3 MNS Blood Group System

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	

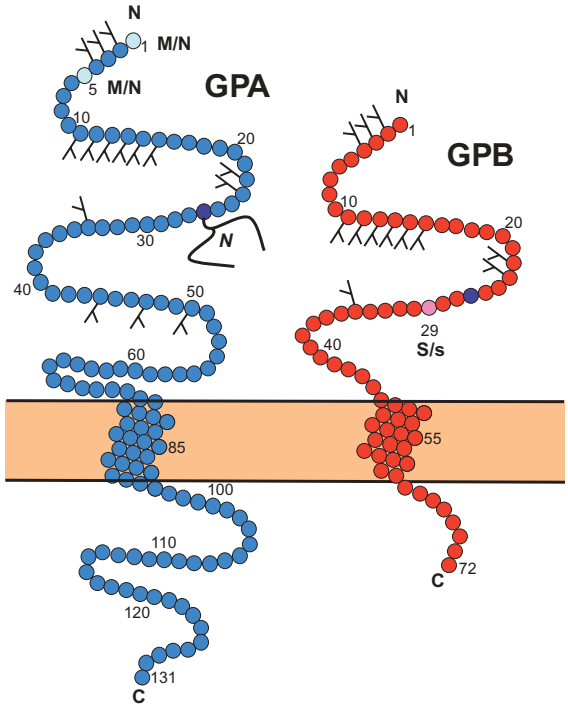

Figure 3.1 Diagrammatic representation of glycophorin A (GPA) and glycophorin B (GPB), and their situation in the red cell membrane, showing the positions of the M/N polymorphism at positions 1 and 5 of GPA, the S/s polymorphism at position 29 of GPB, and the *N*-glycan at Asn26 of GPA.

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].

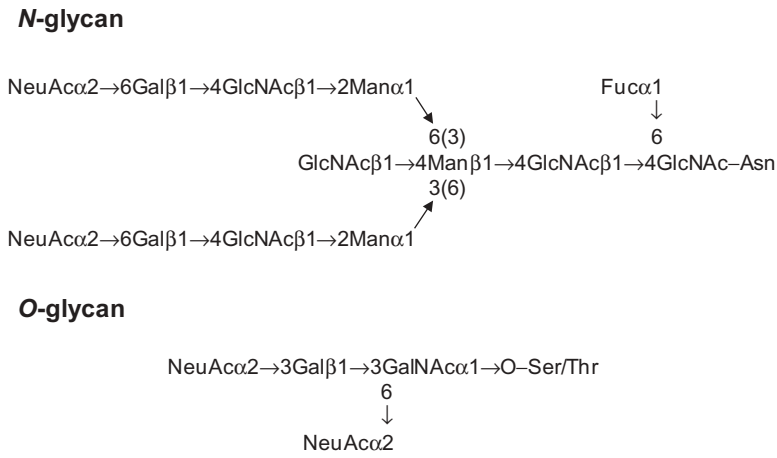

Figure 3.2 Predominant *N*-glycan of GPA and *O*-glycans of glycophorins. For abbreviations see Table 2.4.

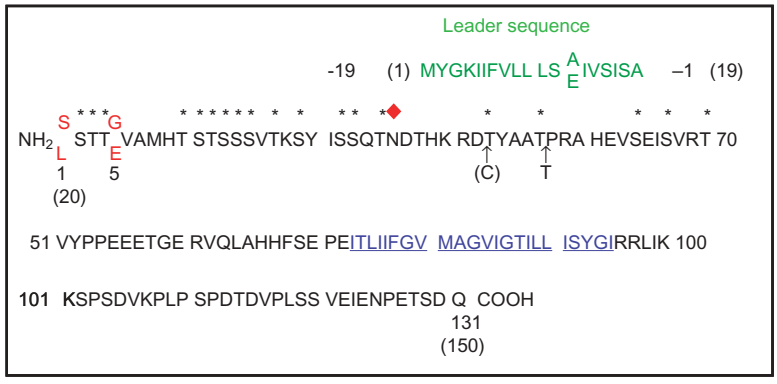

Figure 3.3 Amino acid sequence of glycophorin A (see Table 1.2 for code). Amino acids are numbered from the N-terminal residue of the mature protein with the numbers from the N-terminal Met of the nascent protein in parentheses. The leader sequence is cleaved after insertion of the protein into the membrane. 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

