10 Diego Blood Group System

10.1 Introduction

The Diego system consists of 22 antigens, which includes three pairs of antithetical antigens – Di^a and Di^b, Wr^a and Wr^b, Wu and DISK – plus 16 antigens of very low frequency (Table 10.1). Di^a is a useful anthropological marker because it is polymorphic in most Mongoloid populations, but virtually absent from other ethnic groups. Di^a represents Leu854 and Di^b Pro854 in the red cell anion exchanger, band 3 or AE1 (CD233). Low frequency Wr^a (DI3) and high frequency Wr^b (DI4) represent Lys658 and Glu658 in band 3, respectively. Glycophorin A (GPA)-deficient red cells are Wr(a–b–), as Wr^b requires the presence of both band 3 and GPA for expression. The other low frequency Diego system antigens are all associated with amino acid substitutions in band 3. No true Diego-null phenotype has been reported, reflecting the functional importance of band 3. Band 3 is associated with the Rh proteins and RhAG, GPA, and many other proteins, as part of at least two red cell membrane protein macrocomplexes.

SLC4A1, the gene encoding band 3, is located on chromosome 17q12-q21.

10.2 Band 3, the red cell anion exchanger (AE1), and the gene that encodes it

Band 3, anion exchanger 1 (AE1), or CD233, is a major intrinsic red cell membrane glycoprotein, with approximately 1.2 million copies per red cell. After SDS PAGE of red cell membranes, band 3 is easily detected by Coomassie blue staining. It migrates as a diffuse band of about 100 kDa. Band 3 exists in the red cell membrane mostly as tetramers and dimers. For reviews on band 3 see [34–37].

The band 3 gene (*SLC4A1*) covers 18 kb of DNA and contains 20 exons [38] (Table 10.2). Cloning and sequencing of band 3 cDNA confirmed that the protein consists of three domains: a cytoplasmic N-terminal domain of 403 amino acids; a hydrophobic transmembrane domain of 479 amino acids; and a C-terminal cytoplasmic tail of 29 amino acids [39,40] (Figure 10.1). In the original model the transmembrane domain has 14 α-helical membrane-spanning domains and cytoplasmic N- and C-termini [34,40], but this model has been challenged [41] (Figure 10.1). The single *N*-linked oligosaccharide on Asn642, on the fourth extracellular loop, carries H, A, B, I, and i activity. Variation in the number of repeating *N*-acetyllactosamine units accounts for the broadness of the band on SDS PAGE. Associations of band 3 with other red cell membrane proteins and with components of the cytoskeleton are discussed in Section 10.7.

10.3 Di^a and Di^b (DI1 and DI2)

Di^a was first described in a Venezuelan family by Layrisse *et al.* [1] in 1955. It soon became apparent that Di^a is relatively common in South American Indians, but rare in people of European origin [1,42]. Two examples of

Human Blood Groups, Third Edition. Geoff Daniels.

Table 10.1 Antigens of the Diego blood group system.

Antigen				Molecular basis			
No.	Name	Frequency	Antithetical antigen	Nucleotides	Exon	Amino acids	References
DI1	Di^a	Low*	Di^b (DI2)	2561C>T	19	Pro854Leu	[1,2]
DI2	Di^b	High	Di^a (DI1)	2561C	19	Pro854	[2,3]
DI3	Wr^a	Low	Wr^b (DI4)	1972G>A	16	Glu658Lys	[4,5]
DI4	Wr^b	High	Wr^a (DI3)	1972G	16	Glu658	[5,6]
DI5	Wd^a	Low		1669G>A	14	Val557Met	[7,8]
DI6	Rb^a	Low		1643C>T	14	Pro548Leu	[9,10]
DI7	WARR	Low		1654C>T	14	Thr552Ile	[11,12]
DI8	ELO	Low		1249C>T	12	Arg432Trp	[13–15]
DI9	Wu	Low	DISK (DI22)	1694G>C	14	Gly565Ala	[15–17]
DI10	Bp^a	Low		1707G>A	14	Asn569Lys	[15,18]
DI11	Mo^a	Low		1967G>A	16	Arg656His	[15,19]
DI12	Hg^a	Low		1966G>T	16	Arg656Cys	[15,20]
DI13	Vg^a	Low		1663T>C	14	Tyr555His	[15,21]
DI14	Sw^a	Low		1. 1937G>A	16	Arg646Gln	[22,23]
				2. 1936C>T	16	Arg646Trp	
DI15	BOW	Low		1681C>T	14	Pro561Ser	[15,24,25]
DI16	NFLD	Low		1287A>T, 1681C>G	14	Glu429Asp, Pro561Ala	[26,27]
DI17	Jn^a	Low		1696C>T	14	Pro566Ser	[28,29]
DI18	KREP	Low		1696C>G	14	Pro566Ala	[25]
DI19†	Tr^a	Low		1653C>G	14	Lys551Asn	[10,18]
DI20	Fr^a	Low		1438G>A	13	Glu480Lys	[30,31]
DI21	SW1	Low		1936C>T	16	Arg646Trp	[32,33]
DI22	DISK	High	Wu (DI9)	1694G	14	Gly565	[33]

*Polymorphic in people of Mongoloid origin.
†Provisional assignment.

antibodies detecting an antithetical antigen, Di^b, were described by Thompson *et al.* [3] in 1967.

10.3.1 Frequencies

Frequency studies on Di^a are prodigious because of its usefulness as an anthropological marker. Di^a occurs almost exclusively among Mongoloid people. Extensive reviews of the frequency data are provided elsewhere [43–45]; studies on selected populations are shown in Table 10.3.

Di^a is most common in native South Americans, where the frequency is variable, but reaches over 70% in some populations. Di^a is also found in most Central and North American native populations, although the incidence in the northern continent is generally not as high as in the southern continent. Surprisingly, Di^a is rare among the Inuit of Alaska and Canada, but relatively common in Siberian Inuits. Di^a also occurs in eastern Asian populations where the frequency varies between about 2 and 12% [59].

