors tested. Where more than one survey is reported, the results are shown separately.
[2]Sera containing several or many antibodies to LFAs.
[3]See Section 27.3.5 for notes on clinical significance.
[4]No anti-Bx[a] in three Bx(a–) mothers of Bx(a+) children [5].
[5]No anti-Milne in two Milne– mothers of Milne+ children [11].
[6]Human monoclonal anti-Kg produced [26].
[7]One anti-SHIN found in sera from 1662 Japanese hospital patients [20].
[8]No anti-SHIN in two SHIN– mothers of SHIN+ children [20].

[7,24,30]. Anti-Pt[a] revealed a membrane component of apparent MW 31.6 kDa by immunoblotting with red cell membranes prepared under non-reducing conditions [32]. This structure might be associated with the membrane skeleton.

29.3.2 Li[a] (700028)

There is a suggestion in one family of linkage between the gene controlling Li[a] and the *LU* locus. If an untested husband is assumed to be Lu(a–), then the lod score is 2.709 at a recombination fraction of 0.0, very close to statistical significance; if the father were Lu(a+) the score is 1.204 [10]. Immunoblotting of Li(a+) cells in order to determine whether Li[a] is on the Lutheran glycoproteins revealed no bands.

29.3.3 HOFM (700050) is associated with Rh

HOFM is associated with depressed C antigen in the only family in which it has been detected [17], but family studies do not provide statistically significant evidence that HOFM belongs to the Rh system (see Section 5.17.1).

29.3.4 Clinical significance of antibodies to LFAs

Most antibodies to LFAs of the 700 series do not appear to be clinically significant. Severe HDFN in the HJK+ baby of a mother with anti-HJK (700049) was treated by three intrauterine transfusions [16]. Anti-Kg (700045) [14,26], anti-REIT (700054) [19], and anti-SARA (700052) [31] were also responsible for HDFN requiring exchange transfusion; HDFN caused by anti-JFV (700044) [13] and by anti-JONES (700047) [15] was treated by phototherapy and blood transfusion. Antibody to the Rh-associated antigens HOFM (700050) caused mild HDFN [17]. Anti-Bi (700005) may also have been responsible for HDFN [3]. Anti-By (700002), -Re^a (700019), and -RASM (700040) caused a positive DAT on cord red cells, but no other signs of HDFN [1,8,12,25].

LFAs do not create a major transfusion problem from the aspect of finding compatible donors, but a potentially dangerous antibody to an LFA could remain undetected if a full crossmatch were not performed. Presence of an unrecognised anti-LFA in a blood-grouping reagent can also prove hazardous.

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30 High Frequency Antigens, including Vel

30.1 Introduction, 500
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30.3 ABTI (VEL2), 502
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30.5 Emm (901008), 502
30.6 AnWj (901009), 502
30.7 PEL (901014), 503
30.8 MAM (901016), 503

30.1 Introduction

High frequency antigens (HFAs) were originally classified together in the 900 series, but when the collections were introduced into the blood group terminology in 1990, the 900 series was decimated and was consequently abandoned and replaced by the 901 series. A red cell antigen may belong to the 901 series if it fits the following criteria:

1 it must have a frequency greater than 90% (though all antigens discussed in this chapter have frequencies in excess of 99% in most populations);
2 it must be inherited;
3 it must not be eligible to join a blood group system, form a new system, or be so closely related to another antigen as to merit collection status;
4 it must have been shown to be serologically distinct from all other antigens of high frequency.

The 901 series currently contains six antigens (Table 30.1). These include Sd^a, which has a frequency of about 91% and is described in Chapter 31. Results of some frequency studies are shown in Table 30.2.

Vel (previously 901001) and ABTI (previously 901015) are serologically related (Section 30.3) and so formed the Vel collection: Vel became VEL1 (212001) and ABTI became VEL2 (212002). The Vel antigens are described in this chapter.

None of the antigens described in this chapter are destroyed by the proteases papain, trypsin, or α-chymotrypsin, by sialidase, or the disulphide bond reducing agents AET and DTT, except that some anti-Vel react only weakly with DTT-treated red cells.

None of the genes responsible for the antigens in this chapter have been identified.

Antibodies to HFAs are a transfusion hazard as compatible blood is often very difficult to obtain. Anti-Vel and -Lan have both caused severe HTRs; anti-MAM has caused severe HDFN.

30.2 Vel (VEL1)

When Sussman and Miller [1] described anti-Vel in 1952, Vel became the first reported public antigen that was not part of an established blood group system. Anti-Vel is a dangerous antibody that has been responsible for HTRs (Section 30.2.3).

30.2.1 Frequency and inheritance

Some of the largest studies of testing random donors with anti-Vel are shown in Table 30.2. The Vel– phenotype was found to have an incidence of about one in 3000 in predominantly white English blood donors [4–6]. There are no reports of studies on large numbers of donors from other ethnic groups. Vel might be polymorphic in some populations: four of 328 Thais [14] and two of 160 Chilcotin Indians from Canada [15] were Vel–.

In the families of 11 Vel– propositi the sibling count, excluding propositi, approached very closely 3:1 Vel+:Vel–, the ratio expected if the Vel– phenotype results from

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homozygosity for a recessive allele [4]. In six families that demonstrate that Vel does not belong to the P1PK system an excess of Vel-negatives were P_2, with a probability of significance of about 1:70. This supports earlier speculations of an association between Vel and P1 [5].

30.2.2 Vel antigen

The strength of Vel shows substantial individual variation and in some people expression is very weak. It is advisable to use a two-stage complement-addition antiglobulin test to confirm a negative result with anti-Vel on red cells of individuals who have not produced anti-Vel. In population studies where apparent negatives were not checked in this way it is likely that donors with very weak Vel antigens have been mistyped as Vel–.

Table 30.1 High frequency antigens.

Number	Name	Symbol
212001	Vel	VEL1
212002	ABTI	VEL2
901003	August	At^a
901008		Emm
901009	Anton	AnWj
901012	Sid	Sd^a
901014		PEL
901016		MAM

Obsolete: 901001 Vel is now VEL1; 901002 Lan is LAN1; 901004 Jo^a is DO5; 901005 Jr^a is JR1; 901006 Ok^a is OK1; 901007 JMH is JMH1; 901010 Wr^b is DI4; 901011 is RAPH1; 901013 Duclos is RHAG1; 901015 ABTI is VEL2.

Vel is generally expressed less strongly on cord red cells than on those of adults [3,16]. Vel has been detected on fetal red cells as early as 12 weeks' gestation [17]. A patient who was Vel– when first tested developed a Vel antigen of normal strength during pregnancy; this Vel antigen persisted for at least 6 months after delivery [5].

Three of 14 anti-Vel failed to react with four Ge:–2,–3,4 samples, but did react with single examples of Ge:–2,3,4 and Ge:–2,–3,–4 cells [18]. This serological association between Vel and Gerbich suggests that the structure expressing Vel could be part of the junctional membrane-protein complex containing glycophorin C (see Section 18.7 and Figure 10.2).

Vel is not destroyed by proteases; on the contrary, treatment of cells with papain or ficin enhances detection of weak Vel antigens. Some anti-Vel react less strongly with red cells treated with DTT than with untreated cells, suggesting that some Vel epitopes are maintained by Cys–Cys bonding [19]. Vel was not detected by flow cytometry on lymphocytes, granulocytes, or monocytes [20].

30.2.3 Anti-Vel

Vel alloantibodies are never 'naturally occurring' and most producers of anti-Vel have been transfused; yet Vel antibodies are predominantly IgM and fix complement. Some anti-Vel haemolyse Vel+ cells *in vitro* and this lytic characteristic is destroyed by heating the serum to 56°C [2–4,6,21–23]. Of two IgG anti-Vel, one was IgG1, another contained IgG1 and IgG3 [24].

