21 Indian Blood Group System and the AnWj Antigen

21.1 Introduction

In^b/In^a, a polymorphism in people from the Indian subcontinent and in Arabs, results from an Arg46Pro substitution in CD44. In^a and In^b are of low and high incidence, respectively. The Indian system also contains two antigens of very high frequency, IN3 and IN4 (Table 21.1). CD44 is a ubiquitous glycoprotein with a variety of functions, mostly associated with its ability to bind hyaluronan, a component of the extracellular matrix. The gene encoding CD44 is on chromosome 11p13.

The high frequency antigen AnWj (901009) has not been assigned to the Indian system, but is either located on an isoform of CD44 or is closely associated with it.

21.2 CD44 and the Indian antigens

21.2.1 CD44

CD44, which is present on cells of most tissues, is a member of the link module superfamily of proteoglycans (for review see [1]). It is a major red cell membrane component of apparent MW 80 kDa [2,3]. The *CD44* gene spans 50 kb of DNA and consists of 20 exons [4,5]. CD44 exists as multiple isoforms, arising partly from alternative splicing of 10 variant exons (Figure 21.1) and partly from variation in glycosylation [4,5]. CD44s (standard), which contains products of none of the alternatively spliced exons, is the isoform present on haemopoietic cells. Exon 1 encodes the signal sequence. The 248 amino acid N-terminal extracellular domain can be divided into two regions:

1 the *N*-glycosylated link module encoded by exons 2–3, which is maintained in a folded configuration by three Cys-Cys bonds; and

2 an *O*-glycosylated mucin-like domain encoded by exons 4–7, which contains sites for covalent linkage of the glycosaminoglycan chondroitin sulphate.

Exon 8 encodes the 21 amino acid membrane-spanning domain and exon 9 the 72 amino acid C-terminal cytoplasmic tail (Figure 21.2) [6–8]. The cytoplasmic domain interacts with the membrane skeleton [3]: CD44 can bind protein 4.1R and ankyrin [9] of the two band 3-macrocomplexes (see Figure 10.2) and 4.1R-deficient red cells lack CD44 [10], yet protein 4.2 deficiency causes elevated CD44 expression [11]. There are an estimated 6000 to 10 000 copies of CD44 per red cell [12].

CD44 was assigned to chromosome 11 by testing a panel of somatic cell hybrids with the original anti-CD44 [13] and localised to 11p13 [14,15].

21.2.2 Indian antigens are located on CD44

Spring *et al.* [3] showed that In^a and In^b are carried on the CD44 glycoprotein. Immunoblotting of membranes from antigen-positive cells under non-reducing conditions with human anti-In^a and -In^b revealed an 80 kDa component of identical mobility to CD44; no such component was detected in membranes from In(a–b+) and

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Table 21.1 Antigens of the Indian system.

Antigen				Molecular basis*		
No.	Name	Frequency	Antithetical antigen	Nucleotides	Exon	Amino acids
IN1	In^a	Low	IN2	137G>C	2	Arg46Pro
IN2	In^b	High	IN1	137G (C)	2	Arg46 (Pro)
IN3	INFI	High		255C (G)	2	His85 (Gln)
IN4	INJA	High		488C (G)	2	Thr163 (Lys)

*Molecular basis of antigen-negative phenotype in parentheses.

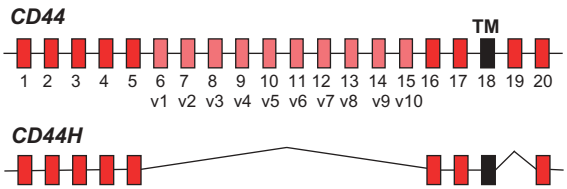

Figure 21.1 Organisation of *CD44* showing the 20 exons, including the 10 alternatively spliced exons, v1–v10, within the region encoding the extracellular domain (exons 1–17) and the exon encoding the transmembrane domain (TM, exon 18). The lower figure (*CD44H*) shows the exons encoding the haemopoietic form of CD44, CD44s.

In(a+b–) cells, respectively. CD44 glycoprotein isolated from human red cell membranes by immunoprecipitation with monoclonal anti-CD44 reacted with anti-In^b on an immunoblot. Anti-In^b was shown, by radioimmunoassay, to bind CD44 glycoprotein isolated from human red cells and leucocytes [3,16] and positive results were obtained in MAIEA assays performed with human alloanti-In^b and mouse monoclonal anti-CD44 [17,18].