Very few Di(a+) white people with no apparent Mongoloid admixture are reported [1,60,61]. Invasion of parts of Poland by Tartars in the thirteenth to seventeenth centuries was used to explain Di^a in 0.46% of Poles [56]. Di^a appears to be absent from Australian Aborigines and many Pacific Oceanic populations, and absent, or extremely rare, among Africans (Table 10.3).

A few large surveys have been carried out with anti-Di^b [43,44,49,62]. All Di(b–) individuals were Di(a+); Di(a–b–) phenotype has not been described (but see Section 10.6).

Table 10.2 Organisation of the band 3 gene, *SLC4A1* [38].

Exon	Size (bp)	Amino acids	Intron size (kb)
1	582		>3
2	83	1–5	0.125
3	91	6–36	0.998
4	62	36–56	0.757
5	181	57–117	0.095
6	136	117–162	0.472
7	124	162–203	0.227
8	85	204–232	0.152
9	182	232–292	0.539
10	211	293–363	0.232
11	195	363–428	0.178
12	149	428–477	0.114
13	195	478–542	1.503
14	174	543–600	0.377
15	90	601–630	0.543
16	167	631–686	1.126
17	254	686–771	1.527
18	170	771–827	0.086
19	174	828–885	0.620
20	2146	886–911	

10.3.2 The molecular basis of the Diego polymorphism

Di^a expression is associated with 2561C>T in exon 19: *DI*A* encodes Leu854; *DI*B* encodes Pro854 [2]. *DI*A* is associated with loss of *Msp*I and *Nae*I restriction sites. According to the 14 transmembrane-domain model, this amino acid substitution occurs in the seventh extracellular loop and amino acid 854 is also exposed at the cell surface in alternative models (Figure 10.1).

When red cells are treated with pronase or chymotrypsin prior to SDS PAGE, a band 3 polymorphism can be detected [63]. In most cases a 60 kDa band is stained representing the N-terminal region of band 3, but in a minority of subjects a second band of reduced mobility (63 kDa) is also apparent. The variant band, band 3 Memphis, results from 166A>G in exon 4 of *SLC4A1*, encoding Lys56Glu within the cytoplasmic N-terminal domain of band 3 [64,65]. Band 3 Memphis was initially detected in 6–7% of random blood samples [64]; a higher incidence has been found in African Americans (16%), Native Americans (17–25%), Chinese (13%), Filipinos (17%), and Japanese (29%) [66,67]. Red cell anion exchange inhibitors diisothiocyanatostilbene (DIDS) and its dihydro derivative H_2DIDS bind covalently to Lys539 of band 3 [39]. In some individuals with band 3 Memphis, the variant band 3 has markedly increased binding of H_2DIDS [68]. This is called band 3 Memphis variant II in order to distinguish it from band 3 Memphis variant I, which has normal H_2DIDS binding. Di(a+) red cells were found to have the band 3 Memphis variant II [69]. Band 3 Memphis from Di(a+) red cells bound about three times the normal quantity of radiolabelled H_2DIDS, whereas the band 3 Memphis from Di(a–) red cells bound normal quantities of H_2DIDS. Di^a is generally associated with band 3 Memphis variant II; Di^b is associated with either normal band 3 and with band 3 Memphis variant I. The enhanced H_2DIDS binding occurring with Leu854 (Di^a) could result from a localised conformational change that affects the rate of covalent cross-linking between Lys851 in the seventh extracellular loop and Lys539 in the fifth transmembrane domain [70].

*Msp*I cleavage of PCR products to determine *DI*A/B* genotype of 70 Parakanã (Amazonian) Indians revealed 27 *DI*A/A*, 26 *DI*A/B*, and 17 *DI*B/B*, giving gene frequencies of *DI*A* 0.57 and *DI*B* 0.43 [46]. Four of the *DI*A* homozygotes were heterozygous for 166G/A, revealing a new allele encoding Di^a, but not band 3 Memphis.

Studies on non-human primates suggest that 2561C (Di^b) *in cis* with 166G (band 3 Memphis) is the ancestral band 3 gene [71].

One Di(a+b–) Japanese donor genotyped as *DI*A/B* had a *DI*B* allele containing a CAC trinucleotide insertion, encoding histidine, at nucleotide 2359 in exon 18 [72].

10.3.3 Effect of enzymes and reducing agents

Di^a and Di^b are resistant to treatment of red cells with papain, trypsin, α-chymotrypsin, pronase, sialidase, and AET.

10.3.4 Weak Di^b

A healthy Mexican woman with many weak red cell antigens initially appeared to have Di(a–b–) red cells, but, on further testing, was shown to have weak Di^b [73]. Weaker than expected Di^b reactions were detected with red cells from several Di(a+) Mexican Americans [49]. All of 784 Hispanic Americans were Di(b+), but 11 had weaker than average Di^b antigens, yet only one of them was Di(a+) [62].

Di^b is depressed in South-East Asian ovalocytosis [74] (Section 10.9).

