5 Rh and RHAG Blood Group Systems

5.1 Introduction

Rh is the most complex of the blood group systems, comprising 54 antigens numbered RH1 to RH61 with seven numbers obsolete (Table 5.1). The Rh antigens are encoded by two homologous, closely linked genes on the short arm of chromosome 1: *RHD*, producing the D antigen, and *RHCE*, producing the Cc and Ee antigens. *RHD* and *RHCE* encode RhD (CD240D) and RhCcEe (CD240CE), highly hydrophobic, non-glycosylated proteins, which span the red cell membrane 12 times. Rh antigens are very dependent on the conformation of the Rh proteins in the membrane and may involve interactions between two or more extracellular loops.

The first discovered and clinically most important antigen is D (RH1). D is often referred to as the Rh or rhesus antigen, because it was initially thought to be the same as the antigen, now called LW, defined by antibodies produced in rabbits immunised with rhesus monkey red cells (Section 5.2). D is present on red cells of about 85% of white people and is more common in Africans and Asians. Before the introduction of anti-D immunoglobulin prophylaxis, anti-D was a common cause of severe haemolytic disease of the fetus and newborn (HDFN) (Section 5.18.1.4). Although most people are either D+ or D–, variants of D exist resulting in weak or partial antigen expression (Section 5.6). D– phenotype occurs from the absence of the RhD protein from the red cell membrane. In white people, D– usually results from homozygosity for a deletion of *RHD*, but in D– Africans inactive *RHD* is common.

C and c, E and e represent two pairs of antithetical antigens; polymorphisms controlled by *RHCE*. As no recombination between D, Cc, and Ee has been disclosed, the alleles are inherited as haplotypes denoted *DCe*, *DcE*, *dce*, etc., where *d* represents an *RHD* deletion or any inactive *RHD* gene. From serological results it is often impossible to determine the true genotype of an individual and phenotypes are often symbolised as the most probable genotype deduced from known haplotype frequencies (Section 5.4).

The term 'haplotype' is used throughout this chapter to represent the haploid complement of Rh genes, even though most D– 'haplotypes' will comprise a single Rh gene.

Human Blood Groups, Third Edition. Geoff Daniels.

Table 5.1 Antigens of the Rh system.

No.	Symbol	Comments
RH1	D	Polymorphic; no antithetical antigen
RH2	C	Polymorphic; antithetical to c
RH3	E	Polymorphic; antithetical to e
RH4	c	Polymorphic; antithetical to C
RH5	e	Polymorphic; antithetical to E
RH6	ce, f	Polymorphic; c and e encoded by the same gene
RH7	Ce, rh_i	Polymorphic; C and e encoded by the same gene
RH8	C^w	Polymorphic; antithetical to C^x and MAR
RH9	C^x	LFA; antithetical to C^w and MAR
RH10	V	Associated with ce^s VS+, but not (C)ce^s VS+
RH11	E^w	LFA associated with E variant
RH12	G	Polymorphic; expressed when either C or D present
RH17	Hr_o	HFA; absent from Rh_{null}, D−−
RH18	Hr	HFA; absent from E− e+ hr^S−, D−−, Rh_{null}
RH19	hr^S	e variant
RH20	VS	Associated with ce^s V+ and (C)ce^s V−
RH21	C^G	C-like antigen of r^G
RH22	CE	LFA; C and E encoded by the same gene
RH23	D^w	LFA; associated with DV
RH26	c-like	Variant of c
RH27	cE	Polymorphic; c and E encoded by the same gene
RH28	hr^H	Variant of VS
RH29	total Rh	HFA; only absent from Rh_{null}
RH30	Go^a	LFA; associated with DIVa, DAU-4
RH31	hr^B	e variant
RH32		LFA; associated with D(C)(e) R^N and DBT
RH33		LFA; associated with DHAR
RH34	Hr^B	HFA; absent from E− e+ hr^B−, D−−, Rh_{null}
RH35		LFA; associated with D(C)(e)
RH36	Be^a	LFA; associated with d(c)(e)
RH37	Evans	LFA; associated with D·· and DIVb
RH39		Anti-C-like autoantibody
RH40	Tar	LFA; associated with DVII
RH41		Ce-like
RH42	Cce^s	Associated with (C)ce^s VS+ V−
RH43	Crawford	LFA; associated with ceCF, d(C)(e)
RH44	Nou	HFA; on DIV(C)− and common phenotypes
RH45	Riv	LFA; associated with DIV(C)−
RH46	Sec	HFA; absent from R^N, D−−, Rh_{null} cells
RH47	Dav	HFA; on D·· and common phenotypes
RH48	JAL	LFA; associated with D(C)(e) and D(c)(e)
RH49	STEM	LFA; associated with some hr^S− and hr^B−
RH50	FPTT	LFA; associated with DFR and DHAR
RH51	MAR	HFA; antithetical to C^w and C^x
RH52	BARC	LFA; associated with DVI-3, -4
RH53	JAHK	LFA; associated with r^G
RH54	DAK	LFA; associated with DIIIa, DOL, R^N
RH55	LOCR	LFA; associated with c+ Rh:−26
RH56	CENR	LFA; associated with (C)C^w(e) NR
RH57	CEST	HFA; antithetical to JAL
RH58	CELO	HFA; antithetical to Crawford
RH59	CEAG	HFA; associated with e variant
RH60	PARG	LFA
RH61	ceMO	HFA; absent from homozygous *RHCE*ceMO*

LFA and HFA, low and high frequency antigens.
Obsolete: RH13 (Rh^A), RH14 (Rh^B), RH15 (Rh^C), RH16 (Rh^D), RH24 (E^T), RH25 (LW, now system 16), RH38 (Duclos, now RHAG1).

Numerous variants exist that involve aberrant expression of one or more Rh antigens. These rare haplotypes often produce one or more characteristic low frequency antigens (Section 5.17) and, in the homozygous state, may result in absence of high frequency antigens.

Abnormal expression of D or CcEe antigens may be caused by missense mutations in *RHD* or *RHCE*, but often involve exchange of genetic material between the two Rh genes.

The Rh-associated glycoprotein (RhAG) is a member of the Rh protein family and is produced by *RHAG* on chromosome 6 and expresses the three antigens of the RHAG blood group system (Section 5.20). Complexes, probably heterotrimers, of RhAG, RhD, and RhCcEe are part of the band 3 red cell glycoprotein macrocomplex (Section 5.5.7). No Rh antigens are expressed in the absence of RhAG.

Red cells of the Rh deficiency phenotype Rh_{null} express none of the Rh system antigens (Section 5.16). Rh_{null} has two genetic backgrounds:

1 homozygosity for an *RHD* deletion together with an inactive *RHCE* gene; and

2 homozygosity for inactivating mutations in *RHAG*.

Functions of the Rh proteins are unknown, although there is evidence that RhAG is involved in transport of neutral gases, ammonium and carbon dioxide (Section 5.22).

5.2 History

In 1939, Levine and Stetson [1] investigated a haemolytic reaction, which resulted from the transfusion of a woman with blood from her husband. She had recently given birth to a stillborn baby. An antibody in the mother's serum agglutinated her husband's red cells and those of 80% of ABO compatible blood donors. Levine and Stetson [1] showed that this new antigen, which they did not name, was independent of the known blood groups, ABO, MN, and P.

In 1940, Landsteiner and Wiener [2,3] made antibodies by injecting rhesus monkey red cells into rabbits and guinea pigs. The antibodies, called anti-Rh, agglutinated rhesus monkey red cells, but also agglutinated the red cells from 85% of white New Yorkers. Studies of 60 families showed that Rh-positive was inherited as a dominant character [3]. In the same year, Wiener and Peters [4] identified antibodies of apparently identical specificity in the sera of patients who had transfusion reactions after receiving ABO compatible blood. Levine and Stetson's antibody also appeared to be the same as anti-Rh [5].

As early as 1942, Fisk and Foord [6] demonstrated a difference between animal and human anti-Rh: red cells from all newborn babies, whether Rh+ or Rh– as defined by human anti-Rh, were positive with animal anti-Rh. It took another 20 years to prove that human and animal Rh antibodies did not react with the same antigen. The name Rh for antigens recognised by human antibodies could not be changed since it appeared in thousands of publications and so Levine [7] proposed that the antigen defined by animal anti-rhesus be called LW in honour of Landsteiner and Wiener. The accumulated information illustrating the differences between LW and Rh (D), and the genetic independence of Rh and LW, is described in Chapter 16.

Meanwhile, the complexity of the Rh groups had increased. By 1943 Race *et al.* [8] had four antisera of different Rh specificities, which defined seven alleles; in New York, Wiener [9] with three different antisera could define six alleles.

When Levine and his colleagues [5,10,11] confirmed that incompatibility between mother and fetus was the cause of HDFN, one of the success stories of prophylactic medicine began. The story culminated in the 1960s with the discovery that primary D immunisation caused by an incompatible pregnancy can be prevented by the passive administration of anti-D immunoglobulin shortly after delivery. Only a quarter of a century had elapsed between the identification of the cause and the introduction of an effective preventive measure for the disease.

5.3 Notation and genetic models

Two symbolic notations were developed to explain the increasing complexity of the Rh groups. These notations were based on different genetic theories: the Fisher–Race theory postulated three closely linked loci, *C*, *D*, and *E*, whereas Wiener's Rh-Hr theory predicted multiple alleles of a single gene.

5.3.1 Fisher's synthesis

In 1943, when Fisher [12] noticed that the reactions of two of the four antibodies being used by Race were antithetical, he suggested that the antigens they detected were encoded by alleles, *C* and *c*. The reactions of the other two antibodies did not suggest an allelic relationship and he called these anti-D and -E. Three closely linked loci

Table 5.2 Eight Rh haplotypes and their frequencies in English, Nigerian, and Hong Kong Chinese populations.

Haplotype			Frequencies		
CDE	Rh-Hr	Numerical	English	Nigerian	Chinese
DCe	R^1	*RH*1,2,–3,–4,5*	0.4205	0.0602	0.7298
dce	*r*	*RH*–1,–2,–3,4,5*	0.3886	0.2028	0.0232
DcE	R^2	*RH*1,–2,3,4,–5*	0.1411	0.1151	0.1870
Dce	R^o	*RH*1,–2,–3,4,5*	0.0257	0.5908	0.0334
dcE	*r″*	*RH*–1,–2,3,4,–5*	0.0119	0	0
dCe	*r′*	*RH*–1,2,–3,–4,5*	0.0098	0.0311	0.0189
DCE	R^z	*RH*1,2,3,–4,–5*	0.0024	0	0.0041
dCE	r^y	*RH*–1,2,3,–4,–5*	0	0	0.0036

Results of testing with anti-D, -C, -c, -E, and -e, red cells from 2000 English donors [13], 274 Yoruba of Nigeria [14], and 4648 Cantonese from Hong Kong [15].

producing *D* or *d*, *C* or *c*, and *E* or *e* were postulated and these could be assembled into eight different gene complexes or haplotypes [12] (Table 5.2). Subsequent identification of anti-e [16] and of the rare haplotype *dCE* [17] supported Fisher's hypothesis, but anti-d has never been found. Although some of the rare haplotypes found later could not be accommodated easily, the Fisher–Race CDE language is the clearest for interpretation of the majority of serological reactions and for the communication of results. Where applicable, it is this notation that will be used in this book.

5.3.2 Wiener's theory

In an alternative theory, Wiener suggested multiple alleles (R^1, R^2, R^o, etc., Table 5.2) at a single locus, each encoding an agglutinogen (antigen) composed of several blood factors (serological determinants). For example, the agglutinogen produced by R^1 expresses at least three blood factors, Rh_o, rh′, and hr″ (D, C, and e in Fisher–Race terminology).

5.3.3 Numerical notation

Rosenfield *et al.* [18] considered that the descriptive notations based on different genetical theories had obstructed critical immunological interpretation. They introduced a numerical terminology that recorded serological data 'free of bias and divorced from speculative implication', and which was ideal for computer storage and manipulation. This system avoided the assumptions often made in the older notations: for example, in CDE language the presumed genotype DCe/DCe is often used to describe D+ C+ c– E– red cells, even though they may not have been tested with anti-e.

D in CDE language, Rh_o in Rh-Hr notation, became Rh1. D+ phenotype was Rh:1 and D– was Rh:–1. The alleles producing these phenotypes were designated R^1 and R^{-1}, respectively. This numerical notation, slightly modified, is now the basis for the ISBT terminology for all blood groups (Chapter 1).

5.3.4 Tippett's two-locus model

In 1986, Tippett [19] proposed a new model, based on a wealth of serological data, proposing only two structural Rh genes: one encoding D, the other encoding the CcEe antigens. Mutation within each gene and recombination between the two genes to produce fusion genes comprising part of the D gene and part of the CcEe gene, were considered as possible explanations for some of the rare Rh phenotypes that involve aberrant expression of Rh antigens.

Within a few years, molecular analysis of the Rh genes disclosed the accuracy of Tippett's two-locus and fusion gene theories (Section 5.5.2). As with the MNS system (Chapter 3), the multifarious Rh variants appear to arise from processes involving mutation, unequal

crossing-over, gene conversion, post-translational modification of proteins, and interaction with unlinked genes.

5.4 Haplotypes, genotypes, and phenotypes

5.4.1 Frequencies

From Fisher's analysis [12], eight different Rh haplotypes were predicted and these have all been identified. The frequencies of these haplotypes vary in different populations (for summaries of data from many populations see [14,20]). Haplotype frequencies tend to differ little among Europeans, with *dce* slightly lower and *DcE* slightly higher in southern Europe than in northern Europe. In sub-Saharan Africa *Dce* dominates; in East Asia, the Pacific area, and among the indigenous people of the Americas, haplotypes lacking *D* are either rare or absent. Estimates for three selected populations are given in Table 5.2.

5.4.2 Genotypes and phenotypes

The eight haplotypes shown in Table 5.2 can be paired into (8/2)(8+1) or 36 genotypes, but, by using anti-D, -C, -c, -E, and -e, only 18 phenotypes can be distinguished (Table 5.3). Only eight of these phenotypes represent a single genotype, the other 10 represent two, three, or six possible genotypes. In the CDE notation, phenotypes are often expressed in the form of genotypes (e.g. DcE/dce). Unless demonstrated by family or molecular analysis this is not a true genotype (and not italicised), but the genotype deemed most likely on the basis of gene frequencies for the appropriate population. For example, a white English donor whose red cells give the reactions D+ C– c+ E+ e+ is 16 times more likely to be *DcE/dce* than *DcE/Dce*, and 180 times more likely than *Dce/dcE*. Consequently, the probable genotype would be DcE/dce. In Africans, however, *Dce* is more frequent than *dce* and the probable genotype for D+ C– c+ E+ e+ would be DcE/Dce.

The phenotype D+ C+ c+ E+ e+ covers six genotypes, but can be subdivided by the use of anti-ce, -Ce, -CE, or -cE (Table 5.3), though these antibodies are rare and in short supply (Section 5.10).

Molecular techniques have made it possible to distinguish *D/D* from *D/d* (see Section 5.7.2). In *D/d* individuals who are also heterozygous for *RHCE*, it is not possible to determine which *RHCE* allele is *in cis* with the active *RHD*.

5.5 Biochemistry and molecular genetics of the Rh polypeptides

5.5.1 Identification and isolation of the Rh polypeptides

In early investigations, Green and his colleagues [21–25] found that D is associated with protein and dependent on phospholipid and intact sulphydryl groups. Although isolation of membrane proteins in deoxycholate led to loss of Rh antigen activity, Lorusso *et al.* [25] noted that immune complexes of D with anti-D remained intact in the presence of the detergent. In 1982, Moore *et al.* [26] in Edinburgh and Gahmberg [27] in Helsinki exploited this protective property of anti-D on the integrity of D in the presence of detergent to isolate D antigen. They sensitised ^{125}I surface-labelled D+ red cells or membranes with IgG polyclonal anti-D, solubilised the membranes in non-ionic detergent, and precipitated immune complexes with protein A-Sepharose. SDS PAGE and autoradiography revealed a major component of apparent MW 30 kDa [26–30]; a very hydrophobic protein which, unlike most mammalian cell surface proteins, is not glycosylated and is not phosphorylated [26,27,31]. The D polypeptide is fatty acylated: palmitic acid chains are attached through thioester linkages to cysteine residues located near to the cytoplasmic leaflet of the lipid bilayer [32,33].

Immunoprecipitation of radioiodinated membrane proteins by polyclonal or monoclonal anti-c or -E, or by a monoclonal antibody to a non-polymorphic epitope associated with CcEe, demonstrated that the CcEe antigens are also associated with a membrane protein of apparent MW about 30 kDa [26,28,30,34]. This component resembles the D polypeptide: it is hydrophobic [26], palmitoylated [32,33], and not glycosylated [30]. Its electrophoretic mobility differs slightly from that of the D polypeptide, with an apparent MW about 2 kDa higher [26,28,30].

Confirmation that D and CcEe antigens are expressed on similar, but distinctly different polypeptides, came from one- and two-dimensional peptide mapping. This involved the use of either iodolabelled peptides produced by protease degradation of MW 30 kDa polypeptides from D+ and D– red cells [35,36] or of Rh polypeptides immunopurified with monoclonal anti-D, -c, or -E [37,38].

RhD and RhCE polypeptides were purified by large-scale immunoprecipitation with monoclonal antibodies [37,39] and amino acid sequences obtained for the N-terminal 41 residues of the two polypeptides were identical [40].

Table 5.3 Rh phenotypes with possible genotypes and their frequencies in an English population. Reactions with some antibodies to compound antigens and with anti-G are also provided.

Antigens									Frequency	Other antigens				
D	C	c	E	e	Phenotype		Genotypes		%	ce	Ce	CE	cE	G
+	+	−	−	+	DCe/DCe	R_1R_1	*DCe/DCe*	R^1/R^1	17.68	−	+	−	−	+
							DCe/dCe	R^1r'	0.82					
+	−	+	+	−	DcE/DcE	R_2R_2	*DcE/DcE*	R^2R^2	1.99	−	−	−	+	+
							DcE/dcE	R^2r''	0.34					
+	−	+	−	+	Dce/dce	R_or	*Dce/dce*	R^or	2.00	+	−	−	−	+
							Dce/Dce	R^oR^o	0.07					
+	+	−	+	−	DCE/DCE	R_zR_z	*DCE/DCE*	R^zR^z	<0.01	−	−	+	−	+
							DCE/dCE	R^zr^y	<0.01*					
+	+	+	−	+	DCe/dce	R_1r	*DCe/dce*	R^1r	32.68	+	+	−	−	+
							DCe/Dce	R^1R^o	2.16					
							Dce/dCe	R^or'	0.05					
+	−	+	+	+	DcE/dce	R_2r	*DcE/dce*	R^2r	10.97	+	−	−	+	+
							DcE/Dce	R^2R^o	0.73					
							Dce/dcE	R^or''	0.06					
+	+	−	+	+	DCe/DCE	R_1R_z	*DCe/DCE*	R^1R^z	0.20	−	+	+	−	+
							DCE/dCe	R^zr'	<0.01					
							DCe/dCE	R^1r^y	<0.01*					
+	+	+	+	−	DcE/DCE	R_2R_z	*DcE/DCE*	R^2R^z	0.07	−	−	+	+	+
							DCE/dcE	R^zr''	<0.01					
							DcE/dCE	R^2r^y	<0.01*					
+	+	+	+	+	DCe/DcE	R_1R_2	*DCe/DcE*	R^1R^2	11.87	−	+	−	+	+
							DCe/dcE	R^1r''	1.00	−	+	−	+	+
							DcE/dCe	R^2r'	0.28	−	+	−	+	+
							DCE/dce	R^zr	0.19	+	−	+	−	+
							Dce/DCE	R^oR^z	0.01	+	−	+	−	+
							Dce/dCE	R^or^y	<0.01*	+	−	+	−	+
−	+	−	−	+	dCe/dCe	r′r′	*dCe/dCe*	$r'r'$	0.01	−	+	−	−	+
−	−	+	+	−	dcE/dcE	r″r″	*dcE/dcE*	$r''r''$	0.01	−	−	−	+	−
−	−	+	−	+	dce/dce	rr	*dce/dce*	rr	15.10	+	−	−	−	−
−	+	−	+	−	dCE/dCE	r_yr_y	*dCE/dCE*	r^yr^y	<0.01*	−	−	+	−	+
−	+	+	−	+	dCe/dce	r′r	*dCe/dce*	$r'r$	0.76	+	+	−	−	+
−	−	+	+	+	dcE/dce	r″r	*dcE/dce*	$r''r$	0.92	+	−	−	+	−
−	+	−	+	+	dCe/dCE	$r'r_y$	*dCe/dCE*	$r'r^y$	<0.01*	−	+	+	−	+
−	+	+	+	−	dcE/dCE	$r''r_y$	*dcE/dCE*	$r''r^y$	<0.01*	−	−	+	+	+
−	+	+	+	+	dcE/dCe	r″r′	*dcE/dCe*	$r''r'$	0.02	−	+	−	+	+
							dCE/dce	r^yr	<0.01*	+	−	+	−	+

*Extremely rare.

5.5.2 Cloning of the Rh genes

In 1990, Avent *et al.* [40] in Bristol and Chérif-Zahar *et al.* [41] in Paris utilised the N-terminal amino acid sequence of D polypeptide to isolate and clone an Rh gene from human bone marrow cDNA libraries. Both teams isolated an identical gene with an open reading frame representing 417 amino acids, but differing from the RhD polypeptide sequence. The encoded amino acid sequence of this cDNA, which represents *RHCE* [42,43], is shown in Figure 5.1.