Anti-Vel is a dangerous antibody and patients with anti-Vel should be transfused with Vel– red cells. The first anti-Vel and other examples since have caused severe immediate HTRs [1,23,25,26]. Furthermore, anti-Vel may be missed in compatibility testing if inappropriate

Table 30.2 Frequencies of high frequency antigens in various populations.

Antigen	Population	No. tested	No. negative	Antigen frequency	References
Vel	American	21 000	8	0.9996	[[1–3]
	British	99 637	25	0.9997	[4–7]
	Swedish	91 605	52	0.9994	[5]
ABTI	Israeli Jews	509	0		[8]
	Israeli Arabs	121	0		[8]
At^a	American*	9600	0		[9,10]
	African American	14 251	1	0.9999	[11,12]
Emm	English	730	0		[13]

*Including about 2200 African Americans [9].

techniques are used [22,23]. Only 3% of radiolabelled Vel+ cells survived 1 hour after injection into a patient with IgG anti-Vel [27] and all of five anti-Vel gave strongly positive reactions in monocyte monolayer assays [24]. In some cases, however, patients with anti-Vel have been successfully transfused with Vel+ red cells despite, in one case, a strongly positive monocyte functional assay [28,29].

Anti-Vel does not usually cause HDFN, although many examples of anti-Vel have been found in pregnant women [1,16,30,31], probably because most anti-Vel are predominantly IgM and Vel antigen is usually expressed weakly on neonatal red cells, especially in babies heterozygous for the Vel gene. IgG anti-Vel has caused neonatal jaundice severe enough to require phototherapy [30,32,33], and in one case the baby also received two transfusions of the mother's red cells [32].

Two examples of autoanti-Vel were responsible AIHA [26,34], although in one case, a nine-week-old infant, the red cells gave a negative DAT [26]. Two other autoanti-Vel did not appear to destroy autologous red cells [6,35].

30.3 ABTI (VEL2)

The first three examples of anti-ABTI were present in multiparous women from an inbred Israeli Arab family [8]. There was no evidence of HDFN in any of their children. Three other members of the family were also ABTI–. Other examples of anti-ABTI were found in two unrelated German women [36]. No other example of ABTI– was found from testing Israeli blood donors (Table 30.2) or from 70 blood samples from a health clinic in the Arab village in which the Israeli propositus lived. Anti-ABTI reacted by an antiglobulin test with anti-IgG. The original anti-ABTI was IgG1 plus IgG3 [8].

Vel– red cells react weakly or give negative results with anti-ABTI [8,36]. In the one case where the results were reported, ABTI– red cells had weak Vel expression [8].

30.4 At^a (August, 901003)

The first anti-At^a was described by Applewhaite *et al.* [9] in 1967 and six more examples were reported in 1973 [37]. Another public antigen, El [10], was later shown to be the same as At^a [38].

30.4.1 Frequency and inheritance

All reported At(a–) propositi are black. Of about 16 450 African Americans tested, only one was At(a–) [11,12,39] (Table 30.2). Of the published families, nine At(a–) propositi had five At(a–) siblings and 16 At(a+) siblings, and all 11 children with an At(a–) parent were At(a+) [9,10,37,40–42]. These figures suggest that At(a–) results from homozygosity for a recessive gene.

30.4.2 Anti-At^a

Anti-At^a may be stimulated by transfusion or pregnancy. No example of 'naturally occurring' anti-At^a is known. At^a antibodies are mostly IgG, reacting by an antiglobulin test, but one example also contained some IgM, which directly agglutinated At(a+) red cells [37]. Of two IgG anti-At^a, one was IgG1, another consisted of IgG1, IgG3, and IgG4 [24].

One anti-At^a caused an immediate HTR with chills and nausea during a red cell survival study [41] and another a severe delayed HTR after transfusion of multiple units of At(a+) red cells [42]. At^a antibodies facilitate rapid destruction of ^{51}Cr-labelled At(a+) red cells *in vivo* and give positive results in *in vitro* functional assays [24,40,41,43]. Ideally At(a–) red cells should be selected for transfusion to patients with anti-At^a, although least incompatible red cell may be suitable for patients with weak examples of the antibody. Despite numerous pregnancies involving anti-At^a, only one of the babies had moderately severe HDFN requiring phototherapy [39].

30.5 Emm (901008)

Four propositi whose red cells lacked the high frequency antigen Emm were described by Daniels *et al.* [13]: a Frenchman born in Madagascar, a Caucasian American, a Pakistani, and a French Canadian. All had anti-Emm in their sera, as did the Emm– brother of the Canadian. Four of the five examples of anti-Emm were produced by men and were apparently 'naturally occurring'; one of the antibodies was IgM, the other four were IgG. Two further examples of anti-Emm in untransfused Emm– males have been identified [44].

Emm antigen is probably a GPI-linked protein. PNHIII red cells from a paroxysmal nocturnal haemoglobinuria patient were Emm–, whilst the PNHI cells were Emm+ (see Chapter 19) [44,45].

30.6 AnWj (901009)

AnWj is an HFA that is expressed only very weakly on the red cells of individuals with an *In(Lu)* gene (Section

6.8). Evidence exists that AnWj is closely associated with CD44 glycoprotein, the membrane structure that carries antigens of the Indian system, so AnWj is described in Section 21.7.

30.7 PEL (901014)

The first two PEL– propositi with anti-PEL were French Canadian women [46]. The second PEL– propositus had three PEL– siblings. Two other examples of antibodies to HFAs that did not react with PEL– cells were found in the propositi of French Canadian families, but the red cells of these propositi and their compatible siblings reacted very weakly with anti-PEL. This weak reaction required adsorption tests for detection. The PEL-like antibodies in these two propositi were provisionally named anti-MTP [46]. Of the 18 siblings of propositi with anti-PEL (or anti-MTP), red cells of six were compatible with the serum of the propositus.

All four producers of anti-PEL (or anti-MTP) had been transfused and three had also been pregnant. Red cells of the baby of the original PEL– propositus gave a negative DAT and there was no sign of HDFN. Radiolabelled PEL+ cells survived normally in the other propositus with anti-PEL, but only 74% survived after 24 hours in one of the patients with anti-MTP [46].

30.8 MAM (901016)

Two MAM– propositi, ascertained through the presence of anti-MAM in their sera during their third pregnancies, were described by Montgomery *et al.* [47]. One propositus was of partial Irish and Cherokee descent, the other was Arabic. The second propositus had a MAM– sister, who had been pregnant once and also had anti-MAM. The parents of the MAM– sisters are cousins. Two other MAM– propositi with anti-MAM were also found during pregnancy [48,49]. None of the MAM– women had a history of transfusion.

The third baby of the second propositus with anti-MAM had severe HDFN, requiring intrauterine transfusion. Red cells of the babies of the other three propositi were DAT+, but there was no HDFN. Two of the anti-MAM contained IgG1 and IgG3, and one also had IgG2 [47]. Monocyte monolayer assays suggested that anti-MAM has the potential to shorten survival of transfused MAM+ red cells substantially [47].

Immunoblotting of red cell membranes with anti-MAM revealed a diffuse band of apparent MW 23–80 kDa, with a discrete band at 18 kDa. MAM is present on lymphocytes, granulocytes, monocytes, and probably platelets from peripheral blood and on the majority of leukaemia, fibroblast, embryonic kidney, and endothelial cell lines, but not on an epithelial cell line [47,48]. Treatment of cells with *N*-glycosidase F, which cleaves *N*-glycans, resulted in loss of reactivity with anti-MAM [50].