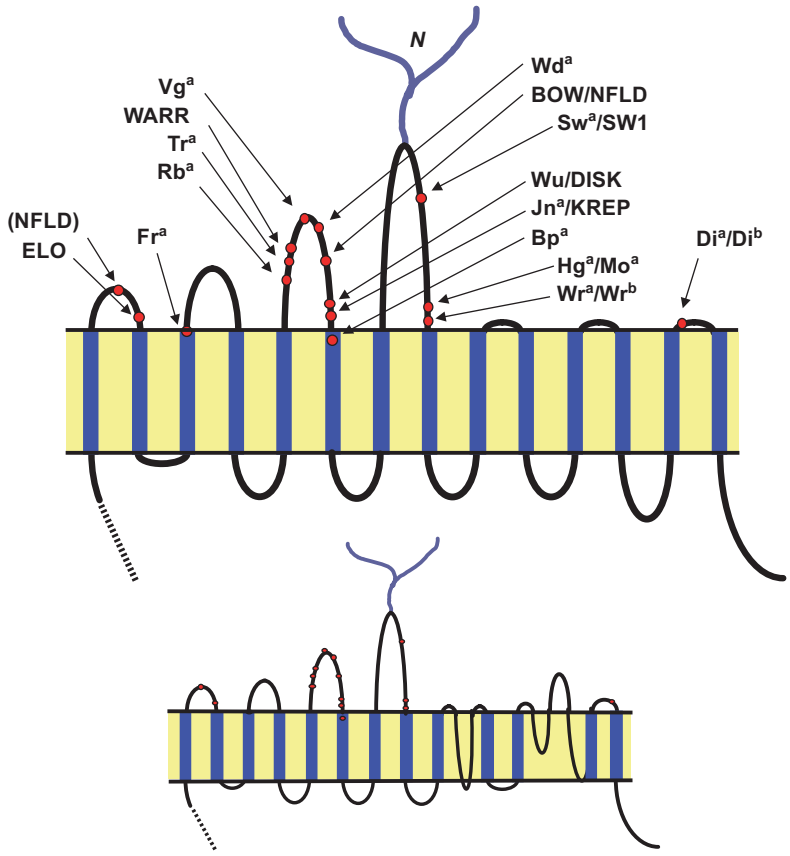

Figure 10.1 Two models for the topography of the membrane domain of band 3 glycoprotein. In the conventional model (above) the protein spans the membrane 14 times and has cytoplasmic N- and C-terminal domains [34,40]. Positions of the amino acid substitutions associated with the Diego system antigens are shown. *N* represents the single *N*-glycan at Asn642. The large cytoplasmic N-terminal domain is not shown. Below is an alternative model [41].

10.3.5 Anti-Di^a and -Di^b

The original anti-Di^a was responsible for fatal HDFN [42] and other cases of severe HDFN caused by anti-Di^a have been reported since [55,56,61,75]. Most anti-Di^a have been stimulated by pregnancy. One example of anti-Di^a, in an Australian Caucasian, was apparently 'naturally occurring' [60]. Anti-Di^a is implicated in a delayed HTR [76] and may have caused an immediate HTR, but the presence of anti-c and incomplete information confused the picture [77]. Red cell panels used for antibody screening often do not contain Di(a+) cells and examples of anti-Di^a remain undetected, even in regions where anti-Di^a is relatively common. In Brazil, four of 112 (3.6%) multitransfused patients had anti-Di^a [78], and in Singapore introduction of Di(a+) cells to an antibody screening panel revealed 19 anti-Di^a in 1383 samples referred for antibody investigation [79].

One of the original two anti-Di^b may have been responsible for a delayed HTR [3]. An HTR caused by anti-Di^b, in which the haemolysis appeared to have been largely intravascular, was considered to be a contributory cause of the patient's death [80]. Mochizuki *et al.* [81] list 27 published cases of HDFN caused by anti-Di^b, of which 10 required exchange transfusion, six received phototherapy (with or without top-up transfusion), one was treated with high dose intravenous immunoglobulin, and 10 required no therapy. In monocyte monolayer assays with anti-Di^b, significantly higher scores of adherence and phagocytosis were obtained with Di(a–b+) red cells than with Di(a+b+) cells [82]. No examples of 'naturally

Table 10.3 Results of some selected studies on the frequency of Di^a.

Population	No. tested	No. Di(a+)	Di^a frequency	References
Carib Indians (Venezuela)	121	43	0.3554	[42]
Arawaco Indians (Venezuela)	152	8	0.0526	[42]
Kainganges Indians (Brazil)	48	26	0.5416	[42]
Parakanã Indians (Brazil)	70	53*	0.7571	[46]
Chippewa Indians (USA)	148	16	0.1081	[47]
Penobscot Indians (USA)	249	20	0.0803	[48]
Inuits (Alaska, Canada)	1477	2	0.0014	[43]
Inuits (Siberia)	86	18	0.2093	[43,44]
Mexicans (USA)	1685	172	0.1021	[49]
Japanese	2427	244	0.1005	[50]
Chinese	617	32	0.0519	[43]
Chinese	1766	125*	0.0708	[51]
Chinese (Taiwan)	1000	32	0.0320	[52]
Koreans	277	17	0.0614	[53]
Indian (North India)	377	15	0.0400	[43]
Europeans	4462	1	0.0002	[42,49,54,55]
Poles	9661	45	0.0047	[56]
White Americans	1000	0	0	[42]
African Americans	827	1	0.0012	[43]
Ghanaians	107	0	0	[57]
Australian Aborigines	1374	0	0	[58]
Papua New Guineans	1741	0	0	[58]

*Predicted from molecular genotyping.

occurring' anti-Di^b are reported. Two of 74 sera containing red cell autoantibodies contained autoanti-Di^b together with other autoantibodies [83]. One of the autoanti-Di^b may have been responsible for AIHA.

Although anti-Di^a has been detected in sera containing multiple antibodies to low incidence antigens, anti-Di^a and -Di^b are most often found alone. They usually require antiglobulin to agglutinate cells, but directly agglutinating anti-Di^a [60] and -Di^b [84] have been described. Anti-Di^a and -Di^b are often IgG1 and IgG3 [85] and occasionally anti-Di^a binds complement and may haemolyse untreated cells [56].

Monoclonal anti-Di^a and -Di^b have been produced by human-mouse heterohybridomas incorporating lymphocytes derived from immunised individuals [50,86].