Figure 5.1 Amino acid sequences of the RhCE and RhD polypeptides as deduced from cDNA nucleotide sequences [40,41,44–47]. Where the amino acid of both polypeptides is the same, that of the D polypeptide is shown by a dash (-). The N-terminal methionine (m at position 1) is cleaved from the mature proteins. Cc and Ee polymorphisms are shown.

In 1992, cDNA representing *RHD* was cloned by Le Van Kim *et al.* [44] after further screening of a human bone marrow cDNA library with *RHCE* cDNA. The nucleotide sequence also predicted a 417 amino acid polypeptide, with 92% sequence identity with the RhCE polypeptide (Figure 5.1).

5.5.3 The Rh polypeptides

Although the *RHD* and *RHCE* cDNA open reading frames encode 417 amino acid polypeptides, the N-terminal methionine, which represents the mRNA translation-initiation signal, is cleaved from the mature proteins [40,41]. The RhD and RhCcEe polypeptides differ by between 31 and 35 amino acids, depending on the *RHCE* allele. The N- and C-terminal regions are well conserved (Figure 5.1).

The calculated MW of the Rh polypeptides is 45.5 kDa [40,41,44]. The much lower MW of 30 kDa estimated from SDS PAGE probably results from the abnormally high level of SDS binding to these very hydrophobic proteins. Results of hydropathy analyses on the amino acid sequences suggested that the Rh polypeptides traverse the membrane lipid bilayer 12 times [40,45]. The sequence of the N-terminal domain, together with the lack of a cleaved N-terminal signal sequence, indicated that the N-terminus is located within the cytoplasm [40,41] and immunochemical analyses on intact cells and on leaky ghosts with rabbit antibodies directed at the C-terminal domain confirmed the cytoplasmic location of the C-terminus [42,48,49]. Subsequently, homology modelling based on crystal structures of bacterial homologues of the Rh proteins and on the human non-erythroid Rh family glycoprotein RhCG, have supported the model of 12 membrane-spanning domains and six extracellular domains [50–54] (Figures 5.2 and 5.3). These models predict the presence of an extracellular vestibule in the regions of the third and fourth external loops and sixth, seventh, and eighth membrane-spanning domains that penetrates the cell membrane permitting access to IgG antibodies [50,55,56].

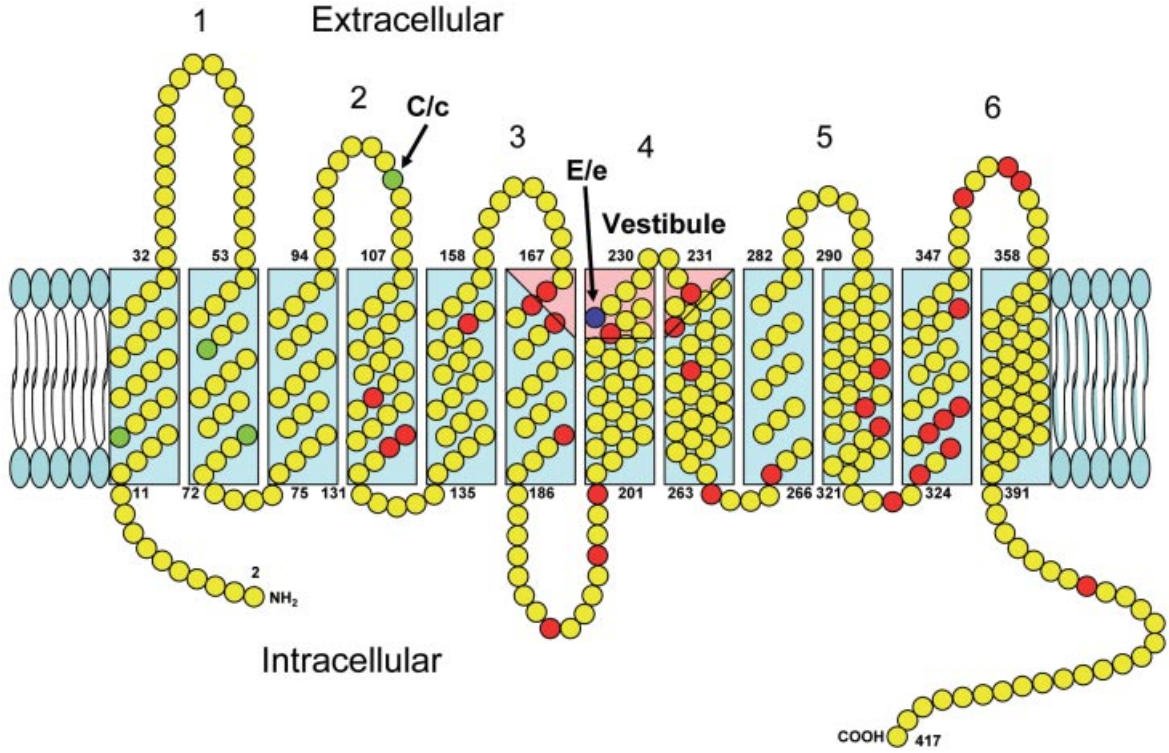

Figure 5.2 Diagrammatic representations of the possible topology of the RhD and RhCE polypeptides within the red cell membrane showing 12 membrane-spanning domains, cytoplasmic N- and C-termini, and the proposed extracellular vestibule region (pink) (after [55]). The yellow circles represent sites where the amino acid residues of the RhD and RhCE polypeptides are the same and the red circles where they may differ; those in green and blue represent the positions of the Cc and Ee polymorphisms, respectively, with the most important C/c residue arrowed.

The RhD and RhCE polypeptides have five and six cysteine residues, respectively. Three of the cysteine residues in the RhCE polypeptide and two in the RhD polypeptide form Cys-Leu-Pro motifs at the point of entry of the polypeptide into the cytoplasmic leaflet of the lipid bilayer and probably represent the major sites for attachment of palmitic acid [32,33]. Expression in erythroleukaemic (K562) cells of RhD polypeptide with the cysteine residues at positions 12, 186, 315, and 316 converted to alanine by site-directed mutagenesis demonstrated that intracellular cysteine residues are not essential for translocation and membrane assembly of RhD [57]. Palmitoylation may play a role, however, in maintaining the tertiary structure of the protein as 11 of 20 monoclonal anti-D showed reduced reactivity with the mutated protein.

The D– phenotype results from absence of the RhD polypeptide from the membrane (Section 5.6.1). Most other Rh antigens are dependent on the conformation of the Rh protein in the membrane and may involve interactions between more than one extracellular domain. Their expression can be affected by relatively minor changes to the protein, such as a single amino acid substitution in a membrane-spanning domain of the protein, which could be remote from the sequence that is primarily responsible for expression of the antigen.

5.5.4 Chromosomal assignment and organisation of the Rh genes

The second autosomal linkage reported in humans was that between the Rh blood group polymorphism and a gene for elliptocytosis, which results from mutations in *EPB41*, the gene encoding the cytoskeletal protein band 4.1 [58,59]. When the gene for peptidase C (*PEPC*) was assigned to chromosome 1 by somatic cell hybridisation [60], it was already known to be syntenic with the linkage

group containing the Rh genes, the gene for the Scianna blood group (*ERMAP*), *PGM1*, *UCK2*, and *FUCA1*. *In situ* hybridisation and somatic cell hybrid analysis located *RHCE* at 1p36.1 [61,62].

The two Rh genes, *RHD* and *RHCE*, have almost identical genomic organisation, each consisting of 10 exons, with exons 1–7 encoding 50–60 amino acids each and exons 8–10 encoding the last 58 residues [63,64] (Table 5.4). The regions of the Rh polypeptides encoded by the exons, according to the 12 membrane-spanning domain model, are shown in Figure 5.4. *RHD* and *RHCE* share 93.8% homology over all introns and coding exons [65]. The most notable difference is in intron 4, where *RHD* has a deletion of about 600 bp, relative to *RHCE* [46,65–67]. Four other intronic insertions or deletions over 100 bp were detected [65].

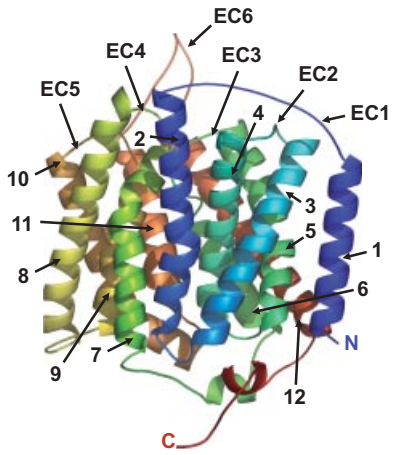

Figure 5.3 Homology model of an Rh protein (RhD or RhCE), showing the intracellular N and C termini, the 12 membrane-spanning domains, and the six extracellular (EC) domains. (Figure provided by Nicholas Burton.)

The transcription initiation site of *RHCE* is located 83 bp upstream of the translation initiation codon [63]. The 5′ flanking regions of *RHD* and *RHCE* contain at least two GATA-1 and Sp1, Ets, E2F, and putative Aly/REF transcription-factor binding sites, and GATA-1, which could play a key part in expression of the Rh genes [63,68,69].

RHD and *RHCE* consist of 57 295 and 57 831 bp, respectively [65], and are separated by about 30 kb, which contains the gene *TMEM50A* (previously *SMP1*) [70] (Figure 5.5). They are very unusual for genes in 'tandem' as they are in opposite orientation: *pter–5′RHCE3′–5′TMEM50A3′–3′RHD5′–cen* (Figure 5.5) [62,70]. *RHD* is flanked by two 9 kb regions of 98.6% homology, the *Rh boxes*. Deletion of *RHD*, the usual cause of the D– phenotype in white people, appears to have occurred between a 1463 bp region of identity in each of the *Rh boxes* [70] (Figure 5.5).

5.5.5 Genomic rearrangement of *RHD* and *RHCE*

Unequal crossing-over and gene conversion, as mechanisms for generating genomic recombination between

Table 5.4 Organisation of *RHD*, *RHCE*, and *RHAG*.

	Codons		3′ intron size (bp)		
Exons	*RHD & RHCE*	*RHAG*	*RHD*	*RHCE*	*RHAG*
1	1–49	1–52	11 857	11 758	17 700
2	50–112	53–114	5269	5575 *C* 5 318 *c*	1000
3	113–162	115–164	10 131	10 437	2300
4	163–211	165–213	426	1075	800
5	212–267	214–269	1627	1627	2100
6	268–313	270–315	3134	3133	1200
7	314–358	316–356	10 276	10 268	3800
8	359–384	357–379	4843	4826	200
9	385–409	380–404	6942	7918	900
10	410–417	405–409			

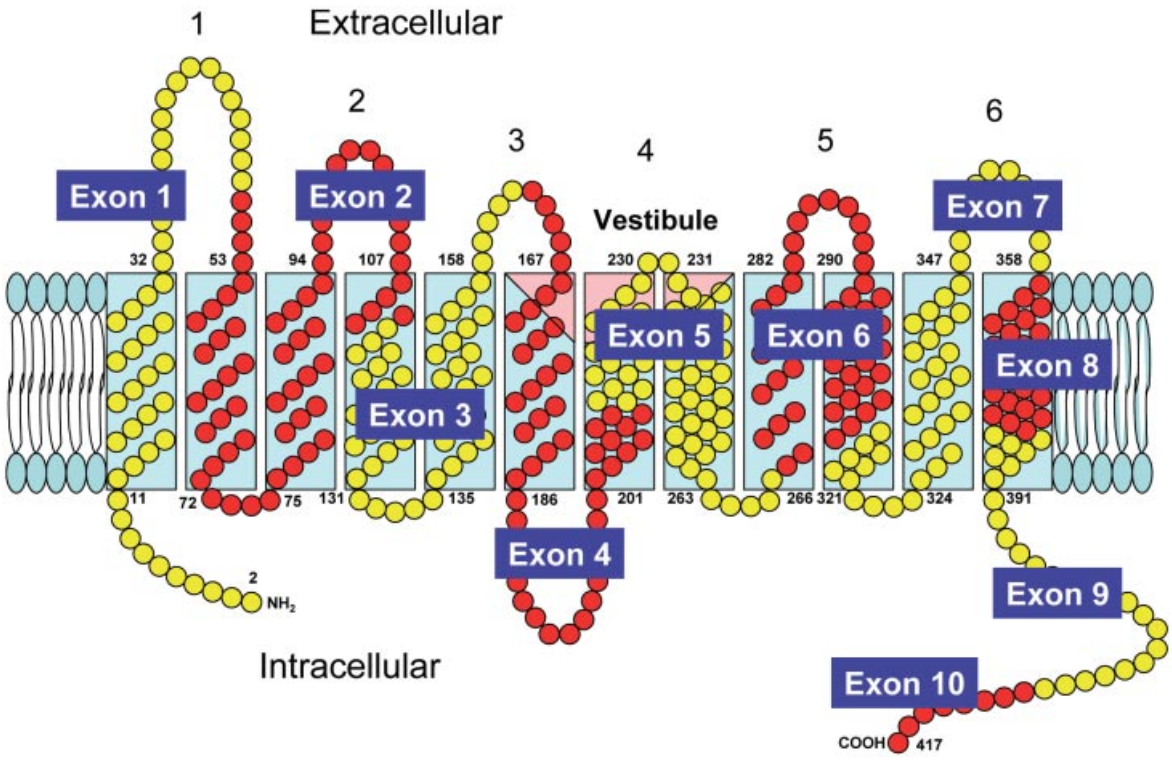

Figure 5.4 Model of the RhD and RhCE polypeptides in the membrane showing the regions encoded by the 10 exons.

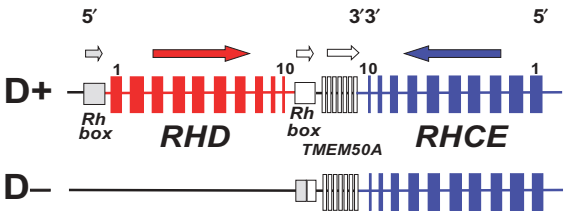

Figure 5.5 Genomic organization of the Rh genes in typical D+ (above) and D– (below) haplotypes, showing *RHD*, the two homologous *Rh boxes*, and *TMEM50A* in 5′ to 3′ orientation, and *RHCE* in 3′ to 5′ orientation. In the D– haplotype, there is a deletion of part of each *Rh box* and of *RHD*.

closely linked homologous genes, are described in Section 3.9 for *GYPA* and *GYPB*. Genomic rearrangements are associated with many examples of variant Rh phenotypes, with gene conversion appearing to be the predominant mechanism and resulting in the creation of *RHD–CE–D* and *RHCE–D–CE* hybrids. Macroconversion events give rise to an Rh gene in which a substantial segment is replaced by the equivalent segment from its homologue, whereas microconversion events can result in exchange of one or more small regions, often leading to single amino acid changes. In addition, gene conversion events are often associated with untemplated mutations, which change nucleotides to those not derived from either gene. *D–CE* or *CE–D* breakpoints for *RHD*DVI*, *RHD*DFR*, R^N, and *Dc–* all occur within a recombination hotspot located in an *Alu*-S sequence and the 100 basepairs immediately downstream of it, within intron 3 (see Figures 5.8 and 5.10) [71].

RHD and *RHCE* are in opposite orientation, so it is likely that the gene conversion events responsible for the generation of Rh hybrid genes occurred following the pairing of *RHD* and *RHCE*, *in cis* (Figure 5.6). Consistent with this supposition, mutations in exons 5 and 7 of the *d(C)ce*^{s} haplotype are present both in the *RHCE* gene and the *RHD–CE(3–8)–D* gene (Section 5.13.2). Likewise, in the DVI-1 haplotype, exons 5 of both *RHCE* and *RHD–CE(4–5)–D* encode Pro226, characteristic of an *RHCE*E* allele (Section 5.6.4.5).

5.5.6 The Rh-associated glycoprotein (RhAG, CD241)

During the original isolation of the 30 kDa Rh polypeptides, Moore *et al.* [26] noticed co-precipitating minor components of lower electrophoretic mobility. These components, with an apparent MW varying between 35 and 100 kDa were shown to be heterogeneously *N*-glycosylated glycoproteins with endo-β-galactosidase-degradable carbohydrate moieties that carry ABH determinants [30,39,72]. Treatment with endoglycosidase-F, which cleaves *N*-glycans, reduced their apparent MW to about 30 kDa [72,73]. This glycoprotein is now named the Rh-associated glycoprotein (RhAG).

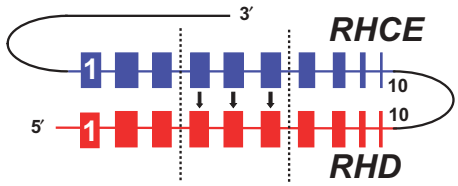

Figure 5.6 Probable mechanism for Rh gene conversion, with pairing between *RHD* and *RHCE*, *in cis*. In this example exons 4–6 of *RHD* are being replaced by the equivalent exons from *RHCE* to produce the *RHD–CE(4–6)–D* gene characteristic of DVI-2 (see Figure 5.8).

Nucleotide sequences of cloned PCR products, amplified from genomic DNA with degenerate primers derived from the N-terminal amino acid sequence of RhAG, predicted a very hydrophobic, 409-amino acid integral membrane protein with two potential extracellular *N*-glycosylation sites, no sites for palmitoylation, and a high degree of sequence similarity with the Rh polypeptides. Hydropathy analysis and *in silico* homology modelling suggested that RhAG closely resembles the Rh polypeptides with 12 membrane-spanning domains [74,75] (Figure 5.7). Only Asn37, on the first extracellular loop, is *N*-glycosylated [49].

Based predominantly on sedimentation velocity through sucrose gradients of membrane components isolated by solubilisation in non-ionic detergent, the Rh core complex was considered to be a tetramer comprising two molecules of Rh polypeptides and two molecules of RhAG [33]. More recent homology modelling based on crystal structures of bacterial Rh homologues and the human Rh family glycoprotein RhCG, the Rh and RhAG proteins appear to form trimers in the membrane (Figure 5.7). These most likely comprise homotrimers of RhAG and heterotrimers of RhAG with RhD or RhCcEe, but least likely to be heterotrimers of RhAG with RhD and RhCE [50–54].

Organisation of *RHAG*, the gene encoding RhAG, is very similar to that of *RHD* and *RHCE* (Table 5.4)

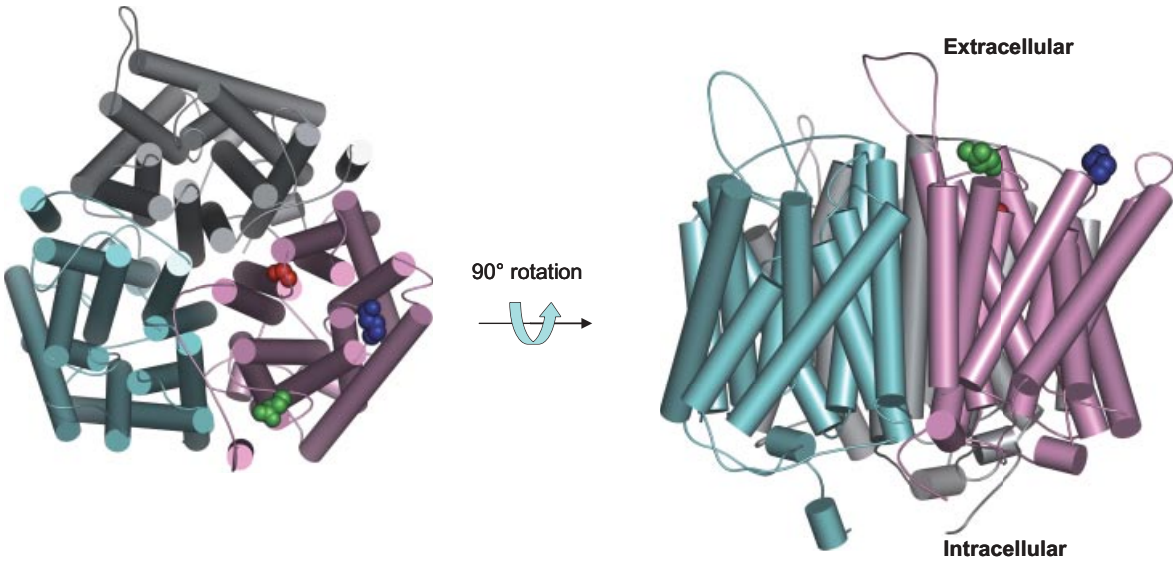

Figure 5.7 Homology model of a heterotrimer of RhAG (pink), RhCE (cyan), and RhD (grey) (reproduced from Tilley *et al.* [76], with permission from John Wiley & Sons). Also shown are the residues associated with Ol^a (red), Duclos (blue), and DSLK (green). In fact, this heterotrimer is unlikely to exist, with heterotrimers containing two molecules of either RhD or RhCE, with RhAG, more probable.

[78–80]. Most of the sequence homology is confined to exons 2–9. The 5′ promoter region contains several putative *cis*-acting elements including an inverted GATA sequence at position −58 to −53, which acts as an erythroid-specific promoter [78,81]. In addition, there is an erythroid-specific hypersensitive site 10 kb upstream from the translation-initiating codon, with powerful *RHAG*-transcription enhancing activity in K562 cells [82]. This site could be responsible for the erythroid-dominant expression of *RHAG*. In contrast to the Rh genes, which are on chromosome 1, *RHAG* is located at 6p12.3 [83].