21.3 Indian antigens

21.3.1 In^a and In^b (IN1 and IN2)

In 1973, a new antigen present on red cells of about 3% of Indians from Bombay was named In^a by Badakere *et al.* [19,20]. Two years later Giles [21] found that an antibody to a public antigen, Salis, was antithetical to anti-In^a and the antibody became anti-In^b.

The In^b/In^a polymorphism results from Arg46Pro at the N-terminal of an α1 helix within the link module of CD44 [22] (Table 21.1, Figure 21.1). This was confirmed by cDNA transfection and site-directed mutagenesis experiments with Jurkat human leukaemia cell line. Other changes detected in some In(a+b–) individuals, encoding Tyr109Ser and Glu239Gly, do not appear to affect expression of the Indian antigens.

Of 1749 Bombay Indians, 51 (3%) were In(a+) [20]. This gives a frequency for *IN*A* of 0.0147, and the following genotype frequencies can be deduced: *IN*A/A* 0.0002; *IN*A/B* 0.0290; *IN*B/B* 0.9708. A higher frequency of In^a was found in some Arabs: 10.6% of Iranians and 11.8% of Arabs in Bombay were In(a+) [23]. In^a is virtually unknown in other populations. Two of 700 Indian blood donors were In(b–) [24], far in excess of the number expected from the calculated genotype frequencies given above. Of 251 members of the Asian immigrant population of northern England, two were In(a+b–), 8 In(a+b+), and 241 In(a–b+); again an excess of In(a+b–) [25].

21.3.2 IN3 (INFI) and IN4 (INJA)

IN3 and IN4 are two antigens of high frequency, shown to be located on CD44 by immunoblotting and by MAIEA analysis [26]. Anti-IN3 was found in three pregnant Moroccan women. IN:–3,4 phenotype in the three propositi was associated with His85Gln in the β4 strand of the link module of CD44 (*IN*02.–03*). Anti-IN4 was found in two pregnant Pakistani women. IN:3,–4 phenotype in the two propositi and in the father and two siblings of one of the propositi was associated with Thr163Lys in the mucin-like C-terminal extension (*IN*02.–04*) (Table 21.1, Figure 21.1).

IN:–3 and IN:–4 red cells were In(a–b+) but showed weakness of In^b in titration studies [26]. Observation of three-dimensional models of the link module indicates that Arg46 (IN2) and His85 (IN3) may be in close proximity on the folded protein, but that Thr163 (IN4) would

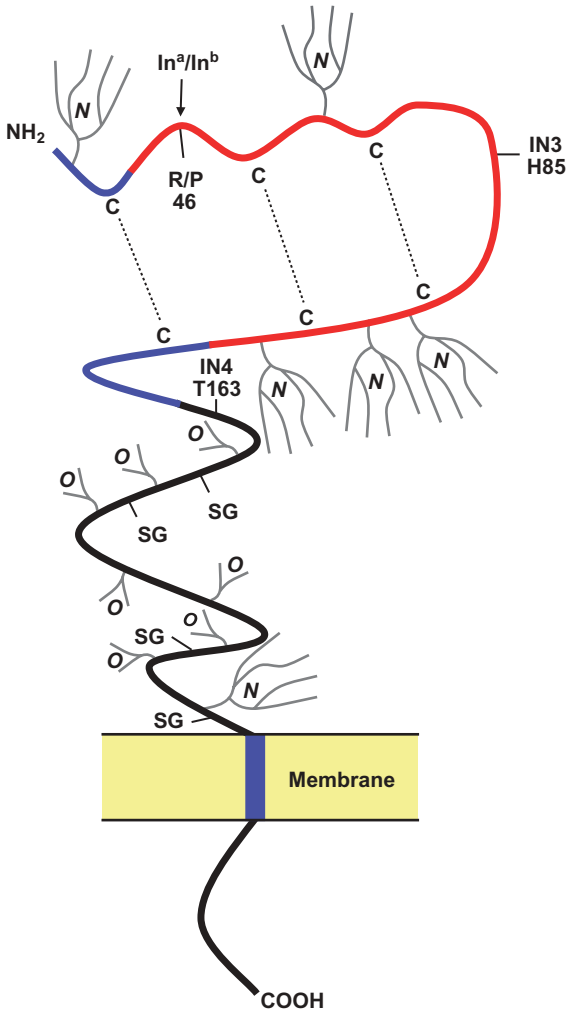

Figure 21.2 Model of CD44s. The extracellular domain consists of two regions: a membrane-proximal region (black) containing one *N*-glycosylation site (*N*), several *O*-glycosylation sites (*O*), and several Ser-Gly chondroitin sulphate linkage sites (SG); and a distal region containing five *N*-glycosylation sites and six cysteine residues (C), which suggests the presence of three disulphide bonds (dotted lines), the link module (red), and flanking extended lobes (blue). The position of the amino acid substitution responsible for the In^a/In^b polymorphism and IN3 and IN4 antigens are shown.