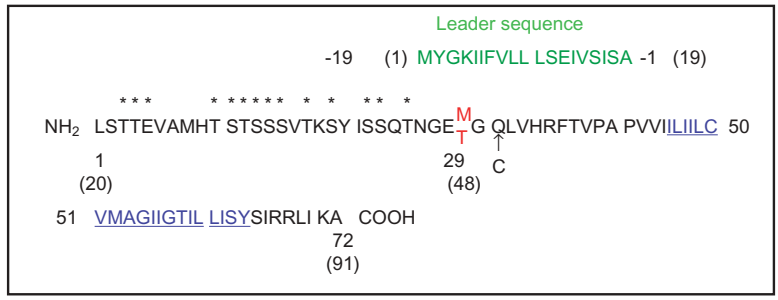

Figure 3.4 Amino acid sequence of glycophorin B (see Table 1.2 for code). Amino acids are numbered from the N-terminal residue of the mature protein with the numbers from the N-terminal Met of the nascent protein in parentheses. The leader sequence is cleaved after insertion of the protein into the membrane. 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].

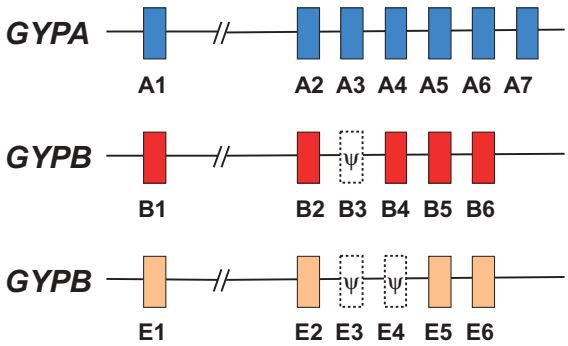

Figure 3.5 Genomic organisation of *GYPA*, *GYPB*, and *GYPE*. Boxes represent exons and pseudoexons (ψ). The pseudoexons are numbered so that homologous exons maintain the same number in all three genes [18].

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+.

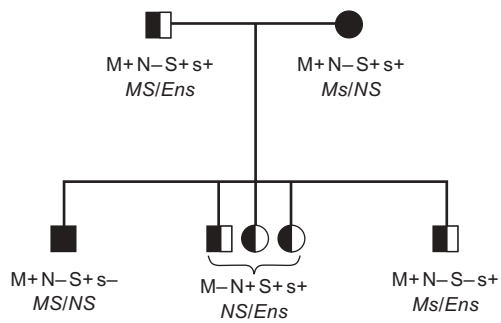

Figure 3.6 Family demonstrating how the presence of an *En* allele can explain an M+ N– father with three M– N+ children and associated red cell membrane modifications. Red cells of all family members are En(a+). The genotype of the mother is deduced from her parents and sibs (not shown). ■●, no modification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

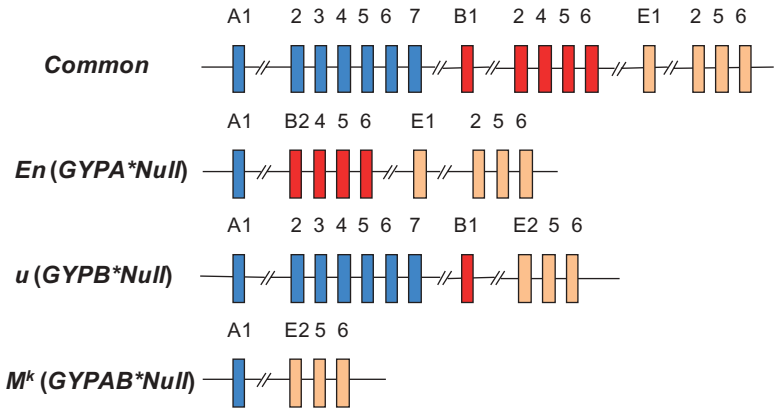

Figure 3.7 Diagram to show the extent of deletions of *GYPA*, *GYPB*, and *GYPE* responsible for En(a–) (*GYPA*Null* or *En*), U– (*GYPB*Null* or *u*), and M^K (*GYPAB*Null*) phenotypes [57,62]. In each case the deletion breakpoints occur within the very long first introns of these genes. *GYPA*Null*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