Presence of RhAG in the red cell membrane is a requirement for expression of Rh antigens. Homozygosity for inactivating mutations in *RHAG* is the most common cause of the rare Rh_{null} phenotype, in which none of the antigens of the Rh system are expressed (Section 5.16.2). Knock-down of RhAG during early erythropoiesis was accompanied by reduced expression Rh proteins at the cell surface [84]. Red cells of *Rhag* knockout mice lack the Rhd protein [85]. Dependency on RhAG for expression of Rh antigens was also demonstrated by transfection experiments in K562 erythroleukaemic cells and human embryonic kidney cells [86–88]. Rh_{null} cells resulting from inactive Rh genes have detectable levels of RhAG [81].

During erythropoiesis *ex vivo*, RhAG appears on early erythroblasts. Initial reports suggest that RhAG appears before the Rh antigens [89–91], whereas a later study shows that they appear concomitantly [84]. The complex of RhAG and the Rh proteins is assembled within the membrane at this early stage of erythropoiesis [84]. The *N*-glycan of RhAG is smaller in Rh_{null} cells, however, inferring that RhAG moves to the surface faster in the absence of the Rh polypeptides [73].

RhAG carries the antigens of the RHAG blood group system described in Section 5.20.

Human homologues of RhAG, named RhBG and RhCG, have been recognised in non-erythroid cells, and many other members of this family are present in other species [80,92–94]. Thus, the Rh family in humans comprises five proteins encoded by the genes *RHD*, *RHCE*, *RHBG* (chromosome 1), *RHAG* (chromosome 6), and *RHCG* (chromosome 15).

5.5.7 The band 3 macrocomplex

RhD, RhCE, and RhAG are part of a macrocomplex of red cell membrane proteins with tetramers of band 3 at its core [95] (see Section 10.7 and Figure 10.2). Other constituents of this complex are the LW glycoprotein, glycophorins A and B, and CD47, and the complex is linked to the red cell cytoskeleton through attachment of band 3 to ankyrin R and protein 4.2.

RhAG and Rh proteins are co-precipitated with band 3 from solubilised membranes [95]. Red cells with band 3 Coimbra, which contain only trace levels of band 3 (~2% of normal), have about 7% and 21% of normal levels of RhAG and Rh proteins, respectively. Band 3 knockout mice have much reduced RhAG and little or no Rh [95]. K562 cells transfected with *RHD* or *RHCE* cDNA express Rh antigens, and subsequent transfection of these cells with cDNA encoding band 3 substantially increased levels of Rh antigens and of endogenously produced RhAG [96,97]. This enhancing effect of band 3 expression appears to be greater on RhCE–RhAG complexes than on RhD–RhAG complexes. The effect is reduced when K562 cells are transfected with band 3 cDNA containing the South-East Asian ovalocytosis (SAO) mutation, providing a possible explanation for reduced expression of Rh antigens on SAO red cells (see Section 10.9). The interaction between RhAG and band 3 in erythroblasts appears to occur inside the cell, followed by transfer to the membrane and subsequent interaction between RhAG and the Rh proteins at the cell surface [84]. Protein 4.2-deficient red cells have normal quantities of the proteins, although hyperglycosylation of RhAG in these cells suggests delayed trafficking from the Golgi [98,99]. There is evidence that direct interaction between ankyrin R and the C-terminal cytoplasmic domains of Rh and RhAG is important for the stability of the Rh complex in the red cell membrane [100]. Amino acid substitutions in those regions of RhD and RhAG could be responsible for weak D and Rh_{null} phenotype, respectively.

Another protein complex first recognised in mice, known as the junctional or 4.1R complex, contains band 3 dimers, Rh proteins, but not RhAG, plus Kell, Xk, and Duffy proteins, attached to the cytoskeleton through glycophorin C, protein 4.1R, and p55 [101] (see Section 10.7 and Figure 10.2).

Monoclonal anti-LW co-precipitates LW glycoprotein and Rh polypeptide [102]. LW glycoprotein is totally absent from Rh_{null} cells (Section 5.16.5) and LW antigens have a higher level of expression on D+ than D− cells (Chapter 16).

Rh_{null} cells have a 60–70% reduction in glycophorin B (GPB) [103], reflected as reduced levels of S, s, and U antigens (Section 3.20). GPB might facilitate transport of RhAG to the cell surface membrane. The RhAG of GPB-deficient (S− s− U−) red cells is more heavily glycosylated

than normal, probably as a result of RhAG remaining in the intracellular membrane system longer, permitting more glycosylation [73], and red cells of individuals homozygous for *GYP*Mur*, in which GPB is replaced by a GP(B–A–B) molecule, have significant reductions in cell surface expression of RhAG and Rh antigens [104].

CD47 is a widely distributed glycoprotein of the immunoglobulin superfamily (IgSF), with an apparent MW of 47–52 kDa in red cell membranes, five membrane-spanning domains, and an extracellular N-terminal domain containing a single IgSF V domain [105]. *CD47* is located on chromosome 3q13.1-q13.2 [106,107]. CD47 is present in reduced quantity (75% of normal) on Rh_{null} red cells [72,106,107] and also on cells expressing RhD, but no RhCE (D–– and related phenotypes, Section 5.15) [98]. CD47 is almost absent from band 3-deficient and protein 4.2-deficient red cells [95,98,99], suggesting that it is closely associated with RhCE in the band 3 macrocomplex. In contrast to mice, alteration or deficiency of CD47 in 4.1R-deficient red cells suggests that CD47 may also be located in a putative human junctional protein complex [108]. CD47 functions as a marker of self by binding signal regulatory protein α (SIRPα) on macrophages, generating a negative signal that prevents phagocytosis of the red cells, prolonging red cell survival in the circulation. This interaction could be important in limiting the destruction of host cells in AIHA [109]. There is evidence that CD47 expression decreases on circulating red cells throughout their lifespan and during storage as blood products [110]. In contrast, however, CD47 on stored red cells may undergo a conformational change, bind thrombospondin 1, and act as an "eat me" signal [111].

Fy5 antigen of the Duffy system (Section 8.4.4) is absent from Rh_{null} cells, though there is no evidence of reduced expression of the Duffy glycoprotein or other Duffy antigens. This supports the presence of a human junctional complex containing Rh proteins and the Duffy glycoprotein (see Section 10.7 and Figure 10.2).

5.6 D and variants of D

D is the most immunogenic of the Rh antigens and is the most important clinically. About 80% of D– healthy volunteers and 20–30% of D– patients who received large volumes of D+ blood made anti-D [112–115]. Until the introduction of immunoglobulin prophylaxis, anti-D was a common cause of severe HDFN.

D+ and D– phenotypes are often referred to as Rh+ and Rh–. Between 82 and 88% of Europeans and North American Caucasians are D+; around 95% of black Africans are D+ [14,20]. D is a high frequency antigen in East Asia, reaching 100% in some populations. By normal blood grouping techniques, 99.7% of Hong Kong Chinese [16] and a similar proportion of Japanese [14] appear D+, but a substantial proportion of those classified as D– have a very weak D antigen called DEL (Section 5.6.4.13).

D antigen expression varies quantitatively, with a continuum of antigen strength from the greatly enhanced expression associated with D–– to weak D, the most extreme of which is DEL. Even among the common phenotypes there is readily detectable quantitative variation of D. Less D is expressed in the presence of C [116–122]: in titrations with anti-D, DcE/DcE cells give higher scores than DCe/DCe cells. Fluorescence flow cytometry with monoclonal and polyclonal anti-D demonstrated the following decreasing order of strength of D antigen: DcE/DcE > DCe/DcE > DCe/DCe > DcE/dce > DCe/dce [123–125]. Further discussion on quantitative aspects of D is found in Section 5.6.8.

There is a plethora of *RHD* genes encoding variants of the D antigen and some producing no D antigen at all. It is not possible to list them all here, but they can be found in the web sites of Wagner and Flegel [126].

5.6.1 Molecular genetics of the D– phenotype

D– phenotype represents a total absence of D polypeptide from the red cell membrane and, consequently, absence of all epitopes of the D antigen. This explains why d, an antigen allelic to D, has never been found.

The most common cause of the D– phenotype in people of European origin is homozygosity for a complete deletion of *RHD* (Figure 5.5); D+ Caucasians are either homozygous or hemizygous for the presence of *RHD* [44,46,127]. The deletion occurs between two 1463 bp regions of identity within the *Rh boxes* that flank *RHD* [70]. Other causes of D– in Caucasians are rare.

In black Africans a common molecular background for D– is homozygosity or hemizygosity (with an *RHD* deletion) for a complete, but inactive *RHD* called the *RHD* pseudogene or *RHD*Ψ*, which contains a 37 bp sequence duplication consisting of the last 19 nucleotides of intron 3 and the first 18 nucleotides of exon 4 [128]. This duplication could generate a reading frameshift and introduce a premature translation stop codon. Alternatively, if a potential splice site at the 3′ end of the inserted intronic sequence in exon 4 were utilised, the sequence of exon 4 would remain unchanged. *RHD*Ψ* also has a nonsense mutation in exon 6 (Tyr269stop), which ensures that no

RhD protein is present in the red cell membrane. No transcript derived from *RHD*Ψ* was detected. *RHD*Ψ* is usually *in cis* with *RHCE*ce*.

Other abnormal genes that are relatively common in D– Africans are called *RHD-CE-D*^{s}. These hybrid genes usually comprise exons 1, 2, and the 3′ end of exon 3 of *RHD*, the 5′ end of exon 3 and exons 4–7 of *RHCE*, and exons 8–10 of *RHD* (type 1), but occasionally the whole of exon 3 is derived from *RHD* (Type 2) [129–132]. These genes produce no D, but probably produce abnormal C. They are associated with the VS+ V– phenotype and the *d(C)ce*^{s} (*r*′^{s}) haplotype (Section 5.13).

In black South Africans and Malians, respectively, the following frequencies of the D– alleles were estimated: *RHD* deletion, 0.10 and 0.14; *RHD*Ψ*, 0.07 and 0.065; *RHD-CE-D*^{s}, 0.04 in both [128,131,133]. Of 82 D– black Africans, 67% had *RHD*Ψ*, 15% had *RHD-CE-D*^{s}, and 18% had no *RHD*. For 54 D– African Americans, the corresponding figures were 24%, 22%, and 54% [128].

In East Asia D– phenotype is rare. Between 62 and 77% of East Asian (Japanese, Chinese, Korean) individuals whose red cells are negative by routine D typing methods are homozygous for the *RHD* deletion [67,134–138]. Most of the remainder, almost all of whom are C+, have a gene for DEL, a variant of D so weak that it can only be detected by very sensitive techniques (Section 5.6.4.13). Between 3 and 8% have an *RHD–CE–D* hybrid gene, *RHD–CE(2–9)–D*$_2$, containing exons 2–9 of *RHD* and producing no D antigen [136–138].

In addition to the *RHD* deletion, *RHD*Ψ*, *RHD-CE-D*^{s}, and *RHD–CE(2–9)–D*$_2$ there are numerous rare *RHD* genes and hybrid genes, which contain exons of *RHD* but produce no RhD protein and, therefore, no D antigen (catalogued in [126]). As a result of screening DNA from 8442 apparent D– European blood donors, 50 (0.59%) contained *RHD*, including three *RHD*DEL* alleles, two missense mutations (encoding Trp16stop or Tyr330stop), a splice site mutation (IVS8+1g>a), *RHD*Ψ*, *RHD-CE-D*^{s}, and various *RHD–CE–D* hybrids, the most common of which was *RHD–CE(2–9)–D*$_2$ [139]. All were linked to either *RHCE*Ce* or *RHCE*cE*, with the exceptions of *RHD*Ψ* and *RHD-CE-D*^{s}, which were linked to *RHCE*ce*.

5.6.2 Weak D (D^{u}) and partial D: a false dichotomy?

Stratton [140] first coined the term D^{u} for a D antigen detected by only some anti-D. The definition of D^{u} evolved to become the D of those red cells that are not agglutinated by IgM anti-D, but which react with IgG anti-D in an antiglobulin test. With the introduction of more potent anti-D reagents, most red cells that would previously have been classified as D^{u}, would not now be considered as having an abnormal D by routine testing. D^{u} has been considered a purely quantitative variant of D, differing from normal D purely on the number of antigen sites per red cell [141–145]. Consequently, there can be no D^{u} antigen and no anti-D^{u}, so 'D^{u}' was replaced with the term 'weak D' in the 1990s [146]. The definition of weak D has often depended on the anti-D reagents and techniques used and so it is difficult to provide frequencies for the weak D phenotype. One estimate gave frequencies for weak D as 0.3% and 1.7% in white and black North London donors, respectively [147].

If weak D is considered a purely quantitative variant of D, then another type of D variant, now usually referred to as partial D, is a qualitative variant. Since the publication by Argall *et al.* [148] in 1953, it has been clear that rare D+ individuals can make a form of alloanti-D. Many different types of partial D antigen have been identified. The D antigen can be regarded as a mosaic of epitopes; individuals whose red cells lack part of the D mosaic can, when exposed to a complete D antigen, make antibody to the missing epitopes. This antibody behaves as anti-D when tested against normal (complete) D.

This distinction between weak D and partial D has come to assume an inappropriate level of importance in transfusion medicine as it is often used as the basis for making the decision on whether to transfuse a patient with D+ or D– red cells and whether or not to give a woman anti-D immunoglobulin after delivery of a D+ baby. The definitions of these two terms are, however, imprecise and the terminology used to identify individual variants is confusing, often representing a mixture of phenotype and genotype.

Two types of differentiating definitions are often used, but neither is satisfactory.

1 'Individuals with partial D antigens can make anti-D; those with weak D antigens cannot.' The problem with this is that a D variant might be called weak D because no person identified with that variant has made anti-D. That does not mean, however, that any patient with that D phenotype will not make anti-D following transfusion with D positive red cells or a D positive pregnancy.

2 'In partial D, the RhD proteins have amino acid changes (from normal RhD) within one or more of the predicted external loops of RhD polypeptide, whereas weak RhD proteins have one or more amino acid substitutions within either the membrane-spanning domains

or the cytoplasmic loops of the protein, but not exposed to the outside of the membrane.' One major problem with this definition is that the precise locations of the amino acid residues of the RhD protein within the membrane are not known. Also, this definition is not functional from the point of view of transfusion practice as *RHD* genotyping is still not performed routinely in many hospitals or even reference laboratories.

The so-called partial D phenotypes have been given symbols such as DIIIa, DVI, DBT, and DFR. They can usually be distinguished from each other by serological means as well as by molecular methods and at least one individual with each phenotype has made alloanti-D. So-called weak D types have been numbered weak D type 1 to weak D type 81 (weak D-1 to weak D-81 here), plus a few intermediate numbers (e.g. weak D-4.2), with the numbers continuing to increase [126,149]. These weak D types are assumed to be associated with weakness of D expression, but it is only possible to distinguish them from each other by molecular genetic testing. Furthermore, in some cases, especially weak D-4.2, -15, and -21, they are found in patients who have made alloanti-D [150,151].

In some cases symbols suggestive of variant D antigen activity may represent *RHD* mutations, without any evidence of abnormal D expression. For example, DMA (Leu207Phe) is encoded by an *RHD* gene that has only been found *in trans* with normal *RHD*, so there is no information on the effect on D expression [133], and weak D-19 (Ile204Thr) and -20 (Phe417Ser) have normal D antigens as determined by serological tests, despite reduced numbers of D antigen sites [152].

As an example of the failure of the current terminology, the D variants DAR and weak D-4.2 have both been found in individuals with alloanti-D and both have the same three nucleotide changes encoding three amino acid substitutions [150,153]. They only differ by a single silent mutation, which does not affect the amino acid sequence of the protein. Consequently, the terms DAR and weak D-4.2 represent D variants with identical phenotypes and almost identical genotypes, yet the former is considered partial D and the latter weak D.

The weak D/partial D dichotomy is artificial and obsolescent, and the terminology used to describe these variants is misleading [154]. The terms weak D and partial D should be abolished and replaced with a single collective name, such as D variant. In the current absence of any internationally established terminology, however, the commonly used terminology will continue to be used in this chapter.

5.6.3 Partial D and the epitopes of D

The pioneering work on the subdivision of D, from which our current understanding of the immunologic profile of the D antigen is derived, was by Tippett and Sanger in the 1960s and 1970s [155,156]. They divided partial D into six categories (I–VI) from the patterns of reactions between the red cells and antibodies of D+ people who had made anti-D. Family studies showed all categories to be inherited. A seventh category was added later and category I is obsolete [157,158]. Associated low frequency antigens have subdivided categories IV, V, and VI, and assist in the definition of DIII, DVII, DFR, DBT, DAU-5, and DHAR (Table 5.5).

In the 1980s, serological analysis of D antigens was revolutionised by the introduction of monoclonal antibody technology. A plethora of monoclonal anti-D was produced. Testing these antibodies against red cells with partial D antigens led to different patterns of reactions, considered to represent different epitopes of the D antigen (epD). Lomas *et al.* [206] defined seven D reaction patterns (epD1 to epD7) by testing 29 monoclonal anti-D against red cells representing most of the category D antigens, and two more epitopes were added later [207–209]. Although epD6 and epD7 had been shown to differ by inhibition studies with radiolabelled antibodies [210], they could not be distinguished by agglutination techniques and were referred to as epD6/7. Eighteen of the 29 antibodies were anti-epD6/7 [206].

In 1994, Lomas *et al.* [187] gave the name DFR to a new partial D antigen, identified with the assistance of monoclonal antibodies. They refrained from naming DFR category VIII because it was no longer possible to carry out all the necessary cross-testing as some of the defining anti-D made by D category members were no longer available. Since then, numerous other partial D antigens have been identified (Table 5.5 [126]), creating many subsplits of the original reaction patterns. The pattern of reactions between partial D antigens and monoclonal anti-D, shown in Table 5.6, was produced in 2001 for the fourth international workshop on monoclonal antibodies to red cell surface antigens [211]. It includes 30 epitopes, with a terminology consisting of the original epD1 to epD9 (excluding epD7) [206,207,209], followed by numbers representing subdivisions of the epitopes (e.g. epD6.4). It is important to remember that the D epitopes really only represent reaction patterns and are not absolute. Reaction patterns may be dependent on antibody concentrations, particularly with epitopes of low affinity, so different batches of the same antibody could produce different results. Other factors, such as

Table 5.5 Selected variant D antigens.