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31 Sid Antigen

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31.1 Introduction

Numerous antibodies of identical specificity were studied in several laboratories for at least 10 years before Macvie *et al.* [1] and Renton *et al.* [2] simultaneously reported the specificity in 1967 and named it anti-Sd[a]. About 91% of white people have Sd(a+) red cells and so Sid qualifies for inclusion in the 901 series of high frequency antigens and has the number 901011. As Sid differs so significantly, both in frequency and characteristics, from the other 901 series antigens, it has been given a separate chapter. Although the gene encoding the β1,4-*N*-acetylgalactosaminyltransferase responsible for Sd[a] expression has been isolated, Sid has not been made a blood group system because the genetic basis of the Sid polymorphism has not been elucidated.

31.2 Sd[a] and Cad

31.2.1 Variation in strength of Sd[a]: the Sd(a++) or Cad phenotype

The strength of expression of Sd[a] on Sd(a+) cells varies from barely recognisable to extremely strong. A 'mixed field' reaction, with small agglutinates among a sea of unagglutinated cells, is a characteristic of reactions with anti-Sd[a]. If the unagglutinated cells are separated and retested with anti-Sd[a], a 'mixed field' picture is again apparent [2]. In haemagglutination tests with a strong anti-Sd[a], about 1% of individuals have strong agglutination with big clumps of cells, about 80% have moderate sized agglutinates consisting of 10–20 cells, about 10% have only occasional tiny agglutinates, and about 9% have no agglutination [1]. Weak examples of anti-Sd[a] react with only a few Sd(a+) samples and may be mistaken for antibodies to a low frequency antigen.

In 1968, Cazal *et al.* [3] described a new private antigen, Cad, in a Mauritian family. Cells of the Cad+ members were polyagglutinable and, despite being group O or B, were agglutinated by *Dolichos biflorus* seed extract, a lectin that generally only agglutinates A_1 cells (Chapter 2). After demonstrating that red cells with very strong Sd[a] expression are also agglutinated by *Dolichos* lectin and that Cad cells are very strongly Sd(a+), Sanger *et al.* [4] concluded that Cad represents the high end of a continuous curve of Sd[a] antigen strength. There is considerable variation in agglutination strength with *Dolichos* and with human anti-Sd[a] of these 'super-Sid' or Sd(a++) cells between individuals and within families [5–11]. As Cad probably represents the expression of an extra strong Sd[a] gene, the term Sd(a++) will be used here to describe all phenotypes in which the red cells have sufficiently strong Sd[a] to be agglutinated by *Dolichos* lectin. The term Cad will be restricted to describe the rare phenotype of members of the Cad family, still probably the strongest Sd(a++) cells encountered.

Sd(a++) cells of the Cad family are polyagglutinable [3]; that is, they are agglutinated by most human sera. Although other Sd(a++) cells are generally polyagglutinable, this is usually less obvious and selected sera and sensitive methods are required to detect the agglutination [9,10]. Sd(a++) polyagglutination differs from all other forms of polyagglutination (Chapter 33).

Weak anti-Sd[a] appears to be present in the sera of most people and responsible for the polyagglutination

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Table 31.1 Frequencies of Sd(a++) in various populations as detected by different preparations of *Dolichos biflorus* lectin.

Population	No. tested	No. positive	Frequency	References
French, Montpellier	250 000	0		[3]
French (four centres)	78 526	56	0.0007	[10]
Canadian, Winnipeg	1425	2	0.0014	[8]
Canadian, Toronto	2191	1	0.0005	[5]
Japanese	51 420	15	0.0003	[7]
Hong Kong Chinese	36 037	110	0.0031	[18]
Thai	14 261	37	0.0026	[12,19]

of Sd(a++) cells. Polyagglutinable Sd(a++) cells are not agglutinated by sera from people with Sd(a++) cells and the stronger the Sd^a antigen on Sd(a++) cells, the greater the number of 'normal' sera that would agglutinate them [12].

31.2.2 Sd^a in body fluids and other tissues

Sd^a can be detected, by haemagglutination inhibition, in the saliva of individuals with Sd(a+) red cells, with greater quantities present in neonatal than adult saliva [1,13]. Sd^a can also be detected in human serum and milk, but by far the greatest abundance is found in meconium and urine [13]. Inhibition of anti-Sd^a with urine is considered the most reliable method of determining Sd^a phenotype.

Sd^a has been found, in variable quantities, in the urine of 12 species of mammals, but not in birds [13]. Guinea pig urine is an extremely concentrated source of Sd^a and a useful tool in blood group antibody identification.

Of the human tissues, kidney is a very rich source of Sd^a [13], with activity localised to the distal convoluted tubules and collecting ducts [14]. Sd^a is also present in colon and stomach, but was not detected in small intestine, muscle, liver, spleen, or brain [15,16].

31.2.3 Frequency and inheritance

The frequency of Sd^a, as determined by testing red cells, is about 90%. Three surveys gave the following results: 290 English blood donors, 91.4% Sd(a+) [1]; 131 English donors, 91.0% Sd(a+) [2]; 1307 Italian donors, 89.3% Sd(a+) [17]. A higher incidence of Sd(a+) is obtained when urine is tested by haemagglutination inhibition. Sd^a was detected in the urine of 96.1% of English blood donors [13] and 93.4% of north Italians [17]. The gene and genotype frequencies in English donors determined by urine inhibition are: Sd^a 0.8030; *Sd* 0.1970; Sd^a/Sd^a 0.6448; Sd^a/Sd 0.3164; *Sd/Sd* 0.0388.

Sd(a++) is very rare in Europeans, but may be less infrequent in the Far East. Table 31.1 shows some frequency determinations on group O and B donors tested with *Dolichos* lectin. These frequencies are somewhat arbitrary as they are partly dependent on the potency of the lectin preparation used.

Tests with anti-Sd^a on 55 families with a total of 168 children, demonstrated that Sd^a is inherited as a dominant character [1]. Sd(a++), as detected by *Dolichos* lectin, is also inherited in a dominant manner [3–8,10–12,20]. Family studies have shown that Sd^a is not part of the ABO, MNS, P1PK, Rh, Lutheran, Kell, Duffy, Kidd, Xg, Dombrock, or XK systems [1,5,8,10]. One Canadian family provided a suggestion of linkage between the genes for Sd(a++) and Wr^a (*SLC4A1* or *DI*), with six non-recombinants and no recombinants, providing a lod score of 1.806 at a recombination fraction (θ) of zero [8]. Both *SLC4A1* and *B4GALNT2*, the Sd^a gene (Section 31.4.3) are on the long arm of chromosome 17.

31.2.4 Sd^a in babies and pregnant women

Red cells of fetuses and newborns are Sd(a–) and become Sd(a+) about 10 weeks post-natally [1,2,15]. Red cells of the son of an Sd(a++) father were non-reactive with *Dolichos* lectin at birth, but were agglutinated by the lectin 6 months later [10]. Saliva, urine, and meconium from neonates generally contain abundant Sd^a substance [13].

An increased frequency of Sd(a–) red cell phenotype is found among pregnant women [1,15,21]. In different studies, 36% and 75% were Sd(a–) at term, and 25% six weeks post-partum had Sd(a–) red cells [15,21]. Sd^a is present in the urine of most Sd(a–) pregnant women [13].

31.3 Sid antibodies and lectins

31.3.1 Anti-Sd^a

Anti-Sd^a is disclosed in about 1% of donor sera when Sd(a+) cells are used for detection [1,2]. The incidence of anti-Sd^a is increased when Sd(a++) cells are used [21], and the exceptionally strong Sd(a++) Cad cells are agglutinated by most sera. Sd^a antibodies are usually IgM and active at 20°C [1,2,15], although IgG anti-Sd^a has been identified [21,22].