10.4 Wright antigens

10.4.1 Wr^a and Wr^b (DI3 and DI4)

Wr^a, first described by Holman in 1953 [4], has an incidence of around one in 1000 in white populations (Table 10.4). Very few large surveys have been carried out on other ethnic groups. No Wr(a+) was found among 2000 Australian Aborigines or 2000 Papua New Guineans [58]. One family gave a suggestion of close linkage between the genes controlling Wr^a and Sd(a++) [97]. Wr^a shows individual variation in strength of expression and is fully developed at birth.

The name anti-Wr^b was provisionally used in 1971 for an antibody, in the serum of a Wr(a+) woman, that detected a high frequency antigen [6]. Her red cells appeared to have a double dose of Wr^a antigen and the antibody reacted more strongly with Wr(a–) cells than with Wr(a+) cells. Wr^a and Wr^b dosage on Wr(a+b+), Wr(a–b+), and Wr(a+b–) cells was confirmed by an enzyme-linked antiglobulin test [98]. Two other examples of Wr(a+b–) phenotype have been described briefly [99,100].

Sequencing of PCR-amplified band 3 cDNA from the original Wr(a+b–) propositus revealed 1972G>A in exon 16, predicting Glu658Lys within the fourth extracellular loop of band 3 (*DI*02.03*) [5] (Figure 10.1). Three Wr(a–b+) individuals had the normal sequence (Glu658); seven

Table 10.4 Frequency of Wr^a in predominantly white populations.

Population	No. tested	No. Wr(a+)	Wr^a frequency	References
English	45 631	36	0.0008	[87,88]
English	5253		0.0004	[89]
Norwegians	5138	0	0	[90]
Norwegians	3140	2	0.0006	[90]
Swiss	3753	2	0.0005	[91]
Spanish	110 000	140	0.0012	[92]
Italians	6350	7	0.0011	[93]
Czechs	1500	1	0.0007	[94]
Americans (Boston)	2784	3	0.0011	[95]
Americans (New York)	5000	5	0.0010	[96]

Wr(a+b+) individuals were heterozygous for Glu658 and Lys658 codons. Another Wr(a+b–) individual was also homozygous for the Lys658 codon [100].

Wr^a and Wr^b are resistant to treatment of red cells with trypsin, chymotrypsin, pronase, papain, and sialidase, and with the reducing agent AET. Neither Wr^a nor Wr^b was detected on peripheral blood lymphocytes, granulocytes, or monocytes.

10.4.2 Association of band 3 with glycophorin A (GPA) and its importance in Wr^b expression

Band 3 and GPA are closely associated in the membrane. A monoclonal antibody to the cytoplasmic C-terminal domain of band 3 precipitated both band 3 and GPA [101], although most antibodies to either band 3 or GPA do not co-precipitate [35]. Binding of GPA antibodies to red cells significantly reduced rotational mobility of band 3 [102–104]. GPA may facilitate the translocation of newly synthesised band 3 to the membrane [105]. GPA is not essential for band 3 expression at the cell surface, but in red cells deficient in GPA, band 3 is imperfectly folded and moves slowly to the surface. In GPA-deficient cells the *N*-glycan on band 3 is of increased molecular weight and, despite normal quantities of band 3, anion transport is impaired (Sections 3.5.1 and 3.23), as a result of altered band 3 structure [106]. Whereas the cytoplasmic C-terminal tail of GPA is responsible for enhancing trafficking of band 3 to the cell surface, extracellular residues 68–70 of GPA increase band 3 anion transport activity [107]. Red cells of band 3 knockout mice are deficient in both band 3 and GPA, suggesting that band 3 plays a chaperone-like role and is essential for expression of GPA at the red cell surface [108]. This is not applicable to humans: GPA appears before band 3 on erythroid progenitor cells during human erythropoiesis [109,110]; a human erythroleukaemia cell line (K562) expresses GPA, but no band 3 [111,112]; and an infant with almost total band 3 deficiency has markedly reduced, but clearly present, GPA [113,114]. Red cells of transgenic mice expressing human GPA had reduced levels of mouse GPA, implying a competition between human and murine GPA for band 3 [115].

GPB, which lacks the cytoplasmic tail of GPA, does not appear to have any affect on band 3 [105]. GP(B-A-B) Mur, however, which resembles GPB but has an additional amino acid sequence in the external domain (Section 3.11.1.2), increases levels of band 3 in the membrane by 25–67% in the GP.Mur phenotype, resulting in superior HCO_3^- transporting capacities [116]. This affect could be attributed to GPA and GP(B-A-B)Mur interaction through the formation of heterodimers.

Despite a single amino acid substitution being the primary cause of the Wr^a/Wr^b dimorphism, Wr^b is not expressed in the absence of GPA. GPA-deficient, En(a–), red cells are Wr(a–b–) [117,118]. The only other Wr(a–b–) cells are those with some rare phenotypes involving hybrid glycophorins, which lack the part of GPA that reacts with antibodies called anti-En^aFR (Sections 3.5 and 3.10). Red cells with GP(B-A-B)Mur had enhanced expression of Wr^b [119]. Red cells of the three Wr(a+b–) patients demonstrated no abnormality in expression of the MNSsU or En^a antigens, of GPA, or of red cell sialic acid levels [100,120]. There was no abnormality in the sequence of *GYPA* cDNA from a Wr(a+b–) person [5].