Name	Molecular basis	Exons	EC domain*	LFA‡	*RHCE**	anti-D	No. D sites/ cell × 10^3	Ethnic group	References
DII	A354D	7	6		*Ce*	Yes	3	White	[158,159]
DIIIa (DIII-5)	L62F, A137V, N152T[§], T201R[§], F223V[§]	2,3,4,5	ECV	DAK	*ce*	Yes	12	Black	[158,160,161]
DIIIb (DIII-7)	*RHD–CE(2)–D*, A137V, N152T[§], T201R[§], F223V[§]	2	2, ECV		*ce*	Yes		Black	[158,162–164]
DIIIc	*RHD–CE(3)–D*	3	0		*Ce*	Yes	22	White	[158,165]
DIII-4	L62F, A137V, N152T[§]	2,3	0			Yes	33	White	[150]
DIII-6	A137V, N152T[§], T201R[§], F223V[§]	3,4,5	ECV		*ce*			Black	[163]
DIII-8	A137V, N152T[§]	3	0			Yes	11	White	[166]
DIII-9	N152T[§], P323H	3,7	0			Yes			[166]
DIVa-1	L62F, N152T[§], D350H[§]	2,3,7	6	Go[a]	*ce*	Yes	9	Black	[158,167]
DIVa-2	L62F, A137V, N152T[§], D350H[§]	2,3,7	6	Go[a]	*ce*			Black	[183]
DIVb	D350H[§], G353W[§], A354N[§], E398V	7–9	6	Evans	*Ce*, *cE*	Yes	4	White	[158,167]
DIV-3	*RHD–CE(6–9)–D*	6–9	6		*Ce*		0.6	White	[149,150]
DIV-4	D350H[§], G353W[§], A354N[§]	7	6		*Ce*		4		[168]
DIV-5	*RHD–CE(7–9)–D*	7–9	6					Japanese	[169]
DV-1 (Kou)	F223V[§], E233Q[§]	5	ECV	D[w]	*Ce*			White, Japanese	[167,170]
DVa-2	*RHD–CE(5)–D*	5	ECV	D[w]	*ce*, *Ce*, *CE*	Yes	9	White, Black, Japanese	[158,167,171, 172]
DVa-4	E233Q[§]	5	ECV	D[w]	*Ce*			Japanese	[170,171]
DVa-6	F223V[§], E233Q[§], V238M[§]	5	ECV	D[w]	*Ce*			Japanese	[170,171]
DVI-1	*RHD–CE(4–5)–D*	4,5	ECV		*cE*	Yes	0.3	White	[158,173]
DVI-2	*RHD–CE(4–6)–D*	4–6	ECV	BARC	*Ce*	Yes	2	White, Japanese	[158,174]
DVI-3	*RHD–CE(3–6)–D*	3–6	ECV	BARC	*Ce*		14	White, Chinese	[175,176]
DVI-4	*RHD–CE(3–5)–D*	3–5	ECV	BARC	*Ce*		8	Spanish	[177]
DVII	L110P	2	0	TAR	*Ce*	Yes	3	White	[158,178]
DAR (weak D-4.2)	T201R[§], F223V[§], I342T	4,5,7	ECV		*ce*	Yes	1	Black	[150,153]
DARE	T201R[§], F223V[§], E223Q[§], I342T	4,5,7	ECV		*ce*			Black	[163]
DAU-1	S230I, T379M	5,8	ECV		*ce*		2	Black	[179]
DAU-2	R70Q, S333N, T379M	2,7,8	0		*ce*		0.4	Black	[179]
DAU-3	V279M, T379M	6,8	0		*ce*	Yes	11	Black	[179]
DAU-4	E233K, T379M	5,8	ECV		*ce*		2	Black	[179]
DAU-5	F223V, E233Q, T379M	5,8	ECV	D[w]	*ce*		10	Black	[180–182]
DAU-6	S333N, T379M	5,8	0					Black	[181]
DAU-7	V279M, S333N, T379M	6,7,8	0					Black	[183]
DBA	L227P	5	ECV				6	White	[126]
DBT-1	*RHD–CE(5–7)–D*	5–7	ECV	Rh32	*Ce*, *ce*	Yes	4	White, Black, Japanese	[184,185]
DBT-2	*RHD–CE(5–9)–D*	5–9	ECV, 6	Rh32	*Ce*			Japanese	[184,186]
DCS-1	F223V[§], A226P[§]	5	ECV		*cE*		3	White	[182]

(*Continued*)

Table 5.5 (*Continued*)

Name	Molecular basis	Exons	EC domain*	LFA‡	*RHCE**	anti-D	No. D sites/ cell × 10^3	Ethnic group	References
DCS-2	A226P§	5	ECV		*cE*		0.8	White	[182]
DCS-3	F223V§, A226P§, E233Q§	5	ECV					Chinese	[176]
DFR-1	M169L§, M170R§, I172F§	4	ECV	FPTT	*Ce*, *cE*	Yes	5	White	[167,187,188]
DFR-2	*RHD–CE(4)–D*	4	ECV						[189]
DFR-3	M169L§, M170R§, I172F§, G180A	4	ECV					White	[189]
DFR-4	M169L§, M170R§	4	ECV					Chinese	[176]
DFV	F223V	5	ECV		*Ce*	Yes		White, Indian	[163,182]
DFW	H166P	4	ECV		*Ce*			White, Sri Lankan	[190]
DHAR	*RHCE–D(5)–CE*	5	ECV	Rh33, FPTT	*ce*	Yes		White	[191–194]
DHK (DYO)	E233K	5	ECV		*ce*			Japanese	[170–172]
DHMI	T283I	6	5		*cE*	Yes	2	White	[195,196]
DHO	K235T	5	ECV		*Ce*		1	White	[197]
DHR	R229K	5	ECV		*cE*		4	White	[198]
DIM	C285Y	6	ECV		*cE*		0.2	White	[150]
DMH	L54P	1	1		*ce*	Yes		White	[199]
DMI	M170I	4	ECV		*Ce*	Yes		White	[190]
DNB	G355S	7	6		*Ce*	Yes	6	White	[168]
DNU	G353R	7	6		*Ce*		10	White	[159]
DOL-1	M170T, F223V§	4,5	ECV		*ce*	Yes	5	Black	[190]
DOL-2	M170T, F223V§, L378V	4,5,8	ECV		*ce*	Yes		Black	[190,340,699]
DVL-1	R229del	5	ECV		*cE*		5	White	[200]
DVL-2	K235del	5	ECV		*Ce*		0.15	White	[200]
DWI	M358T	7	6		*Ce*	Yes	8	White	[201]
DYU	R234W	5	ECV		*ce*		3	White	[180]
Weak D-1	V270G	6	0		*Ce*	Yes	1	White	[149,150]
Weak D-1.1	L18V, V270G	1,6	0		*Ce*		0.6	White	[202]
Weak D-2	G385A	8	0		*cE*	Yes	0.5	White	[149,150]
Weak D-3	S3C	1	0		*Ce*		2	White	[149,150]
Weak D-4	T201R§, F223V§	4,5	ECV		*ce*		2	White	[149,150]
Weak D-4.1	W16C§, T201R§, F223V§	1,4,5	ECV				4	White	[150]
Weak D-15	G282D	6	5		*cE*	Yes	0.3	White	[149,150]
DEL-1	K409K, splice site	9	0		*Ce*		<0.02	E. Asian	[136,203]
DEL-5	*RHD*(IVS3+1g>a)	Intron 3			*Ce*	Yes	<0.02	White	[139,203,204]
DEL-ex 8 del	Exon 8 del	8	0		*Ce*	Yes		Black	[205]

*Extracellular loops involved or proposed extracellular vestibule (ECV).
‡Associated low frequency antigens.
§Encoded by *RHCE* sequence.

Table 5.6 Reactions of monoclonal antibodies defining 30 epitopes of D with partial D antigens (Fourth International Workshop on Monoclonal Antibodies against Human Red Cell Surface Antigens [211]).

Anti-EpD	Partial D antigen																			
	DII	DIII	DIVa	DIVb	DVa1	DVa2	DVa3	DVa4	DVa5	DVI	DVII	DFR	DBT	DHAR	DHMI	DNB	DAR	DNU	DOL	DHK
1.1	+	+	−	−	−	−	−	−	−	−	+	+	−	−	+	+	V	V	V	−
1.2	+	+	−	−	−					−	+	−	−	−	+					
2.1	+	+	−	−	+	+	+	+	−	−	+	+	−	−	+	+	+	+	+	V
2.2	+	+	−	−	+	+	+	+	−	−	+	−	−	−	−	+	−	+	+	V
3.1	+	+	−	−	+	+	+	+	+	+	+	+	−	−	+	V	+	+	+	+
4.1	−	+	+	−	+	+	+	+	+	+	+	+	−	−	+	+	+	+	+	+
5.1	+	+	+	+	−	−	−	−	−	−	+	+	−	+	+	+	+	+	+	
5.2	+	+	+	+	+	+	−	−	−	−	+	+	−	−	+	+	+	−	+	−
5.3	+	+	+	+	−	−	−	−	−	−	+	−	−	+	+	+	−	+	−	
5.4	+	+	+	+	+	−	−	−	−	−	+	−	−	−	+	+	+	+	V	−
5.5	+	+	+	+	−					−	+	−	−	−	−					
6.1	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+
6.2	+	+	+	+	+	+	+	+	+	−	+	+	+	−	+	+	+	+	+	V
6.3	+	+	+	+	+	+	+	+	+	−	+	+	−	−	+	+	+	+	+	V
6.4	+	+	+	+	+	+	+	+	+	−	+	−	+	+	+	+	+	+	+	V
6.5	+	+	+	+	+	+	+	+	+	−	+	−	+	−	+	+	+	+	+	+
6.6	+	+	+	+	+	+	+	+	+	−	+	−	−	+	+	+	+	+	V	V
6.7	+	+	+	+	+	+	−	−	−	−	+	−	−	−	V	+	+	+	+	V
6.8	+	+	+	+	+	+	+	+	+	−	+	−	−	−		+	+	+	+	V
8.1	+	+	+	+	+	+	+	+	+	−	−	−	−	−	V	+	−	−	+	V
8.2	+	+	+	+	+	+	+	+	+	−	−	−	+	−	V	+	+	+	+	+
8.3	+	+	+	+	+	+	−	−	−	−	−	−	+	−		−				
9.1	−	+	−	−	+	+	+	+	+	+	+	+	−	−	+	+	+	−	+	+
10.1	+	+		−	−					−	−	−	−	−	−					
11.1	+	+	+	+	−					−	−	−	−	−	−					
12.1	+	+	+	+	+					−	+	−	+	−	−					
13.1	+	+	+	−	+	+	+	+	+	−	+	+	−	−	−	+	+	+	+	
14.1	+	+	+	−	+					−	+	+	−	−	+					
15.1	+	+	+	−	+	+	+	+	+	+	+	+	−	−	+	+	+	+	+	−
16.1	+	+	+	+	+					+	+	+	+	−	+					

V, variable reactions with different antibodies.
For five types of DVa, see Figure 5.8.

reaction temperature or pH might appear to alter specificity.

Many of the partial D phenotypes have been subdivided on the basis of genotype by molecular sequencing (Table 5.5).

5.6.4 Characteristics of D variants

Listed below and in Table 5.5 are serological and molecular characteristics of selected D variants. Much of the serological information is taken from a variety of publications [163,166–168,172,173,175,177] and from unpublished observations. The molecular bases for some D variants involving gene rearrangements are shown in Figure 5.8.

5.6.4.1 DII, DNU, DNB

DII originally contained three unrelated propositi with *RHD*DII* travelling with *RHCE*Ce* in the two families tested. The rediscovery of the original Category II propositus led to the subdivision of epD3 [209]. DII is associated with Ala354Asp in the sixth extracellular loop of RhD [159]. DNU (Gly353Arg) [159] and DNB (Gly355Ser) [168] are D variants with similar epitope profiles to DII. DNB is common in Central Europe, with frequencies up to 1 in 292 in Switzerland [168].

5.6.4.2 DIII and the DAK (RH54) antigen

DIII red cells react with all monoclonal anti-D (Table 5.6), yet must lack at least one D epitope as some individuals with DIII phenotype make anti-D. There are at least seven sub-divisions of DIII, all having Asn152Thr in the fifth membrane-spanning domain.

DIIIa is quite common in people of African origin: tests on 93 African Americans and 63 African Brazilians revealed frequencies for *RHD*DIIIa* of 0.11 and 0.19, respectively [212], although DIIIa phenotype is not apparent when *RHD* is present on the opposite chromosome. DIIIa was initially associated with three amino acid substitutions [161], but reanalysis plus sequencing of 58 other DIIIa samples revealed six nucleotide changes encoding five amino acid substitutions: Leu62Phe, Ala137Val, Asn152Thr, Thr201Arg, and Phe223Val [213] (also known as DIII-5). None of these amino acids is predicted to be in an extracellular domain, though position 223 could be in the proposed extracellular vestibule (Figures 5.2 and 5.3). *RHD*DIIIa* is usually inherited with variant *RHCE*ce*, usually *RHCE*ce*^{S} (Section 5.13) [213]. Of 39 DIIIa patients in one study, 16 had anti-D and 27 anti-e or anti-hr^{B} [213]. DIIIa cells express the low frequency antigen DAK, which is also expressed by cells

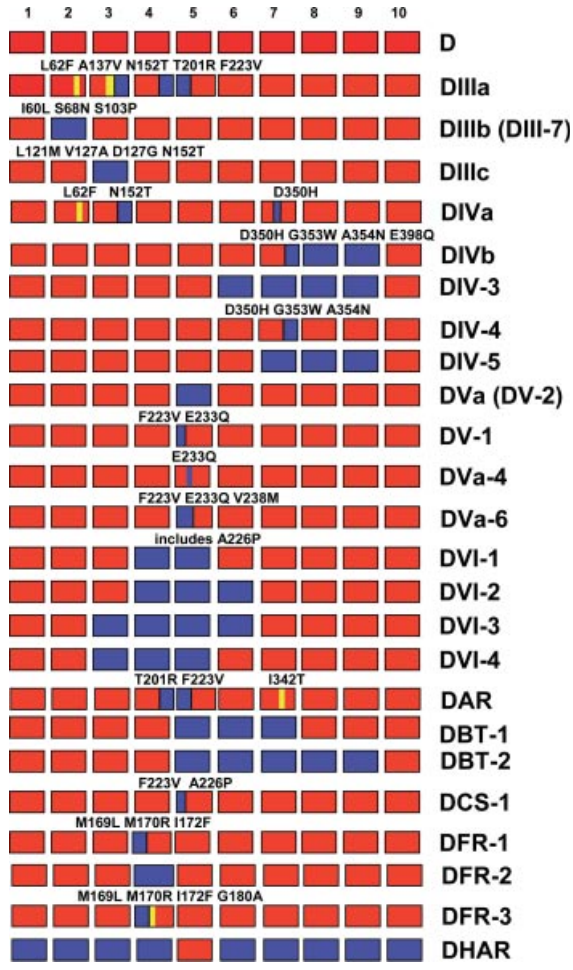

Figure 5.8 Representation of some genes responsible for variant D antigens. Red boxes, exons derived from *RHD*; blue boxes, exons derived from *RHCE*; yellow lines, untemplated amino acid substitutions. All these genes are usually paired with *RHCE*, except *RHD*HAR*.

with the DIIIb (DIII-7) and DOL variants and with the R^{N} phenotype (Section 5.14.1.1) [160,164]. Although Val223 is common to these DAK+ phenotypes, it also occurs in other unusual Rh complexes that do not express DAK. Anti-DAK reacted with red cells of 4% of African American blood donors.

Unlike most D+ red cells, DIIIb cells are G–. Two DIIIb individuals have *RHD* in which exon 2 is replaced by exon 2 from *RHCE*c* [162], but it is likely that DIIIb is identical to the variant named DIII-7, in which, in addition to having exon 2 derived from *RHCE*c*, has Ala137Val,

Asn152Thr, Thr201Arg, and Phe223Val [163,164]. DIIIb has Pro103, as a result of the conversion of *RHD* exon 2 to that of *RHCE*c*, and expresses no G, which is dependent on Ser103 encoded by either normal *RHD* or *RHCE*C* (see Section 5.11).

DIIIc propositi are white and DIIIc is inherited with *RHCE*Ce* in two families [165,214]. In one family, DIIIc is associated with *RHD* in which exon 3 is replaced by exon 3 from *RHCE* [165]. DIII types 4, 6, 8, and 9 are associated with various other amino acid substitutions, but all include Asn152Thr (Table 5.5). DIII type 5 is the same as DIIIa [213].

5.6.4.3 DIV and the Go^a (RH30) antigen

DIV, originally called D^{Cor} [215], appears to be elevated as judged by a few selected incomplete anti-D, which agglutinate saline suspensions of DIV red cells. DIV was initially subdivided by reactions with anti-Go^a, an antibody to a low frequency antigen [216–218]. DIVa and DIVb cells, which are Go(a+) and Go(a–), respectively, can be distinguished by monoclonal anti-D: DIVb, but not DIVa cells, lack epD4 (Table 5.6).

DIVa individuals are mostly black. DIVa travelled with *RHCE*ce* in most families tested, but is also present in the very rare complex DIV(C)– (Section 5.15.5). Three amino acid changes distinguish DIVa-1 from D [167] (Table 5.5): two, encoded by exons 3 and 7, probably represent microconversion events; Leu62Phe (exon 2) represents an untemplated mutation. DIVa-2 has an additional Ala137Val [183]. Asp350His (exon 7) in the sixth extracellular loop is a characteristic of all DIV subcategories.

Approximately 2% of African Americans are Go(a+) [219,220].

DIVb is heterogeneous; there is great variation in the strength of the variant D antigen. At least some DIVb cells have the low frequency antigen Evans (RH37) [221]. All known DIVb individuals have been white. DIVb travelled with *RHCE*Ce* in two families and with *RHCE*cE* in three families [158]. DIVb is associated with an *RHD–CE–D* gene in which the 3′ end of exon 7, and exons 8 and 9 are derived from *RHCE* [167].

DIV-3, -4, and -5 were defined primarily by molecular testing. All have an *RHD–CE–D* gene: in DIV-3 exons 6–9 have the *RHCE* sequence [149]; in DIV-4 only part of exon 7 is exchanged [168]; and in DIV-5, initially considered DIVb in four of 5 million Japanese blood donors [222], exons 7–9 are *RHCE*-derived [169].

DIVb and DIV-3, -4, and -5 have Asp350His, Gly353Trp, and Ala354Asn in the sixth extracellular loop.

5.6.4.4 DV and the D^w (RH23) antigen

DVa red cells are D^w+ [223]. *RHD*DVa* is usually linked to *RHCE*ce* in black families and *RHCE*Ce* in white and Japanese families; *RHD*DVa* linked to *RHCE*cE* is rare [158,224]. The strength of DVa antigen is very variable. The molecular background to DVa is heterogeneous, but always involves replacement of all or part of exon 5 of *RHD* by the equivalent region of *RHCE* (Figure 5.8) [167,170–172,197,224]. All forms involve Glu233Gln, predicted to be in the fourth extracellular loop, and one (DVa-4) has only Glu233Gln. All give rise to a typical DVa phenotype, so the Glu233Gln substitution must be the key to D^w expression. Red cells with the DHK phenotype, in which there is a Glu233Lys substitution, lack epD1 and some epD5 epitopes like DVa (Table 5.6), but are D^w– [170]. DAU-5 red cells, which have Glu233Gln, express D^w, whereas DAU-4 cells, which have Glu233Lys, do not [182]. One of the DVa variants, DVa (E) (Figure 5.8), has Pro226, suggesting that the *RHCE*-derived segment of exon 5 originated from *RHCE*E* [225]. The red cells expressed an abnormal E antigen, despite no *RHCE*E* allele being present, suggesting that the *RHD–CE–D* hybrid produced some E.

Subcategory DVb contained one propositus, whose red cells did not react with any anti-D from DVI individuals and were D^w– [226].

5.6.4.5 DVI and the BARC (RH52) antigen

DVI has very few D epitopes and most monoclonal anti-D do not react with DVI cells (Table 5.6). A minority of anti-D from D– people react with DVI cells, which may reflect a quantitative rather than a qualitative effect [158].

Most DVI propositi are white. DVI travels with *RHCE*Ce* in most families and less commonly with *RHCE*cE*. Anti-BARC is a marker for the *D^{VI}Ce* haplotype: 76 of 78 *D^{VI}Ce* samples were BARC+; all of 21 *D^{VI}cE* samples were BARC– [158,227].

Four types of *RHD–CE–D* encoding DVI have been recognised (Figure 5.8). The gene for DVI-1, which is always linked to *RHCE*cE*, has exons 4 and 5 derived from *RHCE*E* (encoding Pro226) [173,228]. Genes for DVI-2, -3, and -4, which are linked to *RHCE*Ce*, have exons 4–6, 3–6, and 3–5, respectively, derived from *RHCE*e* (Ala226) [173–175,177]. Genes for DVI-2, -3, and -4, but not -1, produce BARC, suggesting that the presence of Ala226 in the hybrid Rh protein is important in BARC expression. Apart from the polymorphism at residue 226, all three types have the same amino acid changes from normal D in predicted extracellular loops

or in the extracellular vestibule: Met169Leu, Met170Arg, and Ile172Phe in or adjacent to loop 3; Glu233Gln in or adjacent to loop 4. Quantitative differences, in terms of numbers of D sites per cell, exist between these DVI types: DVI-1 is low with about 300–500 sites; DVI-2 has about 2000 sites; DVI-4 about 8000 sites; and DVI-3 is about normal for a DCe complex with 12 000–14 000 sites [150,175,177].

By screening with monoclonal antibodies, the incidence of DVI in Europe, the USA, and Australia has been estimated between 0.015 and 0.04% and represents between 5 and 16% of weak D samples [229–233]. Only one DVI was found in over 5 million Japanese donors [222]. DVI-3 is the most common cause of DVI in Chinese [176] and DVI-4 the most frequent in Spain [177].

5.6.4.6 DVII and the Tar (RH40) antigen

Characteristic of DVII is a positive reaction with anti-Tar [234–236]. Of eight DVII individuals with anti-D, two were untransfused males and in four a weak anti-D was accompanied by a strong anti-E [158,237]. Red cells of one of 1585 D+ British donors failed to react with anti-D from a DVII individual; this sample also had DVII phenotype [237]. DVII results from a mutation in *RHD* exon 2 encoding Leu110Pro in the second extracellular loop of RhD [178]. Of over 60 000 German blood donors, 68 had DVII phenotype; of 33 DVII donors analysed, all had the Leu110Pro mutation [238]. An unusual *RHD* (DVII-2) in two unrelated individuals encoded Leu110Pro and Pro103Ser (characteristic of c in *RHCE*), and produced Tar and partial c [239].

5.6.4.7 DFR and the FPTT (RH50) antigen

Red cells with the DFR phenotype react with an antibody to the low frequency Rh antigen FPTT [187,188]. In 23 propositi DFR was associated with *RHCE*Ce* and in two propositi with *RHCE*cE*. Two DFR propositi had produced anti-D. Two of 3967 Australians, but only one of 60 000 Germans, had DFR [187,233].

DFR (DFR-1) is associated with an *RHD–CE–D* gene in which the 5′ end of exon 4 is *RHCE*-derived [167]. The three amino acid substitutions, Met169Leu, Met170Arg, Ile172Phe, are located in the third extracellular loop or extracellular vestibule. The molecular basis of FPTT expression is described in Section 5.17.2. DFR-2 has the whole of exon 4 derived from *RHCE* [189], DFR-3 is the same as DFR-1, but with an additional Gly180Ala substitution [189], DFR-4 only has Met169Leu and Met170Arg, and DFR-5 has segments of exons 3 and 4 from *RHCE*. All have Met169Leu and Met170Arg.

5.6.4.8 DBT and the Rh32 antigen

Rh32 is a low frequency antigen associated with the partial D antigen DBT [184] and with the R^N complex, which has normal D, but weak C and e (Section 5.14.1.1). Of eight DBT propositi tested, five had normal C and c, two had weak C (one of whom had a weak e), and one was C– c+. Three had made anti-D. The propositi were mostly of white European origin, but one was Moroccan, one Thai, and one Japanese.