Anti-Sd^a are not generally considered a transfusion hazard [1,15,21,23], though transfusion of Sd(a+) red cells to patients with anti-Sd^a may evoke a significant increase in IgG antibody titre [21]. Most survival studies with ^{51}Cr-labelled cells in patients with anti-Sd^a have shown insignificant reduction in red cell survival, even when Sd(a++) cells were used [24,25]. A couple of HTRs, allegedly caused by transfusion of Sd(a++) cells to a patient with IgM anti-Sd^a, are reported [24,25].

31.3.2 Lectins and other non-human sources of anti-Sd^a

A single lectin in *Dolichos biflorus* extracts specific for terminal GalNAc agglutinates A_1, Tn-positive, and Sd(a++) red cells [26]. These reactions can be inhibited by GalNAc [4]. Sd(a++) cells express more determinants than A_1 or Tn cells, so an apparently specific anti-Sd(a++) can be produced by dilution of the lectin or by partial adsorption with A_1 or Tn cells; further adsorptions, or adsorption with Sd(a++) cells, removes all activity.

The seed lectins *Salvia horminum*, *S. farinacea*, and *Leonurus cardiaca* [27–29], and the snail lectins *Helix pomatia* and *H. aspersa* [6,30–33] agglutinate Sd(a++) cells and these reactions are inhibited by GalNAc. Sd(a++) and Tn specificity in the *Salvia* lectins can be separated by adsorption [27]. The anti-A_1 lectins *Phaseolus lunatus* and *P. limensis* do not agglutinate even the strongest of Sd(a++) cells [30–32].

Chicken serum contains a 'naturally-occurring' antibody reactive with Sd(a++) cells [34]. Separable anti-Tn and an antibody that agglutinates Sd(a++) cells were identified in the serum of the snake *Python sebae* [35].

31.4 Biochemistry

31.4.1 Sd^a in urine

In 1970, Morton and Terry [36] partially separated Sd^a-active material from human urine by ethanol precipitation. Ten years later, Soh *et al.* [37] showed that Sd^a activity is associated with Tamm–Horsfall protein, now usually called uromodulin, the most abundant glycoprotein in urine [38]. Uromodulin is secreted by the kidney and protects against urinary tract infection (UTI). It is composed of 70% protein and 30% carbohydrate, with eight potential *N*-glycosylation sites. Uromodulin in urine is a key anti-adherence factor for *Escherichia coli*, the principal cause of UTI. Mutations in *UMOD*, the gene encoding the uromodulin protein, lead to rare autosomal dominant diseases, referred to as uromodulin-associated kidney disease, and uromodulin is also a risk factor for chronic kidney disease [38].

Sd(a+) uromodulin contains between 1–2% GalNAc, whereas that from Sd(a–) individuals contains virtually none [37,39]. Sd^a-active uromodulin can be precipitated by *Dolichos* and *Helix pomatia* lectins and by human anti-Sd^a [39,40]. A pentasaccharide isolated from Sd^a-active uromodulin is shown in Table 31.2b [41,42]. This pentasaccharide strongly inhibits the reactivity of anti-Sd^a with Sd(a+) cells and of *Dolichos* lectin with Sd(a++) cells. The pentasaccharide is not present on uromodulin from Sd(a–) urine, whereas a tetrasaccharide lacking the GalNAc in β-linkage with Gal (Table 31.2a), is present on both Sd^a-active and -inactive glycoproteins [43]. Tetrasaccharides prepared by the cleavage of GalNAc or sialic acid residues from the Sd^a-active pentasaccharides lack Sd^a activity [44]. A mucin with high GalNAc content isolated from urine with *H. pomatia* lectin inhibited anti-Sd^a as effectively as uromodulin [45], so at least two substances in human urine carry the Sd^a determinant.

31.4.2 Sd^a on red cells

Most biochemical analysis of Sd^a on red cells has been carried out on the Sd(a++) cells of members of the Cad family, which have exceptionally high levels of Sd^a activity. SDS PAGE revealed that glycophorin A (GPA) and glycophorin B (GPB) from Cad red cells have an increase in apparent MW of 3 and 2 kDa, respectively, compared with normal red cells [46]. This results from the addition of a β-linked GalNAc residue to most of the disialotetrasaccharides characteristic of these glycoproteins [47] (Table 31.2c and d, and Chapter 3). The resulting pentasaccharide (Table 31.2d) inhibited *Dolichos* lectin and anti-Sd^a, and the altered GPA molecules bound to the lectin in affinity columns [46–48]. Red cells from the original Cad propositus had approximately 12 pentasaccharides per GPA molecule, cells from two other Sd(a++) individuals had 2–3 pentasaccharides per GPA molecule, and Sd(a+) and Sd(a–) cells had no pentasaccharides

Table 31.2 Some structures associated with different Sd[a] phenotypes (for abbreviations see Table 2.4).

Tetrasaccharide and pentasaccharide isolated from uromodulin

(a) Sd(a–) and Sd(a+)

Galβ1→4GlcNAcβ1→3Gal
3
↑
NeuAcα2

(b) Sd(a+)

GalNAc β1→4Galβ1→4GlcNAcβ1→3Gal
3
↑
NeuAcα2

Tetrasaccharide and pentasaccharide from glycophorin A

(c) Sd(a–), Sd(a+), and Sd(a++)

Galβ1→3GalNAc—Ser/Thr
3 6
↑ ↑
NeuAcα2 NeuAcα2

(d) Sd(a++)

GalNAcβ1→4Galβ1→3GalNAc—Ser/Thr
3 6
↑ ↑
NeuAcα2 NeuAcα2

Sialosylparagloboside and Sd[a] ganglioside

(e) Sd(a–), Sd(a+), and Sd(a++)

Galβ1→4GlcNAcβ1→3Galβ1→4Glc—Cer
3
↑
NeuAcα2

(f) Sd(a+), and Sd(a++)

GalNAcβ1→4Galβ1→4GlcNAcβ1→3Galβ1→4Glc—Cer
3
↑
NeuAcα2

[49]. Increased apparent MW of decay-accelerating factor (CD55) in Cad cells was also assumed to result from the presence of additional GalNAc residues [50].

Thin layer chromatography of Cad red cell membranes demonstrated an unusual profile characterised by reduction in the content of sialosylparagloboside (Table 31.2e), the major ganglioside in normal cells, and the presence of a new ganglioside of lower mobility [51]. The unusual ganglioside, which binds *H. pomatia* lectin and inhibits anti-Sd[a], represents sialosylparagloboside with an additional terminal β-GalNAc residue (Table 31.2f) [51,52]. The structure of the terminal pentasaccharide of this ganglioside is identical to that of Sd[a]-active uromodulin shown in Table 31.2b [52]. Small quantities of this ganglioside were detected in membranes from Sd(a+), but not Sd(a–) red cells, and might represent the major Sd[a]-active structure in Sd(a+) red cells [51].

31.4.3 The Sd[a] glycosyltransferase and the gene that encodes it

The product of the *Sd[a]* gene is a β1,4-*N*-acetylgalactosaminyltransferase that catalyses the transfer of GalNAc in β-linkage from a nucleotide carrier to an appropriate tetrasaccharide acceptor substrate molecule. Such enzymes have been detected in human urine from Sd(a+) but not Sd(a–) individuals [43,53], in human kidney [54], in human large intestine [55], and in guinea pig kidney [56,57]. Sd(a–) uromodulin was the best acceptor for the human urine enzyme, but the disialotetrasaccharides of GPA were also suitable acceptors [53]. Preparations from human kidney catalysed the transfer of GalNAc to sialosylparagloboside, but not to native GPA, although tryptic peptides of GPA were good acceptor substrates [51,54].