not be [27,28], suggesting that amino acid substitutions responsible for the rare IN:–3 and IN:–4 phenotypes affect the conformation of the link module and reduce binding of anti-In^b. Furthermore, binding of a monoclonal antibody to CD44 appeared to block binding of anti-In^b, -IN3, and -IN4, suggesting either that binding of the monoclonal antibody affects the conformation of the whole link module or that In^b, IN3, and IN4 are in closer proximity than suggested by the three-dimensional models.

21.3.3 Antigen characteristics

Indian antigens are destroyed by the proteases papain, pronase, trypsin, and chymotrypsin, but are resistant to treatment of red cells with sialidase. They are also destroyed by the disulphide bond reducing agents AET and DTT. Higher concentrations of the reducing agents than generally used for treating red cells may be required to destroy antigen expression.

Red cells from cord samples and from pregnant women show reduced expression of In^a, with about 25% of the adult number of In^a antigen sites [29]. Red cells of pregnant women have about 38% of the normal adult number, the number of sites returning to normal 3–6 months after delivery. No weakness of In^b was detected on cord red cell samples by serological titration [25].

CD44 is present in serum [30,31] and In^b can be detected in serum by haemagglutination inhibition.

21.4 Effects of *In(Lu)* on CD44 and Indian antigens

Telen *et al.* [30,32] demonstrated by flow cytometry that a CD44 monoclonal antibody (A3D8) showed markedly reduced levels of binding with red cells of the In(Lu) phenotype. In(Lu) is associated with low expression of Lutheran antigens and some other red cell antigens, and with mutations in *EKLF* (see Section 6.8). One example of In(Lu) cells bound between 25 and 39% of the quantity of anti-CD44 bound by normal cells [32]. Reduced binding was not seen with Lu$_{null}$ cells or with Lu$_{mod}$ cells of the X-linked type (Section 6.8.3), and the Lu$_{null}$ cells may even have had enhanced binding of CD44 antibodies [33]. With some CD44 antibodies the reduced binding to In(Lu) cells can also be detected by conventional serological techniques [3,34]. In inhibition experiments, normal human sera reduced binding of anti-CD44 to red cells by 67% whereas serum from an In(Lu) individual reduced binding by only 33% [30]. Immunoprecipitation and immunoblotting of membranes from In(Lu) cells revealed only a trace of CD44 [2,3,12,34], whereas Lu$_{null}$ and X-linked Lu$_{mod}$ cells had normal, or even slightly enhanced, CD44 expression [33,35].

Anti-In^b has a reduced titre with In(Lu) cells, compared with cells of normal Lutheran type and with Lu_{null} and X-linked Lu_{mod} cells [3,36], and anti-IN3 and -IN4 reacted weakly or not at all with In(Lu) cells [26]. A band of markedly reduced intensity was seen with membranes from In(Lu) cells blotted with anti-In^b [3].

Red cells of a girl with a novel form of congenital dyserythropoietic anaemia associated with heterozygosity for an *EKLF* mutation (Glu325Lys) had a gross deficiency of red cell CD44 and were In(a–b–): they were also IN:–3,–4 (as determined by immunoblotting [26]), Co(a–b–), AnWj–, and weak for LW^{ab} [37–39] (see Section 15.4 for further information).

21.5 Indian antibodies

Indian antigens appear to be good immunogens. Thirty of 39 In(a–) donors immunised for anti-D production with D+ In(a+) red cells made anti-In^a [40]. One anti-In^b and one anti-IN4 were produced in untransfused women during their first pregnancies [26,36]. Anti-In^a and -In^b often agglutinate antigen-positive red cells directly, although the strength of reaction is generally enhanced by antiglobulin [21,25,40].

Radiolabelled In(a+) red cells were eliminated from the circulation of two individuals with anti-In^a within 20 minutes, suggesting potential for a transfusion reaction [40]. There is one case of anti-In^b causing an immediate HTR after infusion of 50 ml of incompatible blood [24]. Reduced *in vivo* survival of In(b+) cells in a patient with anti-In^b was observed at 24 h [36]. Antigen-negative red cells for transfusion to patients with anti-In^b, -IN3, or -IN4, may be very difficult to obtain, though In(Lu) cells would probably be suitable for transfusion. No Indian antibody has been implicated in HDFN and it has been suggested that binding of anti-In^b to CD44 on fetal monocytes and macrophages could have a blocking effect on FcγR1 [41].