In the Moroccan family, DBT-1 is associated with *RHD–CE–D* in which the whole of exons 5–7 are *RHCE*-derived [185]. This gives rise to Glu233Gln in loop 4 or extracellular vestibule and Asp350His, Gly353Trp, and Ala354Asn in loop 6. In the Japanese DBT-2 family, exons 5–9 of *RHCE* are present [186]. The molecular basis of Rh32 expression is described in Section 5.17.2.

5.6.4.9 DAR, DARE, and weak D type 4.2

DAR is a partial D associated with three amino acid substitutions in RhD: two, Thr201Arg and Phe223Val, are *RHCE*-derived; Ile342Thr is untemplated [153]. Val223 is predicted to be in the extracellular vestibule, close to loop 4. DAR is relatively common in Africans: of 326 black South African donors, 16 (4.9%) had *RHD*DAR* and five (1.5%) of these had the DAR phenotype. Of the 16 individuals with *RHD*DAR*, all but two had an *RHCE* variant, *RHCE*ceAR*, in which most of exon 5 and the 5′ end of exon 6 is *RHD*-derived and expresses some D epitopes (Sections 5.6.4.12 and 5.13.2).

The genes encoding weak D-4.2.1, -4.2.2, and -4.2.3 are identical apart from different synonymous mutations, which have no obvious effects on the protein product or phenotype [150]. Both DAR and weak D-4.2 phenotypes are associated with anti-D production, emphasising the difficulties of the partial D/weak D terminology. DARE, a variant found in Ethiopians, has an additional Glu233Gln substitution in the extracellular vestibule [163]. Another variation is DAR with an additional Thr203Ala [77].

5.6.4.10 Variants designated as weak D

As mentioned in Section 5.6.2, weak D is generally considered a complete D antigen, with all epitopes present, but expressed weakly. This, however, is often difficult to determine because monoclonal anti-D could fail to react with weak D cells because of low avidity of the antibody, rather than complete loss of the epitope.

In 1999, Wagner *et al.* [149,150] sequenced the 10 *RHD* exons from 161 weak D samples from Germany, all with between 70 and 4000 antigen sites per cell, and found nucleotide changes encoding amino acid substitutions in all of them. Based on amino acid sequence differences a numerical classification was established, numbering antigens from weak D type 1 to weak D type 81 (abbreviated to weak D-1 to weak D-81 here), and the list is maintained on the Rhesus web site [126]. All the amino acid substitutions associated with these weak D types are considered to be in the predicted membrane-spanning or cytoplasmic domains of the RhD protein; none was extracellular, but in some cases this could be dependent on the conformational model of RhD protein applied and whether the putative RhD vestibule is considered extracellular or intramembranous. Some of the weak D types have been subdivided; for example, weak D-1 has Val 270Gly, whereas weak D-1.1, which has weaker D expression than weak D-1, has Val 270Gly plus Leu18Val [202]. Further subdivisions (e.g. weak D-4.2.1) accommodate synonymous mutations.

Weak D of types 1–3 were the most frequent, representing, respectively, 70%, 18%, and 5% of the weak D samples tested [149]. Of 50 DCe/dce Australian blood donors with weak D, 76% had the type 1 mutation and 6% the type 3 mutation, whereas of 48 DcE/dce donors with weak D, 96% had the type 2 mutation [240]. Frequencies differ in other populations: the very rare weak D-38 (Gly278Asp) is relatively common in Portuguese [241]; weak D-42 is the most common in Quebec, Canada [242] and weak D-3 the most common in the Zagreb region of Croatia [243]. Weak D-4.3 associated with C^x and VS was found in 1 in 854 apparently D– Upper Austrian blood donors [244] (Section 5.12.2). Some of these phenotypes, such as weak D-2 and -38, have very weak expression of D giving very weakly positive results with routinely used anti-D typing reagents, whereas in some others the weakness could be barely detectable with routine reagents.

The definition of these weak D types is molecular rather than practical. They do not fit the early definitions of D^u and weak D, which assume that all epitopes are expressed and consequently cannot be associated with anti-D production. Alloanti-D has been found in individuals with weak D type 15 [150] and weak D type 4.2 is functionally identical to DAR [153] (Section 5.6.4.9), which is often associated with anti-D production. In addition, there are rare cases of patients with the two most common weak D types, types 1 and 2, with anti-D [154,245–248]. Although these antibodies are often dismissed as autoantibodies [249], if the red cells give a negative DAT, no anti-D can be eluted from them, and they do not adsorb the anti-D from the patient's own plasma, they should be considered to be alloanti-D. There are single reports of weak D types 21, 33, and 41 with anti-D [151,250,251].

5.6.4.11 Clusters of D variants

Phylogenetic studies have led to the concept of four clusters of variant *RHD* alleles, each of which is defined by a single allele that differs from the consensus *RHD* allele, and each of which may comprise many alleles that encode additional amino acid substitutions [179,190].

Eurasian Cluster. Contains the consensus or 'normal' *RHD* as its primordial allele. Includes *RHD* alleles encoding DFR-1, -2, and -3, DBA, DFV, DHQ, DMI, DFW, DFL, and weak D-17, -33, and -62 together with *RHCE*Ce* and *RHD* alleles encoding DCS-1 and -2, DHR, DFV, and weak D-16 together with *RHCE*cE* [190].

*African DAU Cluster. RHD*DAU-0* encodes Thr379Met, which has no obvious affect on D phenotype. Seven D variants, numbered DAU-1 to DAU-7, have Thr379Met plus one or two addition substitutions resulting in variant D antigen expression and associated with, at least in the case of DAU-3, anti-D production [133,179–181,183]. DAU cluster alleles are linked to *RHCE*ce*.

African weak D type 4 Cluster. Contains alleles for weak D-4 and its subdivisions, DAR and DARE, DFV, DOL-1 and -2, DTO, and the inactive allele *RHD*Ψ*, all of which encode Phe223Val and are linked to *RHCE*ce* [190].

African DIVa Cluster. Contains alleles for DIVa, DIIIa, and DIII-4, and *RHD–CE–D^S* (type 1), all of which encode Asn152Thr and are linked to *RHCE*ce* [190].

5.6.4.12 DHAR, R_o^{Har}, the Rh33 antigen and other D variants expressed in the absence of *RHD*

R_o^{Har} is a rare complex consisting of a weak partial D (DHAR), c, a very weak e, no G, and two low frequency antigens, Rh33 and FPTT [191–193] (Section 5.14.2.4). Generally, weak D antigens are most efficiently detected by an antiglobulin technique, but DHAR is different: only 7% of anti-D reacted with DHAR cells by the antiglobulin technique, although 27% reacted with enzyme-treated DHAR cells [191]. All four IgM, but only five of 24 IgG human monoclonal anti-D reacted with DHAR cells [207]. Individuals with DHAR have produced anti-D, one of which caused mild HDFN [193,252].

The 'haplotype' responsible for R_o^{Har} consists of only one gene: there is no *RHD* or *RHCE*, but an *RHCE–D–CE* hybrid with exon 5 representing *RHD* [194] (see Figure

5.10). Exon 5 of *RHD* encodes four amino acids, Phe223, Glu233,Val238, and Val245, characteristic of RhD that are in the extracellular vestibule region, and also encodes Ala226, the amino acid characteristic of e, explaining the weak e expression.

Exon 5 of *RHCE*ceCF* encodes two amino acid substitutions characteristic of RhD, Glu233 and Val245. The ceCF complex expresses partial c and e, the low frequency antigen Crawford (RH43), but not its antithetical high frequency antigen CELO (RH58), and some epitopes of D [253,254]. The ceCF phenotype was present in 0.056% of African Americans [253].

Exons 3 of *RHCE*ceRT* and *RHCE*ceSL* each encode a single amino acid substitution, Arg154Thr and Ser-122Leu, respectively, that result in expression of epD6 in the absence of *RHD* [255,256]. Neither Thr154 nor Leu12 is present in RhD and neither is expressed externally. Consequently, it is likely that the substitutions affect the conformation of the RhCE protein so that it mimics epD6. *RHCE*ceAR*, which is an *RHCE–D–CE* gene containing part of *RHD* exons 5 and 6, is usually linked to *RHD*DAR* in people of African origin (Section 5.6.4.9), but red cells of a Caucasian blood donor with *RHCE*ceAR*, but no *RHD*, expressed some D epitopes [257].

5.6.4.13 DEL

DEL is a very weak form of D that can only be detected serologically by adsorption and elution tests. Between 10 and 33% of Japanese, Chinese, and Korean red cell samples shown to be D– by conventional serological techniques were found to be DEL [116,134,135,258]. In eastern Asia the most common *RHD* gene responsible for DEL has 1227G>A in the most 3′ nucleotide of exon 9 [136–138,259–261]. This synonymous mutation in the codon for Lys409 disrupts splicing, resulting in transcripts lacking exon 9 and no normal *RHD* transcript [259,260]. Exon 9 skipping predicts an RhD protein containing 79 new amino acids at its C-terminus. *RHD*DEL* is almost exclusively linked *RHCE*Ce*.

Of 104 Chinese pregnant women with anti-D, none had the DEL phenotype and of 199 apparent D– Chinese women with a history of pregnancy, 44 had the DEL variant and no anti-D and 155 were truly D–, 38 of whom had anti-D [262].

Numerous rare *RHD* alleles are responsible for DEL phenotypes, many of them in Europeans, and almost all linked to *RHCE*Ce* or *RHCE*cE* [126,139,203–205, 261,263]. The most common DEL allele in Caucasians encodes Met295Ile and the second most common has the splice site mutation *RHD*(IVS3+1g>a) [139,204]. Four individuals with *RHD*(IVS3+1g>a) had anti-D and one of the antibodies caused mild HDFN [203,264]. A Lebanese woman with DEL red cells and *RHD* with a deletion of exon 8 also had anti-D [205]. The maximum antigen site density per red cell in DEL was 36, though in most cases there was less than 22 [203]. A deletion of four nucleotides in intron 5 of *RHD* (IVS5-38del4) was initially considered responsible for DEL phenotype [203,265], but was later shown to be a polymorphism with no effect on phenotype [189].

5.6.5 A molecular approach to the structure of D epitopes

The problem of locating D epitopes on a model of the RhD protein was approached by site-directed mutagenesis and the expression of cDNA constructs in K562 cells. Liu *et al.* [266,267] expressed cDNA representing *RHCE*cE* after having changed nucleotides encoding amino acids characteristic of the third, fourth, or sixth extracellular loops of RhCE to those characteristic of RhD. D epitope expression was then evaluated by flow cytometry. The overall conclusions were that there are six epitope clusters, some of which are overlapping, but are located predominantly on the third, fourth, and sixth RhD loops. None of these epitope clusters is more than 25Å in diameter. According to this model, some anti-D recognise a 'footprint' consisting of a single extracellular loop, with others the 'footprint' could comprise two, three, or four loops. More recent modelling of RhD based on crystal structures of homologous proteins has led to the concept of the extracellular vestibule in the region of loops 3 and 4 (Figures 5.2 and 5.3), which may permit access of antibodies to internal regions of the protein, but this does not significantly affect the model of Liu and her colleagues.

Application of phage-display technology, with Fab/phage anti-D libraries from the B cells of an anti-D producer [268], provided an alternative view of the way that monoclonal antibodies define D epitopes. Information from a genetic and serological analysis of 53 unique anti-D chosen from 83 random clones demonstrated extensive genetic homology between antibodies directed against different D epitopes [269]. Chang and Siegel [269] suggested that these antibodies would not have such similar sequences if they recognised spatially discrete and structurally unrelated regions of the D protein. They proposed that antibodies to the various D epitopes bind to an 'identical footprint', which represents most or all of the extracellular expression of the protein, rather

than spatially distinct epitopes. The specificity differences with partial D antigens would result, therefore, from conformational changes within the 'footprint'.

Any further attempts to analyse the nature of the D epitopes must take into account the architecture of the RhD-RhAG heterotrimers [77].

5.6.6 Weak D caused by the *trans* effect of *RHCE*C*

A weak D phenotype that is not inherited in a regular fashion often occurs when the haplotype encoding the D antigen is *in trans* (on the opposite chromosome) with *dCe* or, rarely, *dCE* or *d(C)ce*^{s} [270,271]. That is, there is a *trans* effect from a haplotype encoding C, but not D. When the haplotype producing the weakly expressed D is partnered by a haplotype that encodes neither C nor D (*dce* or *dcE*) in another family member, the D is expressed normally. Some examples of DEL could result from the effect of *dCe* on a haplotype containing a weak D gene [139,272].

5.6.7 Elevated D antigens

Extra strong D antigens are detected by direct agglutination of red cells by incomplete anti-D (IgG antibodies that do not directly agglutinate red cells with normal D expression). Elevation of D associated with *D––* and related haplotypes (*Dc–*, *DC*^{w}*–*, *D••*) results from an increased number of D sites (Section 5.15). Elevation of D is also associated with some *D(C)(e)* haplotypes (Section 5.14.1) and with DIVa (Section 5.6.4.3). Apparent elevation of D antigen, and of other Rh antigens, is observed in red cells with reduced sialic acid levels resulting from glycophorin A deficiency (Chapter 3).

5.6.8 Quantity of D antigen sites

The number of D sites on red cells, estimated by the use of radiolabelled anti-D and by fluorescence flow cytometry, has demonstrated that the site density differs within different phenotypes [122,141,150,273–276] (Table 5.7). Estimates for D site density on red cells with variant D phenotypes are shown in Table 5.5. Some partial D phenotypes, such as DIII and DIVa, have normal numbers of D sites per cell, whereas others, such as DVI-1 and -2, have very low D site density [145,150,273]. DEL red cells usually have less than 22 D sites per cell, with a maximum of 36, whereas weak D-26 cells, with 70 sites, were agglutinated by anti-D in an antiglobulin test [203,204,265]. In any individual, there is a wide range in the number of D sites in each red cell [123,124].

Table 5.7 Estimated number of D antigen sites per red cell for various Rh phenotypes [150,273,274,276].

Phenotype	D sites per cell (range)
DCe/dce	9900–14 600
DcE/cde	12 000–19 700
Dce/dce	12 000–23 200
DCe/DCe	14 500–22 800
DCe/DcE	23 000–31 000
DcE/DcE	15 800–33 300
D––/D––	110 000–202 000

For numbers of C, c, and e sites, see Table 5.8.

5.6.9 D variants and transfusion practice

The recommended practice in the United Kingdom is that patients should be tested in duplicate by direct agglutination with potent IgM monoclonal anti-D [277]. These antibodies are selected to give a negative reaction with DVI red cells. Consequently, patients who have the DVI phenotype and are, therefore, prone to make anti-D, are typed as D–, are transfused with D– red cells, and are given anti-D immunoglobulin following delivery of a D+ baby. Patients with very weak expression of D, including those with DEL phenotype, will also be typed as D– and will receive D– red cells or receive anti-D immunoglobulin, which will not be detrimental and could prevent them from making anti-D. An antiglobulin test for detection of some weak D phenotypes is not recommended for patient testing in the UK or USA [278], but is mandatory in some countries. Some D variants, for example DIII, in which D is expressed strongly, will be typed as D+ and so patients with these variants will be at risk of making anti-D.

If significantly weak or equivocal results are obtained on routine D typing of a patient's red cells, then the patient should be considered D– until the cause of the aberrant result is resolved. Further testing with a panel of D monoclonal antibodies will identify most of the more common variants, especially weak D-1, -2, and -3 and DAR. More definitive identification can be achieved by molecular testing, either with kits or arrays designed to identify many or most of the known variants, or by sequencing *RHD* (which will also identify new variants). Once the variant is identified, then a policy should be in place for transfusion and giving anti-D prophylaxis. It is well established that individuals with weak D-1, -2, and -3 make alloanti-D only extremely rarely, if at all, so it is

a reasonable policy to treat patients with those variants as D+, in order to conserve stocks of D– red cells, and to treat those with any other variant as D–. This will not adversely affect D– stocks as all of the other variants are relatively rare.

For typing blood donors it is essential that red cells capable of immunising a D– patient to make anti-D are labelled D+. Consequently, reagents are selected that give a positive reaction with DVI red cells and those of most other D variants. DVI individuals, therefore, are D+ donors, but D– patients. This leads to the thorny question of those variants, such as DEL, that are not detected by agglutination tests, even in the presence of antiglobulin. There is limited evidence that DEL [265,279] can cause primary immunisation of D– patients, as can other very weak forms of D, including weak D-2 [280], weak D-26 [204], weak D-1 with *dCe in trans* [281], and a D–/D+ chimera with a proportion of D+ cells too low to be detected by routine serological testing [139]. This has led some transfusion services in a few parts of Europe to introduce screening of D– donors for *RHD*, so that transfusion of D– patients with DEL red cells, especially girls and women of childbearing age, can be avoided [263]. In an international forum, however, most contributors were of the opinion that routine testing for *RHD* should not be recommended [282]. Since *RHD*DEL* is usually linked to *RHCE*Ce* or *RHCE*cE*, several contributors to the forum were in favour of transfusing D– C– E– red cells to D– patients, especially girls and women of childbearing age. In a survey from Denmark, testing of 5058 D– donors for *RHD* revealed 13 with *RHD*, all with DEL phenotype [283]. In a subsequent look-back of the 136 transfusions with these red cells to D– patients, only one recipient had made anti-D and that patient had also received D+ platelets. Eleven of the recipients had made antibodies to other blood groups, showing that they are responders. The conclusion from this study is that it would be very difficult to justify the expense of the introduction of screening all D– donors for *RHD*.

DVa stimulated production of anti-D in a D– woman during her first pregnancy and resulted in mild HDFN of her second baby, who also appeared to have the partial D antigen [284]. The mother had received anti-D immunoglobulin after the first pregnancy. A weak D-3 fetus appears to have stimulated anti-D production in a *dCe/dce* mother, causing the neonatal red cells to give a positive DAT [285].

Anti-D in women with variant D red cells has been responsible for severe HDFN [165,184,222,286–290]. Anti-D immunoglobulin should be given to women known to have partial D during and after pregnancy, because the anti-D constituent that does not bind to the mother's own partial D cells should suppress immunisation by binding to the normal D of the fetus [291]. This is particularly important in DVI mothers, whose red cells lack most D epitopes, although in most cases the mother will be typed as D– and receive anti-D automatically.

5.7 Predicting D phenotype from DNA

It is possible to predict, with a high level of accuracy, a person's D phenotype by testing their DNA. Since in most cases the D– phenotype arises from homozygosity for a deletion of *RHD*, at the simplest level this can be achieved by amplifying a region of *RHD* to determine whether the gene is present. Owing to the high level of sequence identity between *RHD* and *RHCE*, primers must be selected so that they only initiate amplification from *RHD*. The regions often used to discriminate between the two genes are exon 7, where there are multiple sequence differences, the 3′ untranslated region of exon 10 where there is a sequence in *RHD* that is not present in *RHCE*, and intron 4, which is 600 bp in *RHD* and 1200 bp in *RHCE*. To avoid false results arising from hybrid *RHD* genes that contain regions with the *RHCE* sequence (see Figure 5.9), at least two different regions must be tested. In addition, any test must also be designed to ensure that *RHD*Ψ*, the D– gene common in people of African origin, is detected [128].

Many of the early publications on PCR-based D phenotype prediction are listed by Flegel *et al.* [292]. There are now several commercial products that will identify all of the most important D variants. These platforms involve multiplex PCR followed by gel electrophoresis or the application of microarray technology, with oligonucleotides probes immobilised on chips or coloured beads.

5.7.1 Fetal D typing

It is beneficial to be able to predict D phenotype from DNA of the fetus of a D– pregnant woman with anti-D: if the fetus is D+ it is at risk from HDFN (Section 5.18.1.4) and the pregnancy can be managed appropriately; if D–, it is not at risk and no further action is required. When the molecular basis for the D– phenotype was first elucidated, fetal DNA was obtained by amniocentesis or chorionic villus sampling, invasive procedures that present a significant risk to the fetus. Since the discovery that cell-free fetal DNA is present in the blood of pregnant women [293], representing between 3 and 6% of cell-free DNA

in the maternal plasma [294], this is the preferred source of fetal DNA in D– pregnant women. Fetal D typing in alloimmunised women is now routine practice in many countries, particularly in Europe. The technology employed is usually real-time quantitative PCR targeting *RHD* exon 4, designed to give a negative result with *RHD*Ψ*, exon 5 or 7, and exon 10, so that *RHD–CE–D*^{S} will be revealed. Lists of published methods can be found in references [295,296].

One potential source of error is a previous solid organ transplant in the mother. Tests on DNA isolated from the plasma of a D– pregnant woman predicted a D+ fetus, whereas DNA isolated from amniocytes gave a D– result. The woman, who had received a kidney transplant from a D+ donor, delivered a D– baby [297].

It is policy in many countries to offer one or two doses of anti-D immunoglobulin to pregnant women at around 28–34 weeks gestation, to protect against antenatal D immunisation. In a predominantly Caucasian population, however, about 38% of these women would be carrying a D– fetus and so receive the treatment unnecessarily. Trials have shown that high-throughput methods for fetal D typing, including robotic isolation of plasma DNA, are accurate from 11 weeks gestation and that fetal D typing of all D– pregnant women is feasible [298–301]. Screening of fetal D type has been introduced as routine practice in Denmark [302] and the Netherlands [700].