In 2003, two groups exploited the sequence of a mouse β1,4-*N*-acetylgalactosaminyltransferase gene to isolate human Sd[a] cDNA from an expressed sequence tag database and from colon carcinoma (Caco2) cells [58,59]. The Sd[a] gene, *B4GALNT2*, is on chromosome 17q23.1 and consists of at least 12 exons encoding two proteins of 506 and 566 amino acids, identical in their sequence with the exception of their cytoplasmic tail. Through alternative splicing of one of two exons 1 most of either a very long (exon 1_L) or very short (exon 1_S) cytoplasmic N-terminal domain is encoded; exon 2 encodes the membrane-spanning domain and the stem region; exons 3–11 the putative catalytic site. Transient transfection of Cos-7 cells with the catalytic domain of the enzyme produced a soluble form of the protein that catalysed the transfer of GalNAc to α2,3-sialylated acceptor substrates to form the GalNAcβ1-4(Neu5Acα2-3)Galβ1-R trisaccharide common to Sd[a] antigens [58]. The short form of the enzyme is associated with a higher level of activity [60]. Northern blot analysis indicated that five transcripts were highly expressed in colon and to a lesser extent in kidney, stomach, ileum, and rectum [58].

31.5 Sd^a and gastrointestinal cancer

Sd^a antigen is expressed abundantly in normal gastrointestinal mucosae [14,61], but neither Sd^a antigen nor the Sd^a-transferase is detected in gastrointestinal cancer cells [14,55]. Transfection of colonic or gastric cell lines with the Sd^a-transferase cDNA resulted in increased cell surface Sd^a expression with concomitant loss of the E-selectin ligands sialyl-Le^x (sLe^x) and sialyl-Le^a (sLe^a) (Section 2.3.4), and significant decreased adhesion to activated human umbilical vein endothelial cells [62]. Kawamura *et al.* [62] propose that the Sd^a-transferase competes with fucosyltransferases, blocking expression of sLe^x and sLe^a biosynthesis and eliminating metastasis. From experiments on colorectal cancer specimens, Malagolini *et al.* [60] found that Sd^a and sLe^x are expressed on different proteins and consider that the effect described by Kawamura *et al.* [62] is an artefact of the experimental model and not applicable to colon cancer tissues.

31.6 Malaria

Sd(a++) cells of the Cad family are relatively resistant to invasion by the malarial parasite *Plasmodium falciparum* [63] (see Section 3.21.1). Sialic acid on glycophorin molecules is required by *P. falciparum* merozoites for invasion of the red cells and this sialic acid might not be available on the *O*-glycans of Cad cells because of chemical interaction with the acetyl group of the extra GalNAc residues [63].

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32 HLA (Human Leucocyte-Associated) Class I Antigens on Red Cells

32.1 Bg antigens, 512
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32.1 Bg antigens

Class I products of the genes of the major histocompatibility complex (MHC), *HLA-A*, *-B*, and *-C*, were initially detected on leucocytes but have since been shown to be present on virtually all nucleated cells. Their primary function is the presentation of foreign antigens to cytotoxic T cells. The very highly polymorphic MHC is located on chromosome 6p21. (For a more detailed description see any textbook on immunology.) Mature human red blood cells are not nucleated and do not generally have easily detectable HLA antigens. On occasion, however, certain HLA antigens are expressed strongly enough on red cells to be detected by conventional blood grouping techniques. Because red cells are unsuitable for HLA phenotyping, HLA is not considered a blood group system and HLA antibodies that react with red cells (often called Bg antibodies) are generally regarded by blood group serologists as unwelcome contaminants of antisera.

The discovery that HLA antigens can be expressed on red cells came when Morton and his colleagues [1,2] showed that an assortment of rather indeterminate and troublesome blood group antigens on red cells, called the Bg (Bennett–Goodspeed) antigens [3–5], were strongly related to the HLA antigens on white cells. Bg^a showed almost complete concordance with HLA-B7 [1], Bg^b correlated with HLA-B17, and Bg^c with HLA-A28 [2]. Bg(c+) red cells may also react with anti-HLA-A2, which is known to cross-react with HLA-28 [6,7], and many other cross-reactions occur. Other HLA antigens have been detected on red cells: HLA-A10 and -B8 are often quite strongly expressed [2,8] and HLA-A9, -B12, and -B15 may also be detected [9]. HLA expression on red cells is always weaker than on white cells [2,8]. Many individuals never express HLA on their red cells even though their lymphocytes carry an appropriate antigen. HLA expression on red cells varies substantially between individuals of the same HLA phenotype and within an individual over a period of time [8]. Red cells may be positive for a particular HLA antigen for months or years and then become negative for that antigen for a similar period. HLA antigen strength on red cells does not appear to be inherited in a conventional manner and an HLA antigen may be detected on the red cells of a person, but not on those of either parent [1,5,10,11]. Radioimmunoassay and flow cytometry revealed that red cells of about 50% of blood donors bound monoclonal antibodies directed at monomorphic determinants on HLA class I molecules [12]. Individuals with HLA-B7 on their lymphocytes always express measurable red cell HLA antigens [12,13]. Red cell-reactive antibodies were detected in all sera containing cytotoxic anti-HLA-B7 [14].

It has been estimated that the number of HLA sites on red cells is in the range of 40–550, compared with 100 000 on T lymphocytes [15]. This explains why it is very difficult to remove haemagglutinating HLA antibodies from sera by adsorbing with red cells even though they are readily removed by white cells [1,2,14,16].

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Red cells of people with HLA-B7 have significantly more HLA Class I molecules on their red cells than those without HLA-B7 [15]. Marked increase in red cell HLA expression is also associated with systemic lupus erythematosus (SLE) and, to a lesser extent, rheumatoid arthritis [17]. HLA-B7 patients with infectious mononucleosis have greatly increased expression of the HLA-B7 on their red cells, which sometimes takes years to return to normal [18]. Certain red cell HLA antigens are elevated in patients with leukaemia and a variety of other haematological diseases [19]. Strong expression of red cell HLA in healthy subjects is rare, but has been reported [10,20].

Class I MHC molecules are heterodimers consisting of a 45 kDa α-chain with three extracellular domains, α_1, α_2, and α_3, non-covalently associated with an 11 kDa polypeptide, β_2-microglobulin. HLA antigenic determinants reside on the α_1 and α_2 domains. β_2-microglobulin is not encoded by the MHC on chromosome 6, but by a gene on chromosome 15. Immunoblotting of red cells with monoclonal antibodies to class I α-chain and to β_2-microglobulin gave bands of apparent 45 kDa and 11 kDa respectively, suggesting that red cell HLA antigens are carried on structures similar or identical to those on nucleated cells [21].

Red cell-reactive HLA antibodies are inhibited by plasma from individuals with the corresponding antigens on their white cells [8]. This, together with the finding that red cell HLA activity is destroyed by treatment of the cells with chloroquine, led to a proposal that HLA antigens might be adsorbed onto the red cell surface from the plasma rather than being intrinsic red cell antigens [22]. There is a weight of evidence against this, however, and supporting the thesis that HLA antigens on red cells represent remnants of those antigens present in greater quantity on nucleated red cell precursors [23,24]. Treatment of red cells with chloroquine only removes the β_2-microglobulin; the α-chain remains intact and loss of antigenic expression probably results from configurational changes [25]. Some HLA activity returns if chloroquine-treated cells are incubated in purified β_2-microglobulin [25]. Furthermore, the number of HLA Class I molecules on red cells decreases with aging of those cells, the opposite of what would be expected if the antigens were acquired from the plasma [15]. The level of expression of HLA Class I and β_2-microglobulin are reduced during erythroid differentiation [26]. Red cell HLA antigens are not destroyed by treatment of the red cells with papain, ficin, pronase, trypsin, chymotrypsin, or with the sulphydryl reducing agents AET and DTT [8,25].