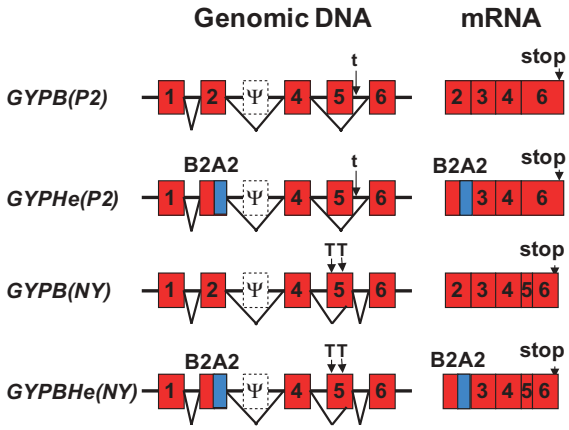

Figure 3.8 Four genes responsible for S– s– U+^{w} phenotype, showing genomic DNA with B2A2 hybrid exon 2 in genes encoding He and mis-splicing of exon 5 and resultant aberrant mRNA. There is no evidence that mis-spliced mRNA is represented as a protein in the red cell membrane [83].

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 $M^g+ \times M^g-$ 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.

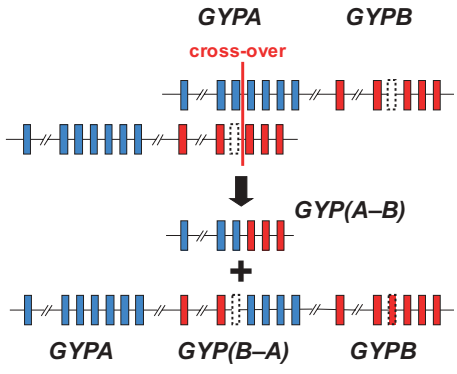

Figure 3.9 Development of hybrid genes involving *GYPA* and *GYPB* by chromosomal misalignment and unequal crossing-over. Two homologous genes become misaligned at meiosis and intergenic crossing-over occurs (red line). Result: one haplotype containing a *GYP(A–B)* fusion gene and another haplotype containing 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.

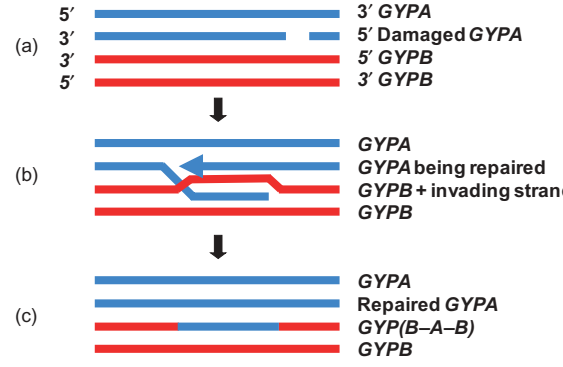

Figure 3.10 Simplified model for gene conversion occurring as the result of damage repair to *GYPA* and involving homologous regions of *GYPA* and *GYPB*. (a) *GYPA/GYPB* heteroduplex, resulting from chromosomal misalignment, with a nick in one *GYPA* strand. (b) An extra copy of one strand of the *GYPA* 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