The main advantage of carrying out fetal RhD screening is that it eliminates unnecessary treatment of pregnant women with blood products and the associated inconvenience, discomfort, and perceived risks of infection from pooled donor blood products that such injections entail. Fetal *RHD* screening provides a way of significantly reducing the quantity of blood products given routinely to pregnant women. In addition anti-D immunoglobulin is in short supply and produced by immunising volunteers with blood products. There may also be a small financial saving, with the cost of the test being less than that of the anti-D immunoglobulin, especially if testing takes place early in pregnancy and so avoids unnecessary anti-D injections following sensitising events such as trauma or threatened miscarriage.

A method for testing single blastomeres obtained from cleavage-stage embryos has been developed for preimplantation diagnosis to prevent severe HDFN [303].

5.7.2 *RHD* zygosity testing

When a pregnant woman has anti-D, knowledge of the *RHD* zygosity of the father will assist in predicting fetal D phenotype. If the father is homozygous for *RHD* then the fetus will be D+ and no fetal testing is required; if the father is hemizygous for *RHD*, there is an even chance that the fetus will be D+ or D–. Prediction of *RHD* zygosity from serologically determined probable genotypes is notoriously inaccurate and of little value, but there are molecular technologies available. Identification of the DNA sequence across the breakpoint within the upstream and downstream *Rh boxes* that occurs with the deletion of *RHD* in the D– haplotype (Figure 5.5) has led to the design of PCR-based tests that detect the *RHD*-deletion haplotype directly, either by the use of sequence-specific primers or *Psf*I restriction endonuclease [70,304,305]. Although this may be a suitable test for Caucasians, it is far from accurate in individuals of African origin because of considerable sequence variation in the upstream and downstream boxes [306–308]. Consequently, a better method for determining *RHD* zygosity is quantitative PCR, comparing *RHD* with a gene of known zygosity, such as *RHCE* [304,309]. It is important that a test for *RHD*Ψ* is also carried out.

5.8 C and c

5.8.1 C and c antigens

C and c are the products of alleles. C has a frequency of 68% and c a frequency of 81% in English blood donors, giving gene frequencies of 0.4327 for *RHCE*C* and 0.5673 for *RHCE*c* [13]. In black Africans the frequency of c is much higher and the frequency of C much lower, whereas in eastern Asia the opposite is the case, C approaching 100% and c of low incidence [14,21].

Table 5.8 shows the estimated numbers of C, c, and e antigen sites on red cells of various Rh phenotypes [276,310]. The number of C sites is partially dependent on the nature of the anti-C used. The figures in Table 5.8 were obtained with anti-C serum that had been adsorbed with DcE/DcE cells to remove all traces of anti-G [310].

The *DCE* haplotype produces weak C. DCE/dce cells react weakly with most anti-C sera and DCE/DcE cells have a low number of C antigen sites [310] (Table 5.8). K562 cells transfected with cDNA representing *RHCE*Ce* reacted strongly with three monoclonal anti-C, but when cDNA representing *RHCE*CE* was used, two of the anti-C did not react and the other reacted weakly [311]. It appears, therefore, that the Pro226Ala (E/e) polymorphism causes conformational changes to the protein that affect C expression.

Table 5.8 Estimated numbers of C, c, and e antigen sites per red cell [276,310].

	Antigen sites per cell (range)		
Phenotype	C	c	e
DCe/DCe	45 700–56 400		
dCe/dCe	42 200		
DCe/DcE	25 500–39 700	40 000–53 000	14 500
DCe/DCE			13 400
DCe/dce		37 000–42 000	24 400
DCe/dce C^w+	21 500–40 000		
dCe/dce	31 100		
DcE/DCE	8500–9800		
DcE/DcE		78 000–80 000	
dce/dce		70 000–85 000	18 200
d(C)ce^s/dce	7200		

For numbers of D antigen sites, see Table 5.7.

Table 5.9 The C/c and E/e polymorphisms: amino acid substitutions in the RhCcEe polypeptide deduced from DNA sequences. The sequence of the D polypeptide is shown for comparison.

Polypeptide	Amino acid residue				
	16	60	68	103	226
ce	Trp	Leu	Asn	Pro	Ala
Ce	Cys	Ile	Ser	Ser	Ala
cE	Trp	Leu	Asn	Pro	Pro
CE	Cys	Ile	Ser	Ser	Pro
D	Trp	Ile	Ser	Ser	Ala

Amino acid residue 16 is encoded by exon 1 of *RHCE*; residues 60, 68, 103 by exon 2; residue 226 by exon 5.

5.8.2 The C/c polymorphism

The C/c polymorphism is usually associated with six nucleotide substitutions in *RHCE* resulting in four amino acid changes (C/c): Cys16Trp (48C/G) encoded by exon 1; Ile60Leu (178A/C), Ser68Asn (203G/A), and Ser103Pro (307T/C) encoded by exon 2 [43,47] (Table 5.9). Only residue 103 is predicted to be outside the membrane, in the second extracellular loop (Figure 5.2).

Ser103 is essential, but not sufficient, for C specificity. Exons 2 of *RHD* and *RHCE*C* are identical and both encode Ser103. Any antibody specific for Ser103 would react with the products of *RHD* and *RHCE*C*, so would be called anti-G, not anti-C (Section 5.11). C, therefore, is a very conformational antigen and anti-C are heterogeneous. For full expression of C, the protein must have Ser103, Cys16, and some other downstream amino acids characteristic of the RhCE protein. Cys16 is not a requirement for all epitopes of C. *RHD–CE–D^s*, part of the *d(C)ce^s* haplotype that is relatively common in Africans, has exons 1, 2, and the 5′ end of exon 3 derived from *RHD*, and so encodes Trp16 and Ser103 from *RHD*, but Thr152 and the amino acids of the third extracellular loop from *RHCE* [130]. *RHD-CE-D^s* produces weak partial C [129–131,312]. Likewise, the aberrant *RHCE* gene associated with r^G, which also produces weak partial C, encodes Ser103, but Trp16 [313]. Conversion of the codon for Cys16 to Trp by site-directed mutagenesis of cDNA representing *RHCE*Ce*, and expression of the construct in K562 cells, resulted in about 50% reduction in binding of two anti-C, compared with wild type *RHCE*Ce* cDNA [311]. The abnormal C associated with Ser103 and Trp16, could represent the antigen called C^G (RH21) [314,315].

The c antigen is determined, almost entirely, by the presence of Pro103, which is encoded by *RHCE*c*, but not *RHD* (Table 5.9). The Cys16Trp polymorphism does not affect c expression – 74% of C– c+ black Africans, with normal c, have Cys16 [316] – though Cys16 encoded by an *RHCE*ce* allele does affect expression of some epitopes of e [317]. Cys16 was also present in 68% of white people with the Dce phenotype, but in none with dce/dce or DcE/DcE phenotypes [318]. Non-human primates with *RHCE* encoding Pro103, but no other amino acids characteristic of c, have c+ red cells [319,320]. Site-directed mutagenesis experiments have revealed that different anti-c have different amino acid requirements [267].

5.8.3 Predicting C/c phenotype from DNA

The defining difference between the *RHCE*C* and *RHCE*c* is a 307T/C polymorphism in exon 2. Exons 2 of *RHD* and *RHCE*C* have an identical sequence, so it is not possible to design allele-specific primers for identification of *RHCE*C* (except in D– samples), though it is relatively straightforward to design them for *RHCE*c* [128,318,321]. C expression does not correlate closely enough with 48C in intron 1, encoding Cys16, for use in predicting C phenotype (Section 5.8.2). A size polymorphism in intron 2 of *RHCE* correlates closely with

C/c expression [322]. *RHCE*C/c* genotyping has been achieved by performing two PCRs: one incorporating a primer complementary to a sequence within an *RHCE*C*-specific 109 bp insert in intron 2; the other utilising a primer specific for 307C (*RHCE*c*) in exon 2 [128,321,323]. The *d(C)ce*^{s} haplotype gives a negative result in the intron 2 test despite producing C, because intron 2 from the *RHD–CE–D*^{s} gene that produces the abnormal C is *RHD*-derived (Section 5.13.2). Quantitative real-time PCR has been applied to predicting C/c phenotypes from fetal DNA in maternal plasma [296].

5.8.4 Cc antibodies

The well-known heterogeneity of anti-C sera (lacking anti-D and -G) has been demonstrated by tests against a variety of C+ phenotypes. This heterogeneity can only be interpreted partly in terms of varying quantities of anti-C, -Ce, and -CE [324]. Most immune 'anti-C' made by D+ people are predominantly anti-Ce (Section 5.10.2). Grouping reagents made from IgM anti-C found in sera of D– people, together with 'incomplete' anti-D, often contain anti-G [324].

5.9 E and e

5.9.1 E and e antigens

E and e, another pair of antithetical antigens within the Rh system, are encoded by *RHCE*. In all populations e has a significantly higher frequency than E [14,21]. The following are antigen and gene frequencies in an English population: E 29%; e 98%; *RHCE*E* 0.1554; *RHCE*e* 0.8446 [13]. The figures for most other populations do not differ substantially from these.

E antigen site densities vary considerably, depending both on red cell phenotype and source of anti-E [276]. Masouredis *et al.* [325] recorded 27 500 E sites on DcE/DcE cells and 17 900 on DcE/dce cells. Estimates of e antigen site densities are shown in Table 5.8.

5.9.2 The E/e polymorphism

The E/e polymorphism is associated with 676C/G in exon 5 of *RHCE*, predicting Pro226Ala in the extracellular vestibule of the RhCE protein [43,47] (Table 5.9). The e antigen is not solely dependent on Ala226 because Ala226 is also encoded by *RHD*, yet the *RHD* product does not express e. Many different changes to *RHCE* will affect e expression, as will become apparent in subsequent sections of this chapter. For example, the presence of VS antigen, which results from Leu245Val encoded by an *RHCE*e* allele, is associated with e weakness (Section 5.13) [130,131,326].

When the same *RHCE* gene encodes C and E, as in the relatively rare *DCE* haplotype, the expression of C is often very weak (Section 5.8.1). Pro226, therefore, appears to suppress C expression [311]. The C/c polymorphism, however, has no obvious effect on the expression of E [311].

5.9.3 Predicting E/e phenotype from DNA

The presence of *RHCE*E* can be detected by PCR with an allele-specific primer, but detecting *RHCE*e* is slightly more complex because 676G, the nucleotide characteristic of the *RHCE*e*, is also present in *RHD*. This difficulty is easily resolved by using a primer specific for 676G paired with a primer to an *RHCE*-specific nucleotide in exon 5 (e.g. 787A) [318,327]. Alternatively, *RHCE*E/e* genotyping could be achieved by *Mnl*I digestion of an *RHCE*-specific PCR product. Occasionally anti-E causes HDFN (Section 5.18.2), so methods have been developed for predicting fetal E phenotype from fetal DNA in maternal plasma [296].

5.9.4 E variants

Red cells with the very rare antigen E^{w} (RH11) are reactive with some, but not all, anti-E sera. One E^{w}+ patient has made alloanti-E [328]. All known examples of anti-E^{w} are red cell immune and anti-E^{w} has been responsible for HDFN [329,330]. E^{w} is always associated with *DcE* and, in two E^{w}+ patients, with 500T>A in exon 4 of *RHCE* encoding Met167Lys in the third extracellular loop [328].

Four categories of E variants (EI to EIV) were revealed by testing monoclonal anti-E against red cells with unusual E antigens [331,332]. The variant E proteins all had Pro226. EI had a Met167Lys, and so is identical to the partial E antigen associated with E^{w} [328]. EII had exons 1–3 derived from *RHD*, EIII had Gln233Glu and Met238Val in the extracellular vestibule, encoded by a segment of exon 5 derived from *RHD*, and EIV had an Arg201Thr exchange in a cytoplasmic domain. Screening of over 140 000 Japanese blood donors with monoclonal anti-E revealed 15 (0.011%) with E variants: nine (EKM) with the EIII mutation; one (EKK) with the EII mutation; and five (EKH) with a new mutation, encoding Arg-154Thr close to the third extracellular loop [333]. Both EKK (EII) and EKH were associated with very weak expression of c and are described further in Section 5.14.3 and Table 5.10.

Weak E, in which the red cells failed to react with four of 14 monoclonal anti-E, had an *RHCE*cE* allele

containing 939G>A in the last (3′) nucleotide of exon 6 [334]. Analysis of cDNA derived from cultured erythroblasts revealed a transcript lacking exon 6 and encoding a protein with a loss of 46 amino acids. Whether this abnormal protein is responsible for the aberrant E, or whether a very small quantity of normal protein was produced by an undetected normally spliced transcript, was not determined.

5.9.5 e Variants – hr^S, hr^B, and related antigens

Some e+ people, most of them of African origin, have made antibodies that resemble anti-e in tests with red cells of common Rh phenotypes. The categorisation of e variants has been prevented by the scarcity of avid e reagents, the complexity of the antibody response by e+ people who make 'anti-e', and the large variety of these aberrant e antigens [335]. Two broad categories can be defined: (i) those with partial e who make an e-like antibody; and (ii) those with partial e and partial Hr_o (RH17) who make antibodies that react with all red cells save those of similar unusual phenotypes, Rh_{null} cells, and cells with D−− and related phenotypes. A separable e-like antibody may also be present in the sera of the second type. Cells of the first category come from individuals heterozygous for a rare *Dce* haplotype, encoding a partial e antigen, and for normal *DcE* (or *dcE*), which produces no e, but does produce the related high frequency antigen. Cells of the second category come from individuals homozygous for a rare haplotype, which produces partial e and no related high frequency antigen (or heterozygous for two similar haplotypes of this type). Two of the possible categories, hr^S and hr^B, are well investigated and are described in more detail below. As will become clear from the text below, to consider these antigens strictly as e variants is a gross oversimplification.

5.9.5.1 hr^S (RH19) and Hr (RH18)

The antibody that defined the blood factor hr^S was the first alloanti-e-like found in the serum of a e+ person (Shabalala) [336]. The serum reacted with all cells of common Rh phenotype, but more strongly with E− e+ cells than with E+ e− cells. After adsorption with E+ e− cells, the serum no longer reacted with e− cells, but still reacted with e+ cells, with the exception of the red cells of about 1% of e+ black South Africans, which were considered hr^S−. Shapiro [336] estimated that about 6% of Bantu Rh haplotypes were *Dce* or *dce* encoding no hr^S. The antibody removed by adsorption of Shabalala serum with E+ e− cells defines a high frequency antigen called Hr (also Hr^S and RH18) [335,336]. Hr is present on all red cells except those rare e+ cells that lack hr^S, cells with D−− and related phenotypes, and Rh_{null} cells. Anti-Hr may also be a component of anti-Hr_o, the immune response of immunised D−− individuals (Section 5.15.6).

Other examples of anti-hr^S have been described [337], though many other antibodies labelled anti-hr^S will have been inaccurately identified. The immune response of hr^S− people is highly variable and antibodies behaving like anti-e in the serum of e+ hr^S− individuals are often assumed to be anti-hr^S even when the critical cells for correct identification are not available. Anti-hr^S appears to be a component of many anti-e sera [336].

Anti-Hr has caused fatal HTRs [338] and HDFN requiring exchange transfusion [336,337,339].

As might be expected from the serological description, the molecular basis for the e+ hr^S− phenotype is heterogeneous. Four *RHCE* genes (see Table 5.10), *RHCE*ceAR*, *RHCE*ceEK*, *RHCE*ceBI*, and *RHCE*ceSM*, encode Met238Val and weak e, but no hr^S or Hr [338, 340], whereas *RHCE*ceMO* also produces weak e and no hr^S or Hr, but encodes Val223Phe and not Met238Val [341].

5.9.5.2 hr^B (RH31) and Hr^B (RH34)

Description of the blood factor hr^B is very similar to the hr^S story. Anti-hr^B was found in the serum of a black South African (Bastiaan), together with an antibody to a high frequency antigen, anti-Hr^B [342]. Although anti-hr^B and -Hr^B in Bastiaan serum may not have been completely separable by adsorption [343], separable anti-hr^B and -Hr^B are found in other similar sera and sera containing either anti-hr^B or -Hr^B also exist [335,344].

Neither hr^B nor Hr^B is produced by the *d(C)ce*^{s} type 1 or 2, or $D^{IIIa}ce^s$ haplotypes, which produce VS, but not V (Section 5.13.2) [344]. Consequently, individuals homozygous for these haplotypes, or with compound heterozygosity, are hr^B− Hr^B− and can make anti-hr^B and -Hr^B; individuals heterozygous for any of these haplotypes and for *DcE* are hr^B− Hr^B+ and can make anti-hr^B. Almost 40 years after the original study on the hr^B− and Hr^B− index case, another blood sample from Bastiaan became available, which revealed homozygosity for *RHCE*ce* typical of *d(C)ce*^{s} (*RHCE*ceVS.03*, see Table 5.10) [345].

Anti-hr^B was detected in the sera of two patients who had transient loss of hr^B [346]. The apparent alloantibodies were later found to be autoantibodies when hr^B reappeared.

5.9.5.3 Transfusion of patients with e variant red cells

Red cells with variant e antigens, especially hr^S– and hr^B–, are commonly found in patients of African origin and, therefore, in transfusion-dependent patients with sickle cell disease (SCD). Protecting these patients from producing Rh antigens, or providing suitable blood if they have already done so, can prove difficult. Often DcE/DcE red cells are selected for transfusion for prevention of production of anti-hr^S or -hr^B, but this can lead to production of anti-E and, because hr^S– and hr^B– phenotypes are often associated with a D variant (DIII) phenotype that remains undetected by routine serology, with production of anti-D [344,347]. Molecular testing of patients with SCD assists in the selection of the most suitable available red cells for transfusion [348,349].

5.9.5.4 STEM (RH49), a low frequency antigen associated with some hr^S– and hr^B– phenotypes

Further heterogeneity of hr^S– and hr^B– phenotypes was revealed by anti-STEM, an antibody to a determinant present on the red cells of 3–6% of South Africans, but only very rarely present in white people [350]. Family studies suggested that STEM is associated with some *Dce* haplotypes that produce neither hr^S nor hr^B. Red cells of approximately 65% of hr^S– Hr– and 30% of hr^B– Hr^B– individuals are STEM+. People with hr^S+ STEM+ or hr^B+ STEM+ red cells may be heterozygous for an hr^S-deficient or hr^B-deficient *Dce* haplotype. STEM, therefore, may be a marker for some partial e or 'partial Hr_o' antigens.

5.9.6 Ee antibodies

Anti-E occurs more commonly than anti-C in the sera of D+ people. Unlike other Rh antibodies, anti-E often appears to be 'naturally occurring'. Many anti-E are only reactive with protease treated E+ red cells [351–354] (Section 5.18.3).

Anti-e is not a common antibody since only 3% of people are e–. Sera containing anti-e often contain anti-Ce or anti-ce (Section 5.10).

5.10 Compound CE antigens

Some Rh antigens, known as compound antigens, are only expressed when c and e, or C and e, or C and E, or c and E are produced by the same *RHCE* gene. Reactions of antibodies to these antigens are shown in Table 5.3. Antibodies to these compound antigens probably recognise conformational differences in the RhCE protein that result from the amino acid substitutions associated with both the C/c and E/e polymorphisms.

5.10.1 ce or f (RH6)

Anti-ce (also known as anti-f) reacts with the products of *RHCE*ce*. It also reacts with d(C)ce^s cells (Section 5.13). Reactions of the products of some aberrant haplotypes with anti-ce are contrary to predictions based on their reactions with anti-c and -e. For example, some *Dc*– haplotypes express ce antigen, but no e (Section 5.15.3).

The first two examples of anti-ce were detected in multi-transfused DCe/DcE patients [355,356]. Anti-ce is a common component of anti-c and -e sera [355,357], but is only rarely found as a single specificity. Three cases of HDFN caused by anti-ce have been reported [358–360] (although one of the sera also contained anti-c). Anti-ce has also been implicated in a delayed HTR [361]. One autoanti-ce in a DCe/dce patient could only be detected in an acidified antiglobulin test; its role in the patient's transfusion reaction was uncertain [362].

5.10.2 Ce (RH7) and Rh41

Recognition of the compound antigen Ce (also known as rh_i) [363], provided an explanation for the observation that certain anti-C sera distinguish C produced by *DCe* and *dCe* from that produced by *DCE* and *dCE* [364]. The original anti-Ce, together with a weak anti-e, was found in the serum of an immunised DcE/DcE woman; the anti-e was removed by adsorption with Dce/dce or dce/dce cells [363]. Most immune anti-C made by D+ people are predominantly anti-Ce and most anti-C and -C+D sera contain at least some anti-Ce [324].

Anti-Ce has caused HDFN requiring treatment by fetal or neonatal transfusion [365–367] and may have contributed to an HTR [368]. IgA anti-Ce in a DCe/DCe woman was responsible for AIHA [369].

Anti-Rh41, an antibody made by a DCe/DcE C^w+ woman, was similar to anti-Ce in reacting with cells from people carrying C and e produced by the same gene, but differed from anti-Ce by reacting with d(C)ce^s/dce cells, but not with C^w+ red cells of DCe/dce, DCe/DcE, or dCe/dce phenotypes [370].

5.10.3 CE (RH22)

Anti-CE is a very rare antibody that reacts with the products of the rare haplotypes *DCE* and *dCE*. The first example was found together with anti-C in the serum of a dce/dce woman who had never been transfused and had a dce/dce husband and child [371]. A second example of

anti-CE plus a weak anti-C also appeared to be 'naturally occurring'.