Most monoclonal antibodies to GPA and band 3 do not co-precipitate both proteins, but monoclonal

anti-Wr^b either precipitated GPA [121,122] or GPA together with band 3 [123,124]. Six sera containing red cell autoantibodies co-precipitated band 3 and GPA [125]. Three of the sera contained autoanti-Wr^b, but the other three sera contained no anti-Wr^b, suggesting that other epitopes may exist that depend on band 3 and GPA interaction. Haemagglutination with alloanti-Wr^b could be inhibited by purified fragments of GPA, but, like anti-En^{a}FR, inhibitory activity was low and only detectable in the presence of lipid [120]. One monoclonal anti-Wr^b was inhibited by a synthetic peptide representing amino acid residues 65–70 of GPA; alloanti-Wr^b, autoanti-Wr^b, and two other monoclonal anti-Wr^b were not inhibited [126]. Monoclonal anti-Wr^b do not bind to human cell lines unless they express both GPA and band 3 [123]. The erythroleukaemia cell line K562 expresses GPA, but no band 3 or Wr^b, but transfection with band 3 cDNA induces Wr^b expression [112]. During *ex vivo* erythropoiesis, Wr^b appears at the cell surface at the same time as band 3, slightly after GPA [110]. Binding of antibodies to the extracellular domain of GPA causes immobilization of band 3, an effect that is significantly reduced in Wr(a+b–) cells [104].

Amino acid sequences of GPA and the hybrid glycophorin GP(B–A)Sch, which are associated with Wr^b expression, were compared with those of GP(A–B)Hil and GP(B–A)Dantu, which are associated with the Wr(a–b–) phenotype. The results suggested that amino acid residues 55–68, an α-helical region close to the insertion of GPA into the membrane, might be important in Wr^b expression [5,127]. Gln63Lys and Ala65Pro substitutions within GPA are both associated with abnormal Wr^b expression (Section 3.15). Hybrid glycophorins expressing SAT, however, do not express Wr^b, despite having amino acids 1–70 or 1–71 derived from GPA, with a transmembrane domain derived from GPB (Section 3.10.3) [127]. It is likely, therefore, that the association between band 3 and GPA occurs between the single membrane-spanning domain of GPA and the eighth membrane-spanning domain of band 3, and the extracellular regions adjacent to these domains.

Whether GPA is required for Wr^a expression is not known. Attempts to identify any membrane component by immunoprecipitation with monoclonal anti-Wr^a were unsuccessful [124].

10.4.3 Anti-Wr^a

Anti-Wr^a is a relatively common antibody. The reported incidence of anti-Wr^a in the sera of normal donors varies in different studies: the highest frequency was one in 13 sera [128], but other studies have provided figures of between one in 37 and one in 100 [89,92,96,129]. The incidence increases dramatically in patients, post-partum women, and in people with other alloantibodies [89,96]. About one in three patients with AIHA has anti-Wr^a [88,92,96]. Some anti-Wr^a are directly agglutinating, but most require an antiglobulin test for detection. Most anti-Wr^a in healthy donors are IgM or IgM plus IgG; IgG1 and IgG3 were the subclasses most commonly detected in pregnant women and transfused patients [92,129].

Anti-Wr^a is clinically significant. It has been implicated in acute HTRs [91,130–132] and has caused severe HDFN [4,133–135]. Red cells compatible by IAT should be selected for transfusion to patients with anti-Wr^a, although anti-Wr^a in patients are not usually detected because Wr(a+) cells are not generally used for antibody screening. Consequently, in the absence of a serological crossmatch, Wr^a incompatible transfusions will occur at a rate of between one in 40 000 and one in 100 000 and the risk of a resultant HTR has been estimated to be about two in a million [136,137]. Anti-Wr^a is common, so use of Wr(a+) screening cells means that many examples are detected and require identification. Consequently, the consensus opinion is that the expense of using Wr(a+) screening cells cannot be justified.

IgG1 mouse monoclonal anti-Wr^a (BGU1-WR) was produced as a result of immunising mice with Wr(a+) red cells [138].

10.4.4 Anti-Wr^b

Alloanti-Wr^b has been found in the sera of the three Wr(a+b–) patients [6,99,100], plus some Wr(a–b–) individuals with certain rare MNS phenotypes (Sections 3.5 and 3.10). Wr^b is generally considered resistant to treatment of red cells with proteases, but one anti-Wr^b did not react with ficin- or papain-treated red cells [100]. There is little information regarding the clinical importance of alloanti-Wr^b. An En(a–) patient with anti-Wr^b and -En^a suffered a mild delayed HTR after receiving six units of En(a+) blood [139] and red cells of a baby born to a mother with alloanti-Wr^b gave a positive DAT, but transfusion was not required [140].

Anti-Wr^b is a relatively common autoantibody [141,142]. Analysis of eluates from the DAT-positive red cells of 150 individuals revealed 110 antibodies unrelated to Rh, 46 of which contained anti-Wr^b, and 34 of those came from patients with AIHA [142]. Two anti-Wr^b autoantibodies in patients with DAT-positive red cells have been responsible for fatal intravascular haemolysis [143,144].

Many examples of murine and one rhesus monoclonal anti-Wr^b have been reported [86,145–147].

10.5 Other Diego antigens, DI5 to DI22

Diego was a simple system consisting of a pair of allelic antigens, Di^a and Di^b, from 1967 until Wr^a and Wr^b joined the system in 1995. Since 1996, a further 18 antigens have joined the Diego system. All except DISK (DI22), which is antithetical to Wu (DI9), are of very low frequency (Table 10.5) and are associated with amino acid substitutions in or close to extracellular loops of band 3 (Figure 10.1).

The low frequency antigens DI5 to DI21 (Table 10.1) were assigned to the Diego system following recognition of an association between antigen expression and a missense mutation in the band 3 gene [8,10,12,14,15,17,23,25,27,29,31,157]. In most cases at least two unrelated individuals with the low frequency antigen were shown to have the associated mutation. Only one Tr(a+) individual was available [10], so the assignment of Tr^a to the Diego system remains provisional. The WARR mutation was found in a Native North American kindred, the only family containing WARR+ members [12], and the mutations associated with Vg^a [15] and with KREP [25] were only identified in single families.