32.2 Clinical significance of Bg antibodies

Antibodies to HLA antigens on red cells have generally been considered clinically benign, but there is now substantial evidence that such antibodies have been responsible for both immediate and delayed HTRs [27–31]. In one patient with anti-HLA-A2, -A28, -B7, and -B7 cross-reactive group a delayed HTR was followed by an acute HTR after transfusion of two more HLA-incompatible units [28]. The patient was successfully transfused with one red cell unit from an HLA-compatible donor. There have been reports of increased destruction of radiolabelled red cells by HLA antibodies, although this appears to affect only a minority of red cells in the circulation [10,32,33]. Anti-HLA-B7 implicated in an acute HTR gave a negative result in a monocyte functional assay [29].

HLA antibodies have not been implicated in HDFN.

HLA antibodies reactive with red cells are often a nuisance in antibody investigations, but the difficulties can be reduced by stripping the HLA antigens from the red cells with chloroquine [22] or EDTA/glycine-HCl [34].

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33 Polyagglutination and Cryptantigens

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33.1 Introduction

Polyagglutination was defined by Bird [1] as, 'The agglutination of red cells, irrespective of blood group, by many sera, the abnormality being a property of the red cells and not the sera'. Microbial polyagglutination generally results from the effect of bacterial or viral glycosidases revealing concealed antigenic determinants, called cryptantigens. These enzymes catalyse the cleavage of terminal monosaccharides or acetyl groups from the oligosaccharide chains of membrane glycoproteins or glycolipids. Polyagglutination occurs because of IgM antibodies against these cryptantigens present in the serum of most people; sera from those individuals with polyagglutinable red cells usually lack the corresponding antibody. Somatic mutation resulting in incomplete biosynthesis of oligosaccharides may lead to a more persistent expression of cryptantigen. Another type of polyagglutination arises from the inheritance of very rare antigens, which are detected by antibodies in most adult human sera. A classification of polyagglutination is provided in Table 33.1. For reviews on polyagglutination see [1–7].

Lectins are non-immunoglobulin extracts from plants and occasionally animals. They have proved invaluable in the identification and classification of cryptantigens and polyagglutination. Some of the most useful lectins are listed in Table 33.2.

33.2 Acquired polyagglutination and the cryptantigens involved

33.2.1 Microbial polyagglutination

Microbial polyagglutination is associated with septicaemia, bowel or respiratory tract infections, wound infections, and conditions, such as tumours or obstructions, that compromise the integrity of the bowel wall and permit entry of microbial enzymes into the blood [1,2,5–7,17]. Occasionally microbial polyagglutination is detected in apparently healthy individuals, presumably because of subclinical infection or an undetected intestinal lesion. Before microbial enzymes can modify red cell membrane structures they must be present in sufficient quantity to neutralise plasma enzyme inhibitors. Cryptantigens are quite commonly detectable with lectins, but not with human sera, on the red cells of patients with various infections, sometimes before the infection has been diagnosed. Microbial polyagglutination is usually transient, the polyagglutinability of the cells diminishing as infection abates.

Red cells may also become polyagglutinable *in vitro*, either as a result of bacterial contamination of blood samples or by the use of enzymes isolated from micro-organisms.

33.2.1.1 T polyagglutination

The Hübener–Thomsen–Friedenreich phenomenon [18] or T activation of red cells results from microbial

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sialidases catalysing the cleavage of sialic acid residues from the *O*-linked disialotetrasaccharides of the major red cell sialoglycoproteins, glycophorins A and B, and from other glycoproteins and glycolipids. The T determinant (CD176) is basically terminal D-galactose in β1,3 linkage with GalNAc, the core 1 *O*-glycan (Table 33.3 and see Chapter 3). T exposure causes depression of M and N antigens [5] and the reduction in cell surface charge makes T-active cells agglutinable by *Glycine soja* lectin [14]. Sialidases from bacteria such as *Clostridium perfringens*, *Vibrio cholerae*, and pneumococci, and from the influenza virus, have all been responsible for *in vivo* T cryptantigen exposure. T activation is more common in infants than in adults and is particularly associated with paediatric intestinal disorders [17,19,20].

In 1964, Bird [8] found that extracts of peanuts, *Arachis hypogaea*, made a powerful anti-T lectin. The term 'peanut-positive polyagglutination' now refers to the exposure of any of five cryptantigens, T, Tk, Th, Tx, or Tr, that bind *Arachis hypogaea* lectin (Table 33.2). Red cells of 52 (0.5%) of 9672 hospital patients were agglutinated by *Arachis hypogaea* lectin, but in only one patient were the cells polyagglutinable [21].

Almost all normal human sera agglutinate T-activated red cells, but only a very small proportion (2% [22]) are haemolytic *in vitro*. This haemolytic activity is usually weak and dependent on the degree of desialylation [22]. Monoclonal antibodies with T specificity have been produced following the immunisation of rodents with desialylated red cells [23–25]. Some monoclonal anti-Le^a cross-react with T-activated Le(a–) red cells [26].

T polyagglutination is occasionally accompanied by haemolysis [27]. Whether this is due to complement fixing anti-T in the patient's serum or to an effect of the associated infection is unclear. Haemolytic-uraemic syndrome in children following pneumococcal infection may be caused by the effect of bacterial sialidase; T activation of red cells, platelets, and renal glomeruli could be responsible for the characteristic symptoms of haemolytic anaemia, thrombocytopenia, and renal failure [28–30]. Transfusion of plasma or whole blood containing anti-T has been implicated as a cause of severe haemolytic reactions in infants with necrotising enterocolitis

Table 33.1 Classification of polyagglutination.

Acquired
Microbial
- Cryptantigens uncovered by microbial enzymes
  - T, Tk, Th, Tx, Acquired B
- Adsorption of bacteria or bacterial products onto cell surface

Non-microbial (persistent)
- Biosynthetic blockage resulting from somatic mutation
  - Tn, ?Th

Inherited
- Sd(a++) (Cad), CDA II, NOR, Hyde Park

Undetermined
- VA, Tr

Table 33.2 Reactions of lectins with cryptantigens and with Sd(a++) and Hyde Park polyagglutinable cells.

Lectins	T	Th	Tk	Tx	Tn	Sd(a++)	Hyde Park	Tr	References
Arachis hypogaea	+	+	+	+	–	–	w	+	[8]
Vicia hyrcanica	+	+	+		–	–	+		[9]
Vicia cretica	+	+	–	–	–	–	w		[10]
Vicia villosa	+	–	–		+	+	+	+	[11]
Griffonia simplicifolia (GSII)	–	–	+	–	–	–	+	+	[12]
Medicago disciformis	+	+	–	–	–		+		[13]
Dolichos biflorus (B and O only)	–	–	–	–	+	+	–	–	[14]
Salvia sclarea	–	–	–	–	+	–	–	+	[15]
Salvia horminum	–	–	–	–	+	+	w	+	[15]
Leonurus cardiaca	w	–	–	–	–	+	–		[16]
Glycine soja	+	–	–	–	+	+	+/–		[14]

w, weak agglutination; +/–, reactive with strong examples only.

Table 33.3 *O*-linked oligosaccharides of sialoglycoproteins from normal red cells and from T- and Tn-activated cells. See Table 2.4 for abbreviations.

Normal	NeuAcα2→3Galβ1→3GalNAc—Ser/Thr 6 ↑ NeuAcα2
T	Galβ1→3GalNAc—Ser/Thr
(core 1)	
Tn	GalNAc—Ser/Thr
Sialyl-Tn	GalNAc—Ser/Thr 6 ↑ NeuAcα2

(NEC) and T-activated red cells, though this complication is rare [17,31].Whether patients likely to have T-activated cells, such as children with NEC, should be transfused with plasma, is controversial [17,22], and plasma exchange may be considered beneficial in extreme cases [32].