Numerous murine monoclonal antibodies to non-polymorphic epitopes on CD44 have been produced, one of which had In^b specificity [42].

21.6 Functional aspects of CD44

CD44 proteoglycan is a ubiquitous structure: very few tissues or cells lack CD44. It is present on circulating red cells, B and T lymphocytes, granulocytes, and monocytes, but not platelets [3,30,34], and is present on thymus, central nervous system white matter, epidermis, skeletal muscle, and epithelium from stomach, intestine, liver, bladder, lung, and breast [12,43].

Many roles have been attributed to CD44, the functions being regulated by the inclusion of the products of the various alternatively spliced exons. Functions of CD44 include the following: adhesion of leucocytes to endothelial cells, stromal cells, and the extracellular matrix (ECM); participation in T and B cell activation in response to immunological stimuli; lymphocyte–endothelial cell interactions involved in the localisation of lymphocytes to the site of inflammation; modelling of the ECM during wound healing and embryonic development. CD44 has also been implicated in tumour metastasis (reviews in [44,45]). Most of these interactions involve binding of CD44 to hyaluronan, a high molecular weight glycoaminoglycan that is a major component of the ECM and is also present on cell surfaces. CD44 also binds collagen, fibronectin, and laminin; proteins of the ECM that fill the spaces between cells [1]. CD44 appears to play a regulatory role in normal haemopoiesis (review in [46]). Hyaluronan binding by CD44 may be involved in adhesion of haemopoietic progenitors, including burst-forming units-erythroid (BFU-E), to the bone marrow stroma [46,47]. Surface expression of CD44 in mice progressively decreased 30-fold in late-stage erythroblasts [48]. Consequently, CD44 on circulating red cells may be vestigial, its functions being completed during erythropoiesis.

CD44 contains three copies of a hyaluronan binding motif, BX_7B (B = arginine or lysine; X_7 = seven non-acidic amino acids), present in proteins that bind hyaluronan [49]. Experiments involving site-directed mutagenesis identified several amino acid residues that were considered important in the binding of CD44 to hyaluronan, one of which was Arg46 (the second B of a BX_7B hyaluronan-binding motif). Mutating Arg46 to Gly in CD44 constructs abolished hyaluronan binding [49]. In(a+b–) phenotype results from homozygosity for *CD44* alleles encoding Pro46 (Section 21.3.1), yet the In^a Arg-46Pro substitution does not reduce hyaluronan binding to intact CD44s, *in vitro* [22]. A combined modelling and mutagenesis study has subsequently suggested that Arg41, Tyr42, Arg78, and Tyr79 are critical for hyaluronan binding, and Lys68, Asn100, Asn 101, and Tyr105 support binding [50]. All of these residues, except Lys68, come together in a three-dimensional module, as a contiguous linear patch [27]. Glycosylation of Asn25 and Asn120, which are outside the link module, also play a key role in hyaluronan binding.

21.7 AnWj (901009)

Anti-Anton [51] and anti-Wj [52] were names given to alloantibodies and autoantibodies that failed to react with In(Lu) phenotype cells and with cord cells, but did react with Lu_{null} cells. It subsequently became clear that anti-Anton and -Wj had the same specificity [53,54] and the name AnWj was given to the antigen they define.

21.7.1 Inheritance and frequency

The rare AnWj– phenotype is usually acquired and may be transient, but one family demonstrated that it could also be inherited [55]. Two of seven siblings of an AnWj– Arab woman with anti-AnWj were AnWj–. The consanguineous parents and the six children of the propositus were AnWj+, suggesting that the AnWj– phenotype in this family results from homozygosity of a rare recessive gene. The family study demonstrated that AnWj is not controlled by *LU*, or by *ABO*, *MNS*, *RH*, *KEL*, *FY*, *JK*, *XG*, or *XK*.

Anti-AnWj screening of red cells from 2400 American donors revealed three In(Lu) samples, but no AnWj– red cells with normal Lutheran antigens [56].

21.7.2 AnWj and the In(Lu) phenotype

Like In^b and several other red cell antigens outside the Lutheran system, AnWj is expressed only very weakly on red cells of individuals with the dominant gene *In(Lu)* (see Section 6.8). Usually AnWj cannot be detected on these Lu_{mod} cells by direct testing, but anti-AnWj can be adsorbed and eluted from them [55]. Lu_{null} and X-linked Lu_{mod} cells have normal AnWj expression [51,57].