5.10.4 cE (RH27)

Anti-cE (together with anti-E and -S) was first identified in the serum of a DCe/DCe woman [372]. Another example of anti-cE, detected in the serum of a DCe/DCE C^w+ man, appeared to be a 'naturally occurring' antibody and was unusual for an Rh antibody because it bound complement [373].

5.11 G (RH12)

Anti-G reacts with red cells that express D or C, or both D and C. Ser103, encoded by *RHD* and by *RHCE*C*, is the key to G reactivity (see Table 5.9). The importance of Ser103 in G expression has been demonstrated by rare phenotypes that break the rule that all cells that have D and/or C are G+.

DcE producing normal D, but no G, was found in three generations of a white family, studied because of HDFN caused by anti-C+G [374]. In two unrelated individuals with DcE/dce G– red cells, also with apparently normal D, *RHD* had 307T>C encoding Ser103Pro [375].

Red cells with the variant D antigen DIIIb express most epitopes of D, but are G– (Section 5.6.4.2) and their immune response sometimes includes a separable anti-G [156,157]. DIIIb is produced by *RHD* in which exon 2 is replaced by exon 2 from *RHCE*c* and encodes Leu60, Asn68, and Pro103 [162–164].

A phenotype (designated r^G, probably erroneously) in which the red cells are D– C– E–, but G+, and probably have weak c and e, was associated with *RHCE* encoding Trp16 (characteristic of *c* and *RHD*) in exon 1 and Ile60, Ser68, and Ser103 (characteristic of *RHCE*C* and *RHD*) in exon 2 [313]. This gene could represent *RHCE*ce* in which exon 2 is substituted by exon 2 from *RHD* or from *RHCE*C*.

The 'haplotype' inappropriately labelled r''^G produces G, E and possibly very weak C and D, but no c or JAHK [376–378] (see Table 5.10). The r''^G gene represents *RHD–CE–D* in which exons 4–8 derive from *RHCE*E* [375]. This gene encodes Trp16 and Ser103.

The gene named r^G produces G, very weak C detected by about one in three anti-C from D+ individuals (initially called anti-C^G [315]), and weak e [314,315,379]. It also produces the low frequency antigen JAHK (RH53) [377]. Homozygosity for r^G was found in a man whose parents were first cousins [314]. The r^G gene is *RHCE*Ce* with 365C>T in exon 3 encoding Ser122Leu in the fourth membrane-spanning domain, adjacent to the second extracellular loop, which contains Ser103 [380]. It is likely that conformational changes resulting from the substitution of a neutral serine by a hydrophobic leucine is responsible for weakness of C and e and for JAHK expression, but G is expressed strongly because it is much less dependent on protein conformation than C.

Estimates for the number of G antigen sites per red cell [381]: DCe/DCe 9900–12 200; dCe/dCe 8200–9700; DCE/DCE 5400; DcE/DcE 3600–5800; Dce/Dce 4500–5300; DcE/dce 4200; and four unrelated dcE/dce G+ (r''^G/r) 600–3600. Anti-G blocks C and D sites [379], and, on occasion, e sites [382].

Anti-G was originally found in the sera of dce/dce people, together with anti-D and/or -C [379,383,384] and was detected in 30% of single donor 'anti-CD' sera and in all commercial anti-CD and -CDE reagents [324]. Anti-G can be isolated by adsorption/elution techniques with r^G/dce or r''^G/dce cells [379,381]. Since these phenotypes are rare, a double elution method has proved useful: 'anti-CD' is adsorbed onto and eluted from dCe/dce (D– C+ G+) cells to isolate anti-C and -G, then this eluate, which contains no anti-D, is adsorbed onto and eluted from Dce/dce (D+ C– G+) to isolate anti-G [385]. A thorough serological analysis of 27 sera from immunised women with apparent anti-D+C revealed three anti-D+C, 13 anti-D+C+G, seven anti-D+G, and four anti-C+G [386]. The clinical significance of anti-G is discussed in Section 5.18.2.

The concept of G has helped to sort out some previous serological puzzles. It explained why some dce/dce women immunised only by pregnancy appeared to have made anti-C+D, even though their husbands were C–; their antibody was anti-D+G. It provided an explanation for the apparent anti-C+D in the serum of two dce/dce mothers who had delivered dCe/dce children [383,384]; their antibodies were anti-C+G. Recognition of anti-G also explained the apparent ability of Dce/dce cells to adsorb anti-C activity from anti-C+D sera; such sera were anti-D+G. An apparent anti-C+D made by a D– woman transfused with eight units of D– blood was shown to be anti-C+G and one of the donors was identified as dCe/dce [387].

5.12 C^w, C^x, and MAR

C^w and C^x are antigens of relatively low frequency that have an allelic relationship with the high incidence

antigen MAR [388]. C^w and C^x result from single nucleotide changes in exon 1 of *RHCE* (usually *RHCE*Ce*) encoding amino acid substitutions in the first extracellular loop: 122A>G, Gln41Arg in C^w; 106G>A, Ala36Thr in C^x [389]. These amino acid changes probably cause conformational alterations in the protein that are responsible for quantitative and qualitative abnormalities of C.

5.12.1 C^w (RH8)

Callender and Race [390] found the first example of anti-C^w in the serum of a DCe/DCe patient who had been transfused with DCe/DCe C^w+ red cells. In an English population C^w has an occurrence of 2.6% [13]; similar frequencies are found in most other northern European and white American populations, but it is very much lower in most other populations [14,20]. The highest frequency of C^w (7–9%) has been found in Latvians, Lapps, and Finns.

C^w+ red cells are almost always C+, but the C antigen associated with C^w is weaker than normal C, though recognition of this weakness depends on the anti-C used. Although C^w is usually produced by a *DCe* haplotype, C^w associated with *dCe*, *dCE*, and *DCE* have also been found. A person with apparently normal DCC^we/dce red cells made anti-C that did not react with DCC^we/dce or dCC^we/dce cells [391]. Similar antibodies have been detected in the sera of DCC^we/DCC^we and DCC^we/DcE individuals [392,393].

Studies with ^{125}I-labelled human monoclonal anti-C^w provided the following estimates of C^w sites per red cell: DCC^we/DCC^we 32 000; DCC^we/DCe 15 200; DCC^we/DcE 19 800; DCC^we/dce 15 300; dCC^we/dce 26 200 [394]. Similar studies with monoclonal anti-C did not reveal any obvious reduction in C antigen density in C^w+ cells compared with C^w– cells [394].

Very rarely, C^w is produced by *RHCE* that produces c and e [389,395,396]. In one individual with C^w+ C– c+ red cells, the cells were also D– and G– (dcC^we/dce). C^w is usually produced by *RHCE*Ce* encoding Arg41; C^w associated with c is produced by *RHCE*ce* encoding Arg41 and Cys16 [389]. *RHCE*CC^{w}e* and *RHCE*cC^{w}e* alleles, therefore, have exons 1 of identical sequence (see Table 5.9) and *RHCE*cC^{w}e* could have arisen by recombination between *RHCE*CC^{w}e* (exon 1) and *RHCE*ce* (exons 2–10). The *DC^w*– haplotype, which produces C^w but no C, c, E, or e, is described in Section 5.15.4.

Anti-C^w is not an uncommon antibody and often results from no known red cell immunising stimulus. One in 1100 pregnant Manitoban women had anti-C^w [397]. Anti-C^w has been responsible for several of cases of HDFN [397], a very few have been severe [398–402]. Anti-C^w is not usually detected in pregnant women as red cells for antibody screening are often C^w–.

5.12.2 C^x (RH9)

Like C^w, C^x is usually produced by a *DCe* haplotype that produces abnormal C. C^x+ red cells react with some, but not all, anti-C. Very rare haplotypes that encode C^x include *dCC^{x}e*. A haplotype containing *RHD* encoding weak D-4.3 and *RHCE*ce* encoding c, no C, C^x, and VS was found in about 0.12% of Upper Austrian donors [244]. A similar phenotype, but D–, was present in four of 513 unrelated Somalis [403].

Seven C^x-positives were found among 5919 (0.12%) British donors [404,405] and 202 were found among 70 503 (0.29%) Americans [406]. C^x has a much higher incidence in Finland: 37 of 2060 (1.8%) Finns were C^x+ [388].

The first anti-C^x caused mild HDFN, as have other examples since [404,405]. Some anti-C^x appear to be 'naturally occurring' [407]. Anti-C in the serum of a transfused DCC^xe/dce patient reacted with most C+ cells, including DCC^we/dce cells, but not with DCC^xe/dce, DCC^xe/DcE, or DCC^xe/DCC^xe cells [408].

5.12.3 MAR (RH51)

Anti-MAR was found in a Finnish woman whose red cells were C^w+ C^x+ D+ C+ c– E– e+ and who was probably heterozygous *DCC^{w}e/DCC^{x}e* [388]. Testing of 10 045 Finnish donors revealed 21 MAR-negatives: nine were C^w+ C^x– (probably *RHCE*C^w/C^w*), three were C^w– C^x+ (*RHCE*C^x/C^x*), and nine were C^w+ C^x+ (*RHCE*C^w/C^x*). In eight families, all 20 children of MAR– parents were either C^w+ or C^x+. Anti-MAR reacted weakly with many examples of C^w+ C^x– and C^w– C^x+ cells. It did not react with Rh_{null}, D––, or DC^w– cells. As C^w and C^x usually result from Gln41Arg and Ala36Thr in the RhCE protein, respectively [389], it is probable that both Gln41 and Ala36 are required for MAR expression.

Two other antibodies to high frequency antigens, produced in probable *RHCE*C^x/C^x* [406] and *RHCE*C^w/C^w* [409] women, resembled anti-MAR in their serological reactions. However, the antibody from the *RHCE*C^w* homozygote reacted weakly with DCC^xe/DCC^xe cells and the antibody from the *RHCE*C^x* homozygote reacted strongly with DCC^we/DCC^we cells. The antibody of the probable *RHCE*C^x* homozygote caused HDFN [406].

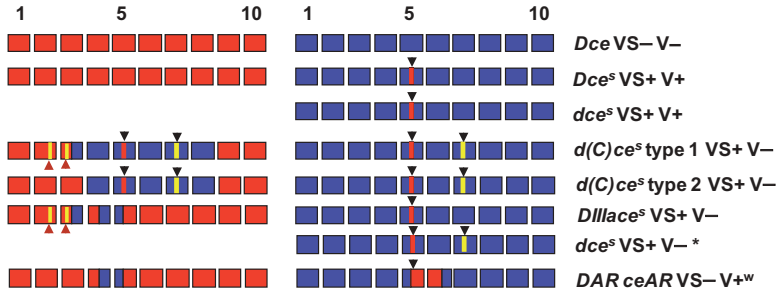

Figure 5.9 Representation of the 10 exons of *RHD* (red boxes) and *RHCE* (blue boxes) in VS+ and/or V+ phenotypes. ▼ Codons for Val245 in exon 5 and Cys336 in exon 7. ▲ Codons for Phe62 (exon 2) and Val137 (exon 3) characteristic of *RHD*DIIIa*. Yellow lines, untemplated amino acid substitutions. * Genotype not confirmed by transcript analysis.

5.13 VS (RH20) and V (RH10)

5.13.1 Serology

Anti-V, first reported in 1955 [410], and anti-VS, described five years later [411], define antigens that are common on the red cells of Africans, but are rare in other populations. V and VS are associated with a partial e called e^s, though e and e^s are often difficult to distinguish. As a general rule, VS is produced by the haplotypes *Dce*^{s}, *dce*^{s}, and *d(C)ce*^{s}, whereas V is produced by *Dce*^{s} and *dce*^{s}, but not *d(C)ce*^{s}.

The haplotype *d(C)ce*^{s} (previously known as *r′*^{s}) produces c, e^s, ce (f), a weak partial C (sometimes called C^G), G, and VS; it does not produce D, Ce, or V. In addition, anti-Rh42, an antibody found in the serum of a DCe/Dce mother of Dce/d(C)ce^s and DCe/d(C)ce^s children, only reacts with the product of *d(C)ce*^{s} [412]. Many individuals with d(C)ce^s phenotype have produced alloanti-C [312,517]. In addition, despite having apparently normal expression, the c and ce might be partial antigens as one *d(C)ce*^{s}*/DCe* patient produced anti-c, which reacted more strongly with ce than c [413]. VS is also associated with the hr^B– phenotype (Section 5.9.5.2): neither hr^B nor Hr^B is encoded by *d(C)ce*^{s} [132,344].

Many examples of anti-VS and -V have been found, often in sera containing other antibodies. The heterogeneity of anti-VS has been thoroughly investigated [414]: some antibodies react preferentially with VS+ V– cells, some prefer VS+ V+ cells, and others react equally well with both types. Heterogeneity of anti-VS probably provides an explanation for the specificity called anti-hr^H (RH28) [415]. Heterogeneity of V antibodies is also observed when they are tested against red cells of unusual phenotypes [414]. No clinically significant anti-VS or -V has been reported. Anti-VS and -V are usually reactive by an antiglobulin technique, but a saline active anti-V is recorded [416].

Some phenotypes do not fit the serological rules described above. A D– C– V– VS+ donor [131] and the VS– V+ (ceAR) phenotype [131,153] are described below (Section 5.13.2). The *DCe*^{s} haplotype, producing an apparently normal C, no c, and VS and V is extremely rare [417].

Of 100 black South African blood donors, 34 were VS+ V+, 9 were VS+ V–, and 4 were VS– V+, with weak V (ceAR) [131]. The incidence of V in two surveys of African Americans was 27% [410] and 39% [418], and was 40% in West Africans [410]. V is not an exclusively African characteristic: there was no trace of African ancestry in two English families with V antigen [410]. In white Argentineans, of 33 *Dce* haplotypes, 12 (36%) were *Dce*^{s}, reflecting the contribution of African alleles in that genetic pool [419].

Weakness of D in red cells of DcE/d(C)ce^s individuals demonstrated that *d(C)ce*^{s} has a depressing effect on *D in trans*, similar to that of *dCe* (see Section 5.6.6).

5.13.2 Molecular genetics

VS is associated with 733C>G in exon 5 of *RHCE*ce* encoding Leu245Val [130,131,326]. In *dce*^{s} and *Dce*^{s} haplotypes, no other mutation has been detected (Figure 5.9). Val245 is present in the RhD protein, so a microconversion event may have been involved in the formation of the VS gene. Amino acid 245 is predicted to be buried in the membrane in the eighth membrane-spanning domain, adjacent to the vestibule region, which contains Ala226, the residue primarily responsible for e expression (see Figure 5.2). It is likely that VS expression and the

associated weakness of e (e^s) result from conformational changes to the protein resulting from Leu245Val. Avent [56] suggested that residue 245 may be involved in stabilisation of the RhCE-RhAG-RhAG trimer.

The typical *d(C)ce*^{s} haplotype (type 1) contains a hybrid gene, *RHD–CE–D*^{s}, consisting of exons 1, 2, part of 3 (including codons 121, 127, and 128), 9, and 10 from *RHD* and the remainder of exon 3 (including codon 152) and exons 4–8 from *RHCE*e* [129,130] (Figure 5.9). As *RHD–CE–D*^{s} (type 1) also encodes Leu62Phe (exon 2) and Ala137Val (exon 3), exons 1–3 are probably derived from *RHD*DIIIa*, so the hybrid could be written *RHDIIIa–CE(4-8)–D*^{s}. The hybrid gene also encodes Leu245Val (exon 5), and Gly336Cys (exon 7, 1006G>T). It is paired with *RHCE*ce* that also encodes Leu245Val (VS) and Gly336Cys. The presence of Ser103 encoded by exon 2 of *RHD–CE–D*^{s} together with downstream sequences characteristic of *RHCE*, is probably responsible for C expression by d(C)ce^s, its weakness being due to tryptophan, and not cysteine, at position 16 (Section 5.8.2). Both *RHD–CE–D*^{s} and its paired *RHCE* gene encode Val245 and Ala226 (e) and are likely to produce VS and e^s. Both genes also encode Gly336Cys, a substitution not present in VS+ V+ phenotypes [131]. It is likely that anti-VS and -V both recognise Val245 in an Rhce protein, but that anti-V is more conformationally dependent than anti-VS and does not react with the protein when Cys336 is also present.

Another *RHD–CE–D*^{s} gene, part of the *d(C)ce*^{s} type 2 haplotype, resembles the type 1 hybrid apart from having the whole of exon 3 derived from *RHCE* (Figure 5.9) [132]. In contrast to *d(C)ce*^{s} type 1, *d(C)ce*^{s} type 2 encodes a much weaker C and does not produce Rh42.

VS, but not V, is also encoded by a haplotype comprising *RHD*DIIIa* paired with *RHCE*ce*^{s} encoding Val245 and Cys336 (typical of *d(C)ce*^{s}) [132].

Pham *et al.* [132] suggest that the origin of the *d(C)ce*^{s} type 1 haplotype is the result of a gene conversion event involving *RHCE*ce*^{s} and *RHD*DIIIa*, whereas the origin of the *d(C)ce*^{s} type 2 involved *RHCE*ce*^{s} and *RHD* (see Section 5.5.5 and Figure 5.6).

*RHCE*ce* encoding Trp16Cys, Leu245Val (typical of VS), Val314Ala, and Gly336Cys (typical of *d(C)ce*^{s}) encoded c, VS (though the V type was not clear), and variant e and hr^B [420]. This allele was found in 5.5% of African Americans. It may be the same as that found in a D– C– c+ E– e+ G– V– VS+ black English blood donor, with an *RHCE* allele encoding Val245 and Cys336 [131] (Figure 5.9). This strongly supports the suggestion that *RHD–CE–D*^{s} produces weak C and that the presence of Cys336, and not the hybrid Rh protein, is responsible for the absence of V in the d(C)ce^s phenotype.

*RHCE*ceAR* encodes partial c and e, weak V, but no VS. Individuals with *RHCE*ceAR* have made anti-c [421,422]. *RHCE*ceAR* is an *RHCE–D–CE* gene with codons for Val238, Val245 (VS), Gly263, and Lys267 derived from exon 5 of *RHD* and Val306 from exon 6 of *RHD* [131,153] (Figure 5.9). *RHCE*ceAR* is usually linked to *RHD*DAR* (Section 5.6.4.9). In a survey of 326 black South Africans, 20 (6.1%) had *RHCE*ceAR* and 14 of these also had the *RHD*DAR* [153].

An Austrian haplotype encoding VS and C^x [244] is described in Section 5.12.2.

5.14 Variants with abnormal Cc and Ee antigens

Gathered together in this section are some haplotypes that result in abnormal expression of C or c and E or e antigens. There are many of these variants known and they are continuing to be found [423], and only selected examples are listed in Table 5.10 and described below. It should be remembered that weak antigens (denoted by parentheses) can only be observed on red cells of people homozygous for the rare haplotype or on those of people with a suitable antithetical Rh haplotype: for example, weak C will not be detected if a normal C is produced by the opposite gene, but will be detected if the opposite gene produces c. Some of these haplotypes also produce low frequency marker antigens. Some details of antibodies to low frequency Rh antigens are provided in Section 5.17 and Table 5.14. Some complexes involving partial e antigens, described in Section 5.9.5, are omitted from this section. Homozygosity for some of the haplotypes described in this section has revealed that they do not encode certain high incidence Rh antigens, such as Hr_o, Rh46, CEST, and CELO, which are produced by all normal Rh haplotypes. Haplotypes producing neither E nor e are described in Section 5.15.

5.14.1 Variant *RHCE*Ce* alleles

5.14.1.1 R^N ($\overline{\overline{R}}^N$) incorporating *RHCE*CeRN;* Rh32 and Rh46 antigens

The symbol $\overline{\overline{R}}^N$ given to a haplotype encoding weak C and weak e in an African American family [424] has been replaced by R^N to avoid word processing complications. In addition to weak C and weak e, R^N encodes D and G, which have been shown to be elevated in some studies

Table 5.10 Some *RHCE* variants.