Leucocytes and platelets of individuals with T-polyagglutinable red cells also express T antigen [33].

33.2.1.2 Th polyagglutination

Th polyagglutination is distinguished from T polyagglutination by the use of lectins (Table 33.2) and probably represents a weak expression or early stage of T activation caused by sialidase from *Corynebacterium aquaticum* [34,35]. Sialidases from *Vibrio cholerae* used under conditions of particularly mild hydrolysis will also produce Th activity, *in vitro*. Release of sialic acid below a critical quantity (20 μg per 10^{10} red cells) leads to Th activity, whereas liberation of greater quantities of sialic acid results in T polyagglutinability [35]. It is possible that Th activity represents the removal of only one sialic acid residue from the tetrasaccharide shown in Table 32.3, exposing terminal Gal, but not the T disaccharide (Galβ1→3GalNAc).

Red cells from 22 of 200 cord samples were Th-activated (but not polyagglutinable); in six of these the mother's red cells were also Th-active [36]. A patient with Th polyagglutination and peritonitis arising from a perforated colonic tumour died as a result of severe intravascular haemolysis [37].

It is possible that a persistent form of Th polyagglutination resulting from incomplete biosynthesis also occurs. Persistent Th activation in the absence of infection was identified in five of seven children with congenital hypoplastic anaemia [38] and in a patient with myelodysplasia whose red cells were also Tn-active [39].

33.2.1.3 Tk polyagglutination

Tk polyagglutination [40] is most readily distinguished from T polyagglutination by the use of GSII, a Tk-specific lectin isolated from the seeds of *Griffonia simplicifolia* [12]. Expression of Tk is enhanced by papain treatment and Tk-activated cells are not sialic acid deficient [40]. *Vicia hyrcanica* seeds contain separable T- and Tk-specific lectins [9]. Monoclonal anti-Tk has been produced by immunising mice with endo-β-galactosidase-modified red cells [41].

Tk polyagglutination is usually associated with *Bacteroides fragilis* infection [42], but *Serratia marcescens*, *Candida albicans*, *Aspergillus niger* and, in children, invasive pneumococcal infection, have also been responsible [3,4,43]. Endo- and exo-β-galactosidases secreted by these pathogens cleave oligosaccharides at Galβ1→4GlcNAc linkages on ABH and Ii active blood group substances (Tables 2.3, 2.5, and 25.1) exposing terminal GlcNAc residues, the Tk receptor [44,45]. Tk-transformed red cells may have reduced expression of ABH and Ii antigens [46,47]. GSII lectin is inhibited by GlcNAc [12,48] and monoclonal anti-Tk was adsorbed by GlcNAcβ1→3Galβ1→4Glcβ–R coupled to silica beads [41]. Endo-β-galactosidases from *B. fragilis, Escherichia freundii*, and *Flavobacterium keratolycus* have been used to produce Tk-activated red cells *in vitro* [41,45,49].

Tk polyagglutination is often associated with acquired B polyagglutination (Section 33.2.1.5) and with T activation in pneumococcal infections in infants [43].

33.2.1.4 Tx cryptantigen

Tx was identified on the red cells of children with pneumococcal infections and could be produced, *in vitro*, by the action on red cells of culture supernatant from pneumococci derived from these patients [50]. Tx is distinguished from T by *Vicia cretica* lectin (Table 33.2). Tx is only slightly weakened by papain treatment [50]. A persistent form of Tx polyagglutination was detected over a period of 5 years in a woman with myelodysplastic syndrome and no symptoms of infection [51].

33.2.1.5 Acquired B

Acquired B phenomenon is described in detail in Section 2.13.1. It is caused by microbial deacetylases converting GalNAc of an A determinant to galactosamine, which

resembles Gal closely enough for the cells to be agglutinated by some anti-B reagents. Acquired B is usually associated with a unique polyagglutination, but is often also associated with Tk and occasionally T or Th polyagglutination.

33.2.2 Non-microbial polyagglutination

33.2.2.1 Tn polyagglutination

In Tn polyagglutination [52] only a proportion of the red cells are polyagglutinable: between 30 and 90% are agglutinated by anti-Tn, the remainder are Tn-negative. Like T, Tn is located on *O*-linked oligosaccharides of red cell glycoproteins, chiefly glycophorins A and B [53]. The Tn determinant (CD175) is GalNAc attached to serine or threonine (Table 33.3) [51–55]. The GalNAc is sometimes sialylated, to form sialyl-Tn (CD175s, Table 33.3) [56].

Tn is exposed by defective oligosaccharide biosynthesis [57,58]. Tn is the biosynthetic precursor of T (core 1), which is a precursor of the disialotetrasaccharide typical of red cell sialoglycoproteins (Table 33.3). In Tn-polyagglutinable cells, conversion of the Tn monosaccharide to the T disaccharide does not occur because of deficiency of T synthase, the core 1 β-3-D-galactosyltransferase [59,60]. This enzyme deficiency arises from a somatic mutation within a pluripotent haemopoietic stem cell and subsequent clonal expansion and proliferation of populations of Tn+ red cells, platelets, granulocytes, and lymphocytes [61–63]. Tn-active red cells are not totally deficient in T synthase as some normal tetrasaccharides are present on the glycophorin molecules [64].

The somatic mutations responsible for Tn phenotype do not occur in *C1GALT1*, the gene encoding T synthase on chromosome 7p21.3, but in *C1GALT1C1*, the gene encoding Cosmc, a molecular chaperone required for effective functioning of T synthase [65–67]. Cosmc interacts with denatured, but not native, T synthase to form a non-covalent and reversible complex that results in the acquisition of T-synthase activity [68]. Four Cosmc mutations have been shown to be responsible for the Tn phenotype in different individuals: 3G>C, Met1Ile; 202C>T, Arg68stop; 454G>A, Glu152Lys; and 577T>C, Ser 193Pro. In one other Tn individual no Cosmc cDNA was detected [66,67]. Cells of the Jurkat human lymphoblastoid T-cell line express Tn and have a mutated Cosmc gene with a deletion introducing a premature stop codon [65]. Transfection of Jurkat cells with Cosmc cDNA restores T-synthase activity and ablates Tn expression, but transfection of Cosmc cDNA containing any of the Tn mutations has no affect on Tn expression [66, 67]. The Cosmc gene is X-linked, located at Xq24 [65]. Owing to the presence of a Y-chromosome in males and X-chromosome inactivation in females, somatic cells contain only one active X-chromosome and consequently only one active Cosmc gene. If a similar process occurs in Tn phenotype as that in PNH (see Section 19.5), then cytotoxic T cells targeting the normal *O*-glycans of blood cells would be expected in individuals having large populations of Tn-positive red cells. Experimental ablation of the Cosmc gene in mice results in embryonic death, with Tn expressed on every observable cell of the embryo [69].

Tn-active red cells have reduced sialic acid content [53,70] and depressed M and N antigens and T cryptantigen [54,71,72]. Tn is destroyed by papain treatment of red cells [70,73].

The immunodominant monosaccharide of Tn is GalNAc, so Tn-activated red cells are agglutinated by lectins that also agglutinate group A cells, such as *Dolichos biflorus* and *Helix pomatia* [57,70,73]. *Salvia sclarea* lectin is more specific for Tn (Table 33.2) [15]. Tn cells are more strongly agglutinated by human sera containing anti-A than those lacking it [70]. Many murine monoclonal Tn antibodies have been produced, though they may cross-react with other carbohydrate epitopes [74], including sialyl-Tn [75]. An scFv–Fc fusion protein, produced by employing a simultaneous positive and negative selection strategy with Tn and A antigens, respectively, had strict Tn specificity [76]. All Tn antibodies and lectins react only with the Tn+ population of cells giving the characteristic mixed field pattern of agglutination with red cells of individuals with Tn polyagglutination.