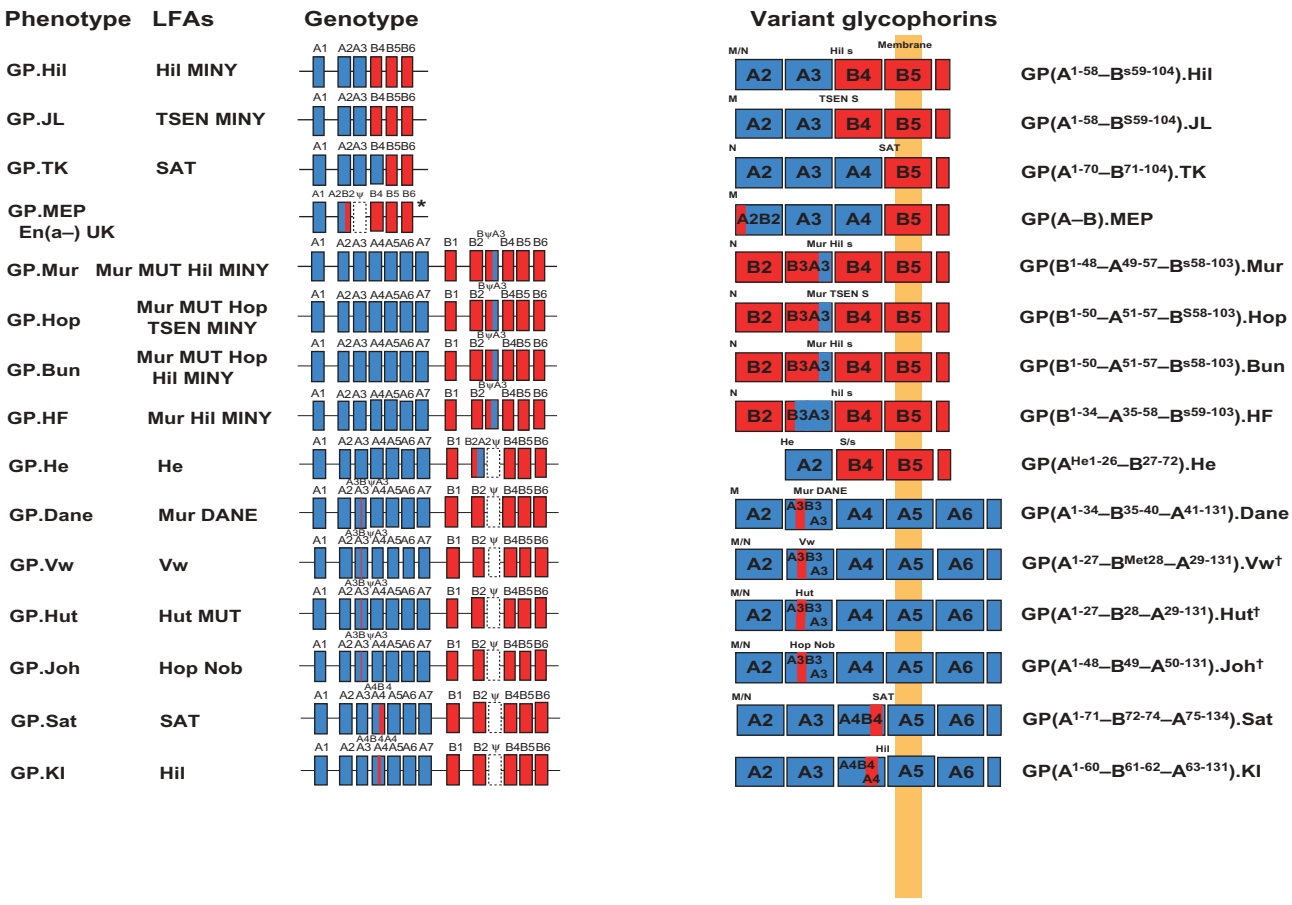

Figure 3.11 Rare MNS phenotypes associated with hybrid glycophorins. *Possible genotypes deduced from serological and biochemical evidence. †Alternatively could result from a point mutation.