All the antigens are associated with a single amino acid substitution, apart from NFLD, in which two substitutions were found, and Sw^a, which represents either of two amino acid substitutions at the same position (Table 10.1). All substitutions are in the putative extracellular

Table 10.5 Frequencies of other antigens of the Diego system.

Antigen			Population	No. tested	No. positive	Antigen frequency	References
DI5	Wd^a	Waldner	North American	4000	0		[7]
			Norwegian	7151	0		[148]
			African Hei//om	114	2*	0.0175	[149]
DI6	Rb^a	Redelberger	English	10 200	1	0.0001	[9]
DI7	WARR	Warrior	North American	8275	1†		[11]
DI8	ELO		English	16 223	1	<0.0001	[13]
DI9	Wu	Wulfsberg	Norwegian	7000	1	0.0001	[16]
			Danish	2021	4‡	0.0020	[150]
			Australian	16 472	4	0.0002	[151]
DI10	Bp^a	Bishop	English	75 000	1	<0.0001	[18]
DI11	Mo^a	Moen	Norwegian	9000	0		[19]
			Belgian	9793	2	0.0002	[19]
DI12	Hg^a	Hughes	Welsh	5434	2	0.0004	[20]
DI13	Vg^a	Van Vugt	Australian	17 209	1	<0.0001	[21]
DI14	Sw^a	Swann	English	55 410	9	0.0002	[18,22,87,88]
			English	17 661	1	<0.0001	[11]
			Swiss	~7000	3	~0.0004	[152]
			Canadian	5000	3	0.0006	[153]
DI15	BOW	Bowyer	English	>55 000	0	<0.0001	[24]
DI16	NFLD	Newfoundland	North American	1125	0		[26]
			Japanese	45 825	2	<0.0001	[154]
DI17	Jn^a	Nunhart	Norwegian	13 824	0	<0.0001	[28,148,150]
DI19	Tr^a	Traversu	English	38 069	2	<0.0001	[18]
DI20	Fr^a	Froese	Canadian	1400	1§	0.0007	[30,155]

*Sisters. Only other Wd(a+) individuals have been Canadian Hutterites with the surname Waldner [7] and a black South African [149].
†Sister of WARR+ propositus.
‡Hov+. Later shown to be the same as Wu [156].
§Mennonite. Most Fr(a+) propositi have been found among Canadian Mennonites [32,155].

loops of band 3 (Figure 10.1), with the exception of that associated with Bp^a, which is in a membrane-spanning domain, but very close to loop 3, so Bp^a may represent a conformational change in the third loop [15]. Most of the amino acid changes are clustered on the third and fourth extracellular domains, but ELO and one of the NFLD substitutions are on the first loop, Fr^a is close to the second loop, and the Di^a/Di^b polymorphism is on loop 7. None of the positions of these amino acid changes challenges any of the models of protein topology (Figure 10.1). There is no evidence that any of the substitutions has an effect on the function of the band 3 molecule or on binding of the red cell anion exchange inhibitor H_2DIDS (see Section 10.7). It is likely, therefore, that the third and fourth extracellular loops are not involved in anion exchange [15]. Most of the substituted amino acids are not well conserved in evolution, but Asn569, which is changed to lysine in the Bp^a-associated substitution, is conserved in 12 anion exchanger homologues [15].

Adsorption of some sera containing anti-Wu, -NFLD, and -BOW with Wu+, NFLD+, or BOW+ cells will often remove all three antibodies [158]. BOW and NFLD represent different substitutions of Pro561, whereas Wu represents a substitution of Gly565 [15,17,25,27]. NFLD is associated with amino acid changes in the first and third extracellular loops [27]. The serological associations of NFLD with BOW and Wu suggest that Ala561 is most important in determining NFLD expression. Similar serological cross-reactivity occurs between Jn^a and KREP, which represent different substitutions of Pro566 [25,29]. Some Sw(a+) red cells are SW1+, whilst others are SW1– [23,32]. Anti-Sw^a appears to recognise changes associated with conversion of Arg646 to Gln or Trp, whereas anti-SW1 is specific for the Arg646Trp substitution [23]. Hg^a and Mo^a represent different substitutions of Arg656 [15], but no serological association is reported.

The high frequency antigen DISK (DI22) is antithetical to Wu. The only example of anti-DISK was produced by a woman who was homozygous for the Wu mutation (1694C) and whose red cells were DISK– and expressed higher levels of Wu than other Wu+ cells [33]. Her heterozygous brother was Wu+ DISK+. An IgM murine monoclonal antibody directed at the third external loop of band 3 did not react with red cells of another probable Wu homozygote and, therefore, behaves like anti-DISK [159].

Band 3 has two chymotrypsin cleavage sites in the third extracellular loop, at Tyr553 and Tyr555. Consequently, the antigens in the third loop are mostly sensitive to α-chymotrypsin, whereas those in the fourth and seventh loop are resistant [160]. This provides further evidence that Pro561Ala is the most important substitution in determining NFLD expression. There are two unexpected effects. One example of anti-ELO did not react with chymotrypsin-treated cells, though ELO is on the first loop. It is possible that the Arg432Trp substitution associated with ELO creates a new chymotrypsin site, giving rise to partial band 3 cleavage and loss of activity with one example of anti-ELO. Diego-system antigens are papain- and trypsin-resistant.

With the exception of anti-ELO, antibodies to the low frequency antigens from DI5 to DI22 have not been found to be clinically significant. A second ELO+ child of a woman with anti-ELO had mild HDFN and her third ELO+ child had severe HDFN, necessitating exchange transfusion [161,162]. The anti-ELO was IgG (mostly IgG3) and haemolytic IgM. Anti-Fr^a caused a positive DAT on cord red cells, but no other signs of HDFN [163].