Tn polyagglutination is often associated with haemolytic anaemia, leucopenia, and thrombocytopenia [27] and, on occasion, is detected in healthy blood donors [70,77]. Although usually persistent, Tn polyagglutination has been known to recede [58]. There are three reports of transient Tn polyagglutination in newborns, possibly resulting from late development of full T-synthase activity [78–80]. Flow cytometry with monoclonal anti-Tn revealed less than $1x10^{-6}$ Tn red cells in the peripheral blood of healthy donors [81].

33.2.3 T, Tn, sialyl-Tn, and Tk in malignancy

T and Tn are cryptantigens on epithelial tissues, in the carbohydrate chains of glycoproteins and glycolipids. Springer and others have shown that T, Tn, or sialyl-Tn are exposed on about 90% of primary and metastatic

carcinomas, probably as a result of incomplete biosynthesis [82]. All human tumour cells examined that express Tn and sialyl-Tn harbour mutations in the Cosmc gene, resulting in loss of T-synthase activity [83]. Increased density of Tn over T is an indicator of high metastatic potential of the tumour. Immunotherapy with a vaccine derived from T- and Tn-activated red cells has been successful in preventing recurrence of breast cancer and has a potential for wider application (reviewed in [84,85]). Monoclonal anti-Tk reacted with 48% of human colorectal carcinomas [41].

Reports of patients with Tn polyagglutination and concurrent acute myeloid leukaemia (AML), or who subsequently developed leukaemia, led Ness *et al.* [86] to propose that Tn polyagglutination may represent a preleukaemic state and that careful clinical observation of individuals with Tn polyagglutination would be circumspect. Polyagglutination vanished during chemotherapy [58,86]. Tn polyagglutination has also been associated with myelodysplasia [39,77].

33.3 Inherited polyagglutination

33.3.1 Sd(a++) (Cad)

Sd^a is a red cell antigen with an incidence of about 91% and of variable strength. Red cells of the very rare individuals with an extra strong form of Sd^a antigen (referred to as Sd(a++) or Cad) are polyagglutinable. The immunodominant structure of Sd^a is GalNAc in β-linkage with Gal. Sd(a++) red cells are agglutinated by *Dolichos biflorus* and *Helix pomatia* lectins, but can be distinguished from Tn cells by *Salvia sclarea* and *Leonurus cardiaca* (Table 33.2). Sd^a and Sd(a++) are described in detail in Chapter 31.

33.3.2 Congenital dyserythropoietic anaemia type II (CDA II or HEMPAS)

CDA II (or hereditary erythroblastic multinuclearity with a positive acidified serum lysis test, HEMPAS) is a rare, autosomal-recessive syndrome found predominantly in southern Italy and Central Europe [87,88]. Most patients have a mild anaemia, although some become transfusion-dependent. Red cells of patients with CDA II are agglutinated at 20°C, or lysed at 37°C, by complement fixing IgM antibodies in about one third of normal sera [89,90]. CDA II red cells have elevated i, normal I, and depressed H [89–92]. They have reduced levels of *N*-glycosylation of cell surface glycoproteins, particularly band 3 and band 4.5 (glucose transporter), which contain repeating *N*-acetyllactosamine (Galβ1→4GlcNAc) units [93], revealing a cryptantigen responsible for the polyagglutination and resulting in excess production of i-active linear *N*-acetyllactosaminylceramides. In addition, 10–35% of erythroblasts in CDA II are binucleated and some red cells have a second membrane close to the outer membrane [87].

CDA II is caused by mutations in *SEC23B*, a gene comprising 20 exons and located on chromosome 20p11.23 [94,95]. *SEC23B* encodes a component of the COPII coat protein complex [96], which is involved in protein-trafficking through membrane vesicles. Abnormalities of *SEC23B* may disturb endoplasmic reticulum-to-Golgi trafficking and interfere with sugar transporters and glycosyltransferases, affecting glycosylation pathways [97]. Homozygosity and compound heterozygosity for numerous *SEC23B* missense or inactivating mutations result in CDA II [94,95,98–100]. Homozygosity or compound heterozygosity for two inactivating mutations has been found only once [100], suggesting that complete lack of the protein could be incompatible with life; compound heterozygosity for a missense and an inactivating mutation tends to result in more severe symptoms than homozygosity or compound heterozygosity for missense mutations [98]. The most common mutations encode Glu109Lys and Arg14Trp and represent 32% and 19%, respectively, of mutations identified [100]. CDAII patients exhibit 40–60% decrease in *SEC23B* mRNA levels, and the patient with a nonsense mutation and a splice site mutation had 30% mRNA level [100].

33.3.3 NOR polyagglutination

NOR is a very rare form of polyagglutination found in only two families. It results from the expression of abnormal glycolipids resulting from a mutation in *A4GALT*, the gene encoding the enzyme responsible for biosynthesis of P1 and P antigens. Consequently, NOR antigen is P1PK4 and is described in Section 4.5.

33.3.4 Hyde Park polyagglutination

The association between a unique form of polyagglutination associated with a rare haemoglobin variant in a large South African family of mixed ethnicity remains a puzzle. Thirty-five members of the family were studied: 12 had the variant haemoglobin M-Hyde Park and polyagglutinable red cells; 23 had neither [101]. Polyagglutination had not been observed previously in association with haemoglobin M-Hyde Park.

Group O, N+ Hyde Park red cells show the following serological characteristics [101,102]:
1 agglutination with a minority of normal human sera (7 of 40);
2 enhanced agglutination with *Vicia graminea* and *Ulex europaeus* lectins and with human anti-I and -i, but normal strength agglutination with rabbit and monoclonal anti-N;
3 agglutination by monoclonal anti-Tn, but not by Tn-specific *Salvia sclarea* lectin;
4 agglutination by *Glycine soja*, *Sophora japonica*, and *Arachis hypogaea* (weakly), lectins that detect desialylated *O*-glycans; and
5 agglutination by the GlcNAc-specific lectins *Vicia hyrcanica* and GSII (weakly) (Table 33.2).
These serological characteristics, together with results of biochemical analyses, suggest that the polyagglutination results from two unrelated anomalies, one associated with heterogeneity of sialylation of the *O*-glycans of glycophorin molecules and the other associated with exposed GalNAc residues on the *N*-glycans of band 3 and band 4.5 [103].

33.4 Polyagglutination of undetermined status

33.4.1 VA polyagglutination

VA polyagglutination is very rare. The red cells are not agglutinated by *Arachis hypogaea* or *Dolichos biflorus* lectins, but give a mixed-field agglutination with *Helix pomatia* lectin [103,104]; they also give a characteristic stippled appearance in immunofluorescence with *H. pomatia* lectin [104]. An associated depression of H antigen has led to the suggestion that microbial α-fucosidase could be responsible for VA exposure [103, 105]. In the original case VA polyagglutination was persistent and associated with haemolytic anaemia [103,104]; in the only other reported example, VA-active red cells were also Tk active [105].

33.4.2 Tr polyagglutination

Tr polyagglutination has been found in one individual [106]. Tr red cells gave a unique pattern of reactions with a panel of lectins (Table 33.2) and reacted with monoclonal anti-T + Tn and -Tk. Results of tests with lectins, reduced periodic acid-Schiff staining of glycophorins A, B, and C, and increased electrophoretic mobility of band 3 suggested a reduction in sialylation of both *N*- and *O*-glycans, exposing β1→4Gal and β1→3Gal residues. It is feasible that Tr results from a defective glycosyltransferase, probably an α1,6-sialyltransferase. If so, that may also account for a bleeding defect, probably resulting from reduced platelet glycoprotein Ib levels, and for other defects suggesting a connective tissue disorder.

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