AnWj differs from Lutheran and Indian system antigens in being resistant to trypsin, chymotrypsin, and the disulphide bond reducing agent AET.

21.7.3 Development of AnWj

Analysis of red cells from 36 infants revealed that the age at which conversion from AnWj– to AnWj+ takes place varies from infant to infant, but occurs between the ages of three days and 46 days and requires less than one day to complete [58]. This rapid, 'all or nothing' phenomenon is unexpected as the red cells in the circulation are not all produced at the same time and no evidence could be found for a conversion factor in the serum. Whatever causes the change from AnWj– to AnWj+ in infants might be reversible on rare occasions, as the adult AnWj– phenotype with concurrent presence of anti-AnWj has been found to be transient in several patients and in a healthy individual [54,58–60].

21.7.4 AnWj as a receptor for *Haemophilus influenzae*

H. influenzae is a commensal bacterium of the throat of most healthy people, but it may also cause respiratory tract infections and, more seriously, is a major cause of bacterial meningitis in young children. Some strains of *H. influenzae* express fimbriae (short, thread-like processes attached to the cell walls), which are probably involved in adherence to nasopharyngeal epithelial cells.

Fimbriae-bearing strains of *H. influenzae* isolated from patients with invasive disease and respiratory tract infections agglutinated most red cell samples from adults, including Lu_{null} cells, but did not agglutinate cord cells, In(Lu) cells, or AnWj– red cells of both acquired and inherited types [61,62]. Anti-AnWj inhibited agglutination of AnWj+ red cells by the bacteria.

H. influenzae bound to buccal epithelial cells, including those from one individual with transient AnWj– red cells, two with *In(Lu)* genes, and several neonates, but did not bind to buccal epithelial cells from three AnWj– members of the Arab family with inherited AnWj– phenotype [62,63]. Adherence of *H. influenzae* to epithelial cells was not inhibited by anti-AnWj [63]. Although the receptors for *H. influenzae* adherence on red cells and epithelial cells may not be identical, they appear to have a common genetic basis.

21.7.5 Anti-AnWj

Anti-AnWj may be autoantibodies or alloantibodies, or apparent alloantibodies in patients, usually with lymphoid tumours, with an acquired AnWj– phenotype. In the only family with more than one AnWj– member, the propositus and her AnWj– sister both had anti-AnWj and both had been pregnant, but not transfused [55]. It is a little surprising that anti-AnWj can be stimulated by pregnancy considering the very low level of AnWj antigen on neonatal red cells.

Anti-AnWj has been incriminated in severe HTRs [60,64–68], although there are several cases where AnWj+ cells have been transfused uneventfully to patients with anti-AnWj. The haemolytic potential of anti-AnWj has been supported by *in vivo* red cell survival studies [64,69] and monocyte monolayer analysis [67,70]. In a patient with autoanti-AnWj and depressed red cell AnWj expression, radiolabelled autologous red cells survived normally, but AnWj+ allogeneic cells had reduced survival

[71]. If available, In(Lu) cells are most suitable for transfusing patients with anti-AnWj.

There was no indication of HDFN in any of the children of a mother with alloanti-AnWj [55].

Two monoclonal antibodies with AnWj specificity were produced from mice immunised with human T-cell lines derived from patients with acute lymphocytic leukaemia [72].

21.7.6 AnWj may be located on CD44 glycoprotein

In(a–b–) red cells of the patient with a novel form of CDA and a gross deficiency of red cell CD44 were AnWj– [37–39]. Positive results were obtained in MAIEA analyses performed with either anti-CD44 or -AnWj murine monoclonal antibodies and anti-In^b or -AnWj human antibodies [17]. The CD44-negative human leukaemia cell line, Jurkat, transfected with *CD44* cDNA reacted with human and mouse monoclonal anti-AnWj [73]. Immunoblotting with anti-AnWj revealed an 80 kDa component in normal red cells and in Chinese hamster ovary cells (CHO) and murine erythroleukaemia (MEL) cells transfected with *CD44* cDNA, but not in non-transfected CHO or MEL cells, or in In(Lu) red cells. The anti-AnWj also bound a 200 kDa structure in red cells and transfected CHO cells. Telen *et al.* [73] suggest that AnWj antigen is located on a trypsin-resistant region of an isoform of CD44 that is not present on red cells of the newborn. AnWj might also be on the 200 kDa chondroitinated isoform of CD44.

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