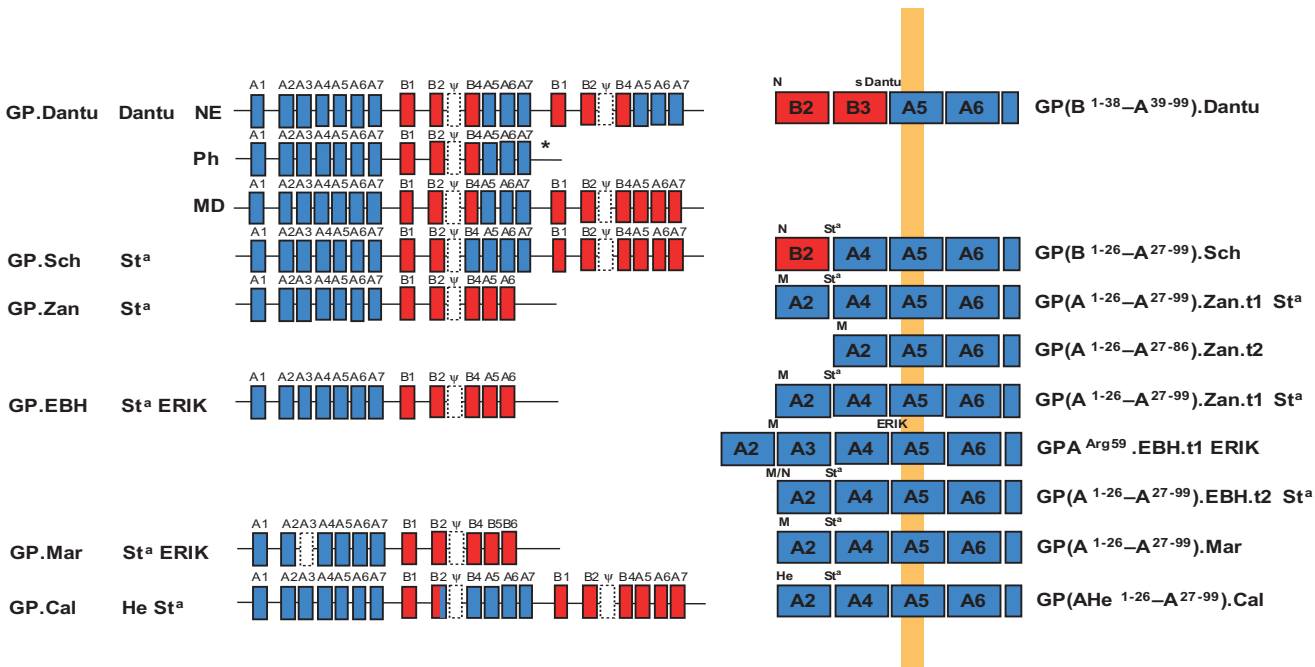

Figure 3.11 (*Continued*)

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).

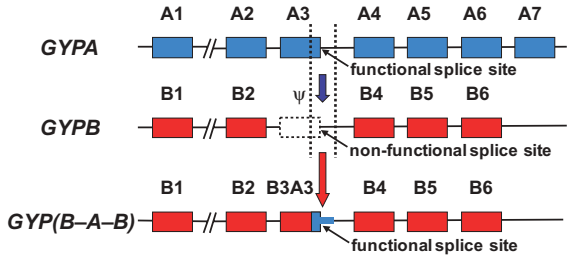

Figure 3.12 Diagram demonstrating the replacement of a small segment of *GYPB* by the homologous region from *GYPA*, including part of exon A3 and part of intron 3, to generate a novel *GYP(B–A–B)* gene; the result of non-reciprocal recombination by gene conversion (blue arrow). The mutated, non-functional 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-GE**T**GQLVHR s	+	–	+	–
PEEET-GE**M**GQLVHR 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,

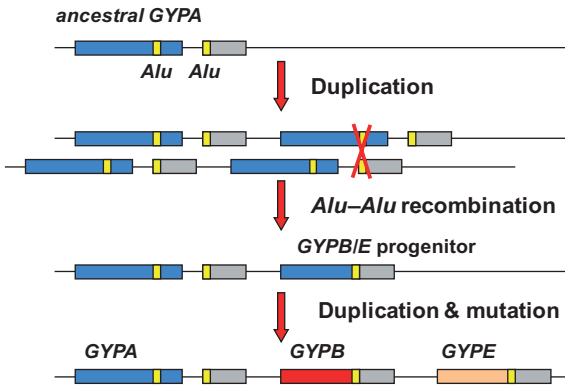

Figure 3.13 Model to explain the evolution of the three glycophorin genes on chromosome 4. Duplication of an ancestral *GYPA* was followed by chromosomal misalignment and unequal crossing-over occurring at an *Alu* sequence within intron A5 of the duplicated *GYPA* ancestral gene and another *Alu* sequence 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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