Some antisera contain antibodies to numerous red cell antigens of low frequency. These antibodies are produced with no obvious immunising stimulus (see Chapter 29). Antibodies to all low frequency Diego antigens (with the possible exception of anti-Di^a) have been found in multispecific sera. Anti-ELO [161], -Sw^a, and -Fr^a [163] have also been produced as a result of immunisation by antigen-positive red cells. No anti-Rb^a was found in five Rb(a–) mothers of Rb(a+) children [9], no anti-Wd^a was found in six Wd(a–) Hutterite mothers of a total of 30 Wd(a+) children [7], and no anti-NFLD was found in an NFLD– mother of three NFLD+ children [154]. The only anti-DISK was a very strong antibody, reactive by direct agglutination [33].

Two cases of severe AIHA were caused by autoantibodies directed at chymotrypsin-sensitive determinants on band 3. One was IgA optimally active at 37°C [164]; the other cold reactive IgM plus IgG [165].

10.6 Band 3 deficiency

No Diego-null phenotype has been reported and it is likely that total band 3 deficiency would be lethal because of the vital importance of band 3 as an anion exchanger (Section 10.7). There is one description of a baby with only trace quantities (2%) of band 3, resulting from homozygosity for a band 3 mutation (band 3 Coimbra, Val488Met) [113,114]. The severely hydropic, anaemic baby, with dramatic erythroblastosis and poikilocytosis, was delivered by emergency caesarean section, resuscitated, and kept alive by blood transfusion. At 3 months

the baby developed distal renal tube acidosis (dRTA) (Section 10.8). At 3.5 years the patient was splenectomised and became largely transfusion independent with no major complications. Two other patients, with less severe symptoms, are homozygous mutations in the band 3 gene: band 3 Neapolis (deletion of 11 N-terminal amino acids) and band 3 Courcouronnes (Ser667Phe), with 88% and 65% band 3 deficiency, respectively [166,167].

Band 3 knockout mice and cattle with a band 3 nonsense mutation were able to survive, despite spherocytosis, haemolytic anaemia, and growth retardation [168–170].

10.7 Functional aspects and band 3 membrane complexes

Band 3 plays an important role in the efficient transport of respiratory gases in the blood. In the presence of H_2O, carbonic anhydrase II (CAII) in red cells hydrates CO_2 to HCO_3^- and H^+. Band 3, an antiporter, transfers HCO_3^- ions out of the cell in exchange for Cl^-. The proton then interacts with haemoglobin promoting the release of O_2. As HCO_3^- is more soluble than CO_2, this process supports the transport of CO_2 and the release of O_2 in the tissues, with the reverse process occurring in the lungs. The short C-terminal domain of band 3 associates with CAII and the N-terminal domain associates with deoxyhaemoglobin, glycolytic enzymes, and hemichromes [34–37]. This has led to the concept that band 3 is part of a metabolon, a functional complex of proteins that catalyse sequential reactions in a metabolic pathway [36,114].

In addition to being a membrane transporter and other metabolic functions, band 3 has a structural role, linking the membrane to the red cell cytoskeleton, a lattice of glycoproteins beneath the red cell membrane that is vital for maintaining the shape and integrity of the red cells (see Section 18.7 for reviews). The N-terminal domain of band 3 tetramers interacts with the red cell cytoskeleton (Figure 10.2). Reduced levels of red cell band 3 resulting from heterozygosity for a variety of mutations within the band 3 gene often causes abnormally shaped red cells, especially spherocytosis, a common, familial haemolytic anaemia characterised by small spheroid red cells [171]. There is no evidence that any of the mutations responsible for the low frequency Diego system antigens affects red cell morphology.

Band 3 tetramers form the core of a macrocomplex of membrane proteins, which includes trimers of the Rh proteins and the Rh-associated glycoprotein (RhAG), ICAM-4 (LW), CD47, GPA and GPB, and is linked to the cytoskeleton through ankyrin-1 and protein 4.2 [114,172] (Figure 10.2 and see Section 5.7). All of these proteins are either dramatically reduced or absent from red cells of the patient with only trace quantities of band 3 (Coimbra) and a similar effect was found in red cells of band 3 knockout mice [114]. Cotransfection experiments in K562 erythroleukaemia cells show that band 3 enhances expression of Rh proteins and RhAG at the cell surface [93]. Bruce *et al.* [114] speculate that RhAG could be a relatively non-specific channel for neutral small molecules and function as a gas channel for CO_2, O_2, and/or NO. As part of the band 3 metabolon, RhAG is ideally located to channel CO_2 to and from CAII, and O_2 to and from haemoglobin. In addition, the adhesion molecules ICAM-4 and CD47 might assist in facilitating transient adhesive interactions between the red cell and the vascular endothelium to maximise gas transfer, and may even have signalling functions, switching on the Rh/RhAG complex at the appropriate sites. The band 3/Rh macrocomplex may also be associated with AQP1 in the membrane, which provides the H_2O necessary for the hydration of CO_2.

Studies on mice deficient in the cytoskeleton protein 4.1R revealed another red cell membrane macrocomplex involving band 3 [174]. Dimers of band 3 are linked to the spectrin-actin junction through adducin and protein 4.1R, together with glycophorin C (Gerbich blood group antigen), which is linked to actin through p55 and protein 4.1R (see Section 18.7) [173,174]. In addition, reduced levels of Rh proteins, Kell, Xk, and Duffy glycoproteins in 4.1R-deficient mice suggest that these structures are also part of this 'junctional' macrocomplex, whereas RhAG does not appear to be involved. Phosphorylation of 4.1R at two sites in human red cells, which weakens the affinity of 4.1R for β-spectrin, renders GPC, Duffy, Kell, and Xk readily extractable by non-ionic detergent [175]. The glucose transporter, GLUT1, which is linked to the spectrin-actin junction through dematin and adducin, may also be part of the junctional complex [176] (Figure 10.2).

In addition to the two complexes, band 3 can also exist in the membrane as freely diffusing dimers [174].

Band 3 also plays a role in removal of senescent and oxidatively damaged red cells from the circulation, through the action of 'naturally occurring' autoantibodies [177,178]. Haemoglobin is denatured to form hemichromes. Hemichromes have strong affinity for the N-terminal domain of band 3 and could cause

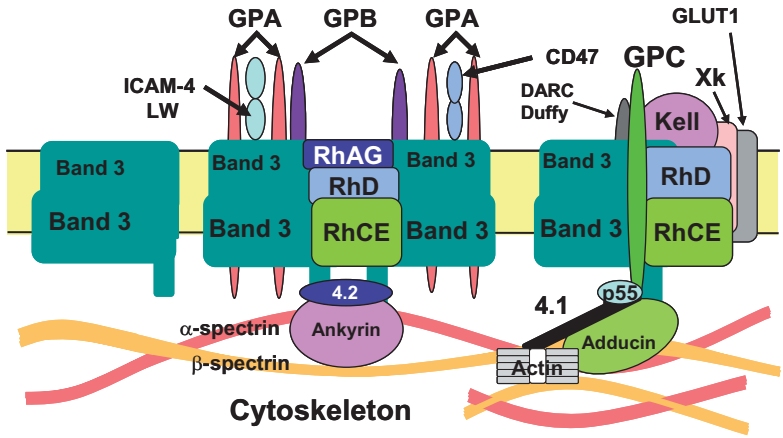

Figure 10.2 Diagrammatic representation of band 3 in the red cell membrane, showing the band 3 macrocomplex containing tetramers of band 3 linked to the spectrin matrix through protein 4.2 and ankyrin-1, the junctional complex linked to spectrin through adducin, and freely diffusing dimers of band 3.

cross-linking and disruption of band 3 attachment to the cytoskeleton, resulting in the formation of band 3 clusters. Binding of autoantibodies to the band 3 clusters may then lead to complement activation and phagocytosis of the aging red cells by splenic macrophages [178]. Similar mechanisms could be involved in the band 3 clustering and exposure of neoantigens in *Plasmodium falciparum*-infected red cells [179].

The sialic acid-rich glycoproteins, GPA, GPB, and GPC, are red cell receptors for the malarial parasite *P. falciparum* (Sections 3.21.1 and 18.8). In addition, interaction between a chymotrypsin-sensitive region of band 3 and *P. falciparum* merozoite surface protein 1 (MSP1) plays a role in a sialic acid-independent invasion pathway [180].

10.8 Tissue distribution

In addition to erythroid cells, band 3 has only been detected in acid-secreting intercalated cells of the kidney collecting duct. The renal isoform of band 3 lacks the N-terminal 65 amino acid residues of erythroid band 3, as a result of transcription initiation from an alternative promoter region in intron 3. It plays an important role in acid secretion by removing H^+ in the form of HCO_3^- ions [181]. Recessive and dominant forms of distal renal tubule acidosis (dRTA), a condition associated with impaired acid secretion in the distal nephron and inability to acidify urine resulting in symptoms including metabolic acidosis, hypokalaemia, nephrocalcinosis, kidney stones, and metabolic bone disease, result from mutations in the band 3 gene (reviewed in [36,171,182]). Mutations responsible for dRTA do not usually affect red cell band 3 function because of the presence of GPA in red cells, which is not present in kidney. The Gly701Asp mutant, for example, which causes recessive dRTA but no red cell defects, is absolutely dependent on GPA for movement to the red cell surface. The reduction in anion transport associated with these mutations in transfected *Xenopus* oocytes is rescued, at least partially, by co-transfection with GPA cDNA [183,184].

Despite the limited band 3 tissue distribution, band 3 was unexpectedly expressed on gastric cancer cells. Expression of band 3 is modulated by the microRNA miR-24, which decreases expression of band 3 through binding to the 3′-untranslated region of band 3 mRNA. Transfection of miR-24 into gastric cancer cells reduced band 3 expression, whereas miR-24 inhibitor co-operated with hemin to induce band 3 expression on K562 erythroleukaemia cells [185].

10.9 South-East Asian ovalocytosis (SAO)

A form of hereditary ovalocytosis, known as South-East Asian Ovalocytosis (SAO), is relatively common in the southern Pacific region and confers protection against cerebral malaria in children (reviewed in [35,36,171]).

SAO results from heterozygosity for a band 3 gene with a 27 bp deletion, which encodes a variant protein (band 3 SAO) with a deletion of amino acids 400–408, the region of the protein at the boundary of the cytoplasmic N-terminal domain and the first membrane-spanning domain (Figure 10.1). Band 3 SAO also has the Memphis I variant and is non-functional as an anion transporter. Despite its common occurrence, no person homozygous for this mutation has been found, supporting the assertion that homozygosity for any mutation that inactivates band 3 would normally be lethal.

Many antigens are depressed on red cells of Melanesians with SAO, including Di^b and Wr^b, but also S, s, U, En^a, D, C, e, Kp^b, Jk^a, Jk^b, Xg^a, Sc1, LW, Ge2, Ge3, Ge4, I^T, and I^F [74,186]. Reduced binding to SAO cells was also detected with rodent monoclonal antibodies to extracellular epitopes on band 3 and with monoclonal anti-Wr^b [187]. Depression of antigens on band 3 in SAO may result from the disturbance of co-operative interactions between the membrane-spanning domains, important in maintaining structural integrity of band 3. Depression of antigens of the Rh membrane complex (RhD, RhCcEe, LW, SsU) could be due to reduced translocation of the complex to the cell surface [188]. In addition there may be wider effects on other membrane proteins, caused by disruption of protein complexes involving band 3 and to an abnormal interaction between band 3 SAO (and dimers of band 3 and band 3 SAO) and the cytoskeleton [187].

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