Name	Antigens	Exons	Amino acid changes or hybrid gene	Paired *RHD*	Ethnic group	References
*RHCE*CeRN.01*, R^N	(C) (e) Rh32, DAK Rh:–46	4	*RHCE–D(4)–CE*	*RHD*	Black	[424,425]
*RHCE*CeRN.02*, R^N	(C) (e) Rh32, DAK Rh:–46	3, 4	*RHCE–D(4)–CE* + T152N	*RHD*	Black	[424,425]
*RHCE*CeJAL* (*RHCE*CeMA*)	(C) (e) (hr^S) (hr^B) JAL CEST–	3	R114W	*RHD*	White	[426–428]
*RHCE*CeVA*	(C) (e) Rh33 FPTT	5	*RHCE–D(5)–CE*	*RHD*	White	[429]
*RHCE*CeNR*	(C) (e) C^w CENR	1, 6–10	*RHCE(1–5)–RHD(6–10)* + Q41R	*RHD*	White	[430,431]
*RHCE*CeJAHK*	(C) (e) G JAHK	3	S122L	None	White	[379,380]
*RHCE*Ce667*	(C) (e)	5	V223F			[423]
*RHCE*ceJAL*	(C) (e) (hr^S) (hr^B) (VS) (V) JAL CEST–	3, 5	R114W, L245V	*RHD*DAU0*	Black	[426–428]
*RHCE*ceBE*	(c) (e) (ce) Be^a	5	P221R	None	White	[432,433]
*RHCE*ceLOCR*	(c) (e) LOCR Rh:–26	2	G96S	None	White	[434–436]
*RHCE*ceHAR*, R_o^{Har}	c (e) (ce) Rh33 FPTT (D)	5	*RHCE–D(5)–CE*	None	White	[191,194]
*RHCE*ceCF*	(c) (e) Crawford VS (D) CELO–	1, 5	W16C, Q233E, L245V	None	Black	[253,254]
*RHCE*ceRT*	(c) (D)	3	R154T	None	White	[255]
*RHCE*ceSL*	(D)	1, 3	W16C S122L	None	White	[256]
*RHCE*ceAR*	(c) (e) (V) (D) VS– hr^S– Hr–	1, 5, 6	W16C, M238V, L245V, R263G, M267K, I306V	Often with *RHD*DAR*	Black	[153]
*RHCE*ceVS.01*, ce^s	(c) (e) VS V hr^B– Hr^B–	5	L245V	*RHD* or none	Black	[130,131,326]
*RHCE*ceVS.02*, ce^s	(c) (e) VS V hr^B– Hr^B–	1, 5	W16C, L245V	*RHD*DIIIa* or none	Black	[130,131,326]
*RHCE*ceVS.03*, ce^s	(c) (e) VS V– hr^B– Hr^B–	1, 5, 7	W16C, L245V, G336C	*RHD–CE–D*^{s} or *RHD*DIIIa*	Black	[130,131,326]
*RHCE*ceC*^{x}*VS*	c e C^x VS	1, 5	W16C, A36T, L245V	*RHD*weak 4.3*	White	[244]
*RHCE*ceMO*	(c) (e) hr^s– hr^B– Hr– Hr^B–	1, 5	W16C, V223F	Often with *RHD*DAU0*	Black	[338,341]
*RHCE*ceEK*	(c) (e) hr^s– Hr–	1, 5	W16C, M238V, R263G, M267K	Often with *RHD*DAR*	Black	[338]
*RHCE*ceBI*	c (e) STEM hr^s– Hr–	1, 5, 6, 8	W16C, M238V, A273V, L378V	Often with *RHD*DOL*	Black	[338,699]
*RHCE*ceSM*	(e) (STEM) hr^s– Hr–	1, 5, 6	W16C, M238V, A273V	Often with *RHD*DOL*		[340]
*RHCE*ceRA*	(c) (e) hr^S–	1, 4	W16C, G180R	None	Indian	[437]
*RHCE*ceAG*	(e) (hr^B)	2	A85G		Black	[438]
*RHCE*ceBP*	c (e) ce–	5	R229del	*RHD* or none	White	[697]
*RHCE*ceTI*	(e) (C)	1, 7	W16C, T342I	Usually *RHD*DIVa-2*		[698]
*RHCE*cEKH*	(c) (E)	3	R154T		Japanese	[333]
*RHCE*EKK*	(E)	1–3	*RHD(1–3)–CE*	With *RHCE(1–3)–D*	Japanese	[333]
*RHCE*cE734C*	(c)(E)	5	L245P	*RHD*	White	[459]

[425,441,442], and the low frequency antigens Rh32 and DAK (also associated with DIIIa) [160]; R^N does not produce any c, E, ce, or, usually, Ce. One individual with R^N produced anti-C [443]. The Hr_o-like high frequency antigen Rh46, produced by most Rh haplotypes, is not produced by R^N [442].

R^N is most commonly found in people of African origin. It was estimated that Rh32 is present on red cells of about 1% of African Americans, but it has only been found as a rarity in white people [443]. Numerous R^N homozygotes are recorded; some found because of immune antibody in their serum (anti-Rh46), others because their weak C and e antigens were detected during routine Rh typing [29,442].

In two R^N homozygotes, R^N represented *RHD* paired with an *RHCE–D–CE* gene (*RHCE*CeRN.01*) with exon 4 derived from *RHD*, the remainder of the gene having the *RHCE*Ce* sequence [425]. One other individual, apparently heterozygous for R^N, had a similar hybrid gene (*RHCE*CeRN.02*), but with the 3′ end of exon 3, encoding Asn152, also derived from *RHD* [425] (Figure 5.10). These hybrid genes encode Leu169Met, Arg170Met, and Phe172Ile changes close to the third predicted extracellular loop of RhCE. The molecular basis for Rh32 expression is described in Section 5.17.2.

Homozygous R^N individuals can make, if immunised by pregnancy or transfusion, anti-Rh46, which reacts with red cells of almost all Rh phenotypes except R^N, Rh_{null}, D−−, and related phenotypes [157,442]. Anti-Rh46 has been responsible for serious HDFN [442]. Mouse and human monoclonal antibodies with Rh46 specificity have been produced [444,445].

5.14.1.2 *RHCE*CeJAL* and JAL (RH48)

A multilaboratory investigation of the serum of a mother whose baby had DAT-positive red cells culminated in the recognition of a new low frequency antigen, JAL (RH48) [426]. In Caucasians JAL is associated with weak expression of C, e, hr^S, and hr^B [427,446,447]. *RHCE*CeJAL* contains 340C>T in exon 3 encoding Arg114Trp in the fourth membrane-spanning domain and is paired with *RHD* [427,428]. It is apparently identical to the previously described *RHCE*CeMA*, though red cells were not tested with anti-JAL [429]. *RHCE*CeJAL* (identified serologically) has a frequency of 0.0003 in French-speaking Swiss; no JAL+ individual was found among more than 50 000 German-speaking Swiss [446].

JAL associated with a haplotype producing weak c and weak e in JAL+ individuals of African origin is described in Section 5.14.2.1.

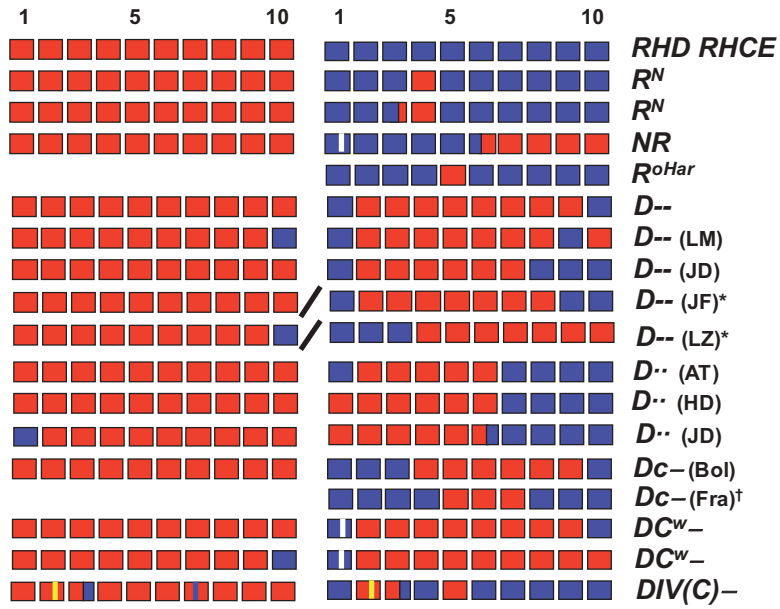

Figure 5.10 Representation of the 10 exons of *RHD* (red boxes) and *RHCE* (blue boxes) in some haplotypes containing rearranged genes encoding either partial Hr_o or no Hr_o. White line, exon 1 encoding Arg41 and C^w. Yellow line, untemplated amino acid substitutions. *Proposed separate haplotypes in two D−− propositi (JF and LZ). †Presence or absence of *RHD* not stated (Fra).

5.14.1.3 FPTT-associated haplotypes

Several new unusual Rh phenotypes were recognised during the investigation of a serum (Mol), which contained antibodies to several low incidence antigens [188]. Results of adsorption and elution tests suggested that a single antibody, anti-FPTT (-RH50), was reacting with cells of three propositi, each with a 'new' phenotype. None of the families was large enough to define the new complexes completely. FPTT is also associated with R_o^{Har} (Sections 5.6.4.12 and 5.14.2.4) and DFR (Section 5.6.4.7).

One haplotype encoded weak C, detected by one of six anti-C, very weak e, and FPTT, but no ce. The haplotype was not paired with *dce* or *DCe*, so whether it produces D and/or G is not known and lack of c and E is not proven. The reactions were similar to those of r^G cells (Section 5.14.1.5), but r^G cells are not FPTT+. The family of the second propositus showed that their FPTT-associated haplotype produced D, G, weak C, weak e, and atypical VS; it did not produce any E, ce, or V. Red cells of the third propositus expressed weak e, but normal C [188].

FPTT antigen is very variable in expression and is inherited in a normal way. FPTT has a frequency of about 0.01% in the South of France [188].

5.14.1.4 Other depressed DCe haplotypes

*RHCE*CeVA* represents an *RHCE-D-CE* hybrid allele in which exon 5 from *RHCE*C* is replaced by exon 5 from *RHD*. It produces weak C and weak e, Rh33, and probably FPTT [429]. Consequently it resembles R_o^{Har} (Sections 5.6.4.12 and 5.14.2.4) apart from producing weak C instead of weak c and by being linked to *RHD*, so it cannot be determined whether *RHCE*CeVA* produces any D.

Red cells of a white woman (NR) with anti-Hr_o, which caused severe HDFN in her second child, were D+ C+ c– E– e+ C^W+, with weak expression of C and e, and were Rh:–32, but reacted with an antibody to a new low frequency antigen CENR (RH56) [430,431]. She was considered to have the genotype *D(C)C^w(e)/D––*, confirmed by molecular analysis. The *RHCE*(C)C^w(e)* allele (*RHCE*CeNR*) is an *RHCE–D* hybrid, with exons 1–5 derived from *RHC*CC^{w}e* and exons 6–10 (or 9) derived from *RHD* [431]. Also present were transcripts representing *RHCE–D*, with only exon 1 derived from *RHCE* (reported previously in *D·· and DC^w–* [448,449]) (Figure 5.10) and *RHD*. A family study showed that *RHCE*CeNR* is paired with *RHD*.

A depressed haplotype similar to R^N, but not producing Rh32, was found in six unrelated Swedes [450]. Selected anti-C and -e sera distinguished the Swedish D(C)(e) from R^N, even before anti-Rh32 had been identified [451]. Four more propositi with the Swedish *D(C)(e)* haplotype were reported [452], but in one propositus and his mother, unlike the others, the depressed haplotype was associated with enhanced D expression. Existence of two Swedish *D(C)(e)* haplotypes was confirmed by the identification of an antibody, anti-Rh35, specific for Swedish D(C)(e) complex with normal D [441]. The original anti-Rh35 is still the only example and very little of it remains.

5.14.1.5 r^G or *RHCE*CeJAHK* and JAHK (RH53)

The inappropriately named r^G is an *RHCE*Ce* allele that produces G, very weak C, weak e, and the low frequency antigen JAHK [314,315,377,379] (see Section 5.11). It contains 365C>T encoding Ser122Leu [380]. It is not linked to *RHD*.

Anti-JAHK was found in several sera containing multiple antibodies to low frequency antigens.

5.14.2 Variant *RHCE*ce* alleles

5.14.2.1 *RHCE*ceJAL* and CEST (RH57)

In contrast to *RHCE*CeJAL* found in Caucasians (Section 5.14.1.2), in people of African origin JAL is produced by a variant *RHCE*ce* allele that produces weak c, e, ce, VS, V, hr^S, and hr^B [426,427,447]. Alloanti-c and -e have been produced by c+ e+ JAL+ individuals [447,453] and an anti-Hr-like antibody, named anti-CEST (-RH57) was made by an *RHCE*ceJAL* homozygote [447]. Like *RHCE*CeJAL*, *RHCE*ceJAL* encodes Arg114Trp, but also encodes Leu245Val, which is responsible for VS expression (Section 5.13.2) [427,428]. Westhoff *et al.* [428] propose that the loss of a hydrogen bond between Arg114 and Ala 226 and the conformational modification caused by the tryptophan side chain are responsible for the weak expression of C, c, and e associated with JAL. *RHCE*ceJAL* is identical to the previously reported ce^s(340) [338], although red cells were not tested with anti-JAL. *RHCE*ceJAL* is usually paired with *RHD*DAU0* [427,428].

A different mutation was present in a JAL+ Caucasian and a JAL+ Asian, who had JAL encoded by *RHCE*ce*: 341G>A, Arg114Gln [427]. This allele was linked to *RHD*.

5.14.2.2 *RHCE*ceLOCR*, Rh26, and LOCR (RH55)

A variant c antigen detected by its failure to react with one strong anti-c reagent was numbered anti-Rh26 [434]. Two further c+ Rh:–26 propositi were found in tests with anti-Rh26 on 1900 C+ red cell samples. A DCe/dce Dutch

woman made anti-Rh26 during her first pregnancy. Screening with the antibody revealed a second Dutch propositus with DCe/dce Rh:–26 red cells [435]. Most anti-c sera appeared to contain mixtures of anti-c and -Rh26. Two of 10 monoclonal anti-c behaved as anti-Rh26 as they did not react with c+ Rh:–26 cells [435]. The c+ Rh:–26 phenotype results from an *RHCE*ce* allele that produces weak c, normal ce, weak e (in one individual), and the low frequency antigen LOCR (RH55) [434–436,454]. *RHCE*ceLOCR* contains 286G>A in exon 2, encoding Gly96Ser in the second extracellular loop [435,436]. It is not linked to *RHD*.

5.14.2.3 *RHCE*ceBE* and Be^a (RH36)

The low frequency antigen Be^a (Berrens) was first described in 1953 [432], but was not shown to be related to the Rh system until 20 years later. Anti-Be^a has been implicated in several cases of severe HDFN [433,455–457]. Be^a is encoded by an *RHCE*ce* allele that produces slightly weakened c, e, and ce and contains 662C>G in exon 5 encoding Pro221Arg in or close to the extracellular vestibule [433].

5.14.2.4 Variant *RHCE*ce* alleles encoding epitopes of D

Several variant *RHCE*ce* alleles – *Ro^{Har}* (*RHCE*ceHAR*), *RHCE*ceCF*, and *RHCE*ceAR* – contain nucleotides in exon 5 characteristic of *RHD* and the encoded protein expresses some D epitopes. Exons 5 of *RHCE*ceRT* and *RHCE*ceSL* encoded amino acid substitutions that are not present in RhD, yet the proteins still express some D epitopes. All of these variants are described in Section 5.6.4.12.

Ro^{Har} (Figure 5.10) produces two low frequency antigens, Rh33 and FPTT. Two Rh:33 individuals, both Germans, appeared to be homozygous for R$_o^{Har}$ [191,458]. Their red cells did not react with about 50% of immune sera from D–– people (anti-Hr$_o$), suggesting they have a partial Hr$_o$ antigen [430]. R$_o^{Har}$ is probably less rare in Germany than elsewhere. Seven of 14 000 apparently D– German donors gave discrepant results with anti-D; all seven turned out to be Rh:33. None of 1060 English blood donors screened with anti-Rh33 was Rh:33 [191]. One of 42 600 donors tested in southern France was Rh:33 and appeared to have the R$_o^{Har}$ haplotype [188].

5.14.2.5 Other variant *RHCE*ce* alleles

A number of variant *RHCE*ce* alleles have been found in people of African origin that do not produce the high frequency antigens Hr or Hr^B (Section 5.9.5). People with these alleles in the homozygous or compound heterozygous state may produce antibodies to high frequency antigens following transfusion, which will make finding blood for future transfusion difficult. This is a particular problem in patients with sickle cell disease. In addition to *RHCE*ceVS* (*ce^s* and *(C)ce^s*, Section 5.13) and *RHCE*ceAR* (Section 5.6.4.9), these alleles include *RHCE*ceMO*, *RHCE*ceEK*, and *RHCE*ceBI* [338,341,423] (Table 5.10).

*RHCE*ceRA*, encoding Gly180Arg, produced profoundly weakened e and no hr^S [437]. Three multiply transfused African American e+ patients made alloanti-e [438]. Their abnormal allele, *RHCE*ceAG*, contained 254G>C in exon 2 encoding Ala85Gly in the third transmembrane domain and absence of the high frequency antigen CEAG (RH59).

5.14.3 Variant *RHCE*cE* alleles

Screening of over 140 000 Japanese blood donors with monoclonal anti-E revealed 15 (0.011%) with E variants [333]. Two of the variants, EKH and EKK (EII) are also associated with weak c (Section 5.9.4 and Table 5.10). The aberrant E of EKK appears to result from a hybrid gene with exons 1–3 derived from *RHD* and exons 4–10 from *RHCE*E* (or *RHCE–D(2,3)–CE*). This gene is linked to the opposite hybrid: exons 1–3 derived from *RHCE*c*, producing the weak c, and exons 4–10 from *RHD* (or *RHD–CE(2,3)–D*) [333].

Two Caucasian men had an *RHCE* allele (*RHCE*cE734C*) encoding Leu245Pro and produced altered c and suppressed E, not detected with standard reagents [459].

5.15 Haplotypes producing neither E nor e; D–– and related phenotypes

This section includes haplotypes that encode neither E nor e; some also produce neither C nor c. All encode D, which is usually exalted in expression. Most of these haplotypes were initially identified in individuals homozygous for the haplotype, who had produced antibodies to high frequency antigens collectively known as anti-Hr$_o$ or -Rh17. When the terms D––, Dc–, DC^w–, etc. are used to denote phenotypes, they usually represent homozygosity for the corresponding haplotype.

These haplotypes often consist of a complete or almost complete *RHD* paired with a hybrid gene containing a

substantial portion of *RHD* (Figure 5.10). This may provide an explanation for the enhanced D expression.

5.15.1 *D––*

The first propositus with the D–– phenotype, in which the red cells expressed D, but no C, c, E, or e, had consanguineous parents and was presumed to be homozygous for a very rare haplotype, *D––* [460,461]. Despite the rarity of this haplotype, many examples of the D–– phenotype have been found in many different ethnic groups. Almost all D–– propositi have been ascertained through the presence of anti-Hr_o in their serum (Section 5.15.6). Many propositi heterozygous for *D––* and a common Rh haplotype have been found, often being disclosed by apparent parentage exclusions [462]. Analysis of a large Canadian family revealed that two sisters with the D–– phenotype, and whose parents were not consanguineous, had the genotype *D––/–––* (heterozygous for *D––* and the Rh amorph gene, Section 5.16.1) [463].

The frequency of the *D––* haplotype was roughly estimated as 0.0005 in Sweden [464], 0.0047 in Iceland [465], and 0.005 in American Hispanics (determined by molecular typing) [439]. Seven of 692 000 (0.001%) Japanese donors had the D–– phenotype, a frequency of 0.0032 for the *D––* haplotype [466].

D–– produces only three Rh antigens: D, G, and Rh29. Red cells of individuals homozygous or heterozygous for *D––* express more D antigen than normal D+ cells and are directly agglutinated by some incomplete (non-agglutinating) anti-D. Most incomplete polyclonal anti-D will directly agglutinate cells of *D– –* homozygotes, but only carefully selected antibodies will distinguish cells of *D––* heterozygotes from DcE/DcE cells. This elevated expression of D is attributed to an increased number of D sites per red cell (Table 5.7).

The molecular genetic background of the D–– phenotype is heterogeneous. Homozygosity for *RHCE–D–CE* hybrid genes appears to account for several examples of D–– (Figure 5.10). In D–– individuals from Iceland, Britain, and Italy, only exons 1 and 10 were *RHCE*-derived [467,468]. Studies on two D–– members of an Italian family (LM) suggested that two hybrid genes were present: *RHCE–D(2–8)–CE–D(10)* and *RHD–CE(10)* [467,469,470]. Transcript analyses on a family (JD) whose propositus was heterozygous for *D––* and *D··* (Section 5.15.2) revealed *RHD* linked to *RHCE–D(*2 or 3–6*)–CE* [448]. Production of D epitopes from both genes of the haplotypes might account for the exalted expression of D.

Homozygosity for a single nucleotide deletion (907delC) in an *RHCE*cE* allele was responsible for D–– in three American Hispanics and heterozygosity for the same haplotype was found in patients and donors from the same ethnic group who were E– yet were predicted to be E+ by molecular testing [439]. One of 100 Hispanic donors had *RHCE*cE(907delC)*.

Two members of a Chinese family appeared to be *D––/Dc–* (Section 5.15.3) [440]. They were heterozygous for *RHD–RHCE(10)* and *RHCE–RHD(4–10)*, with the *RHCE*c* sequence, but neither of these hybrid genes was linked to *RHD* or *RHCE* (Figure 5.10) and the red cells did not have exalted D.

A thorough molecular analysis on a Japanese family (JF) with a D–– propositus, whose parents were not consanguineous, suggested that she was heterozygous for two abnormal Rh haplotypes [471]. In one, inherited from the father, there is *RHCE–D(2 or 3–7)–CE*, but no normal *RHD* (Figure 5.10). The other comprises *RHD*, but no *RHCE*. *D1S80* is a gene marker on chromosome 1, telomeric to the *RH* loci. As the propositus has received no *D1S80* allele from her mother, Okuda *et al.* [471] propose that a region of chromosome 1, containing *RHCE* and *D1S80*, was deleted during maternal gametogenesis.

An *RHCE*cE* allele (*RHCE*cEMI*) in a D+ C– c+ E– e+ black individual contained a 9-nucleotide deletion in exon 3 and appeared to produce no Rh polypeptide at the red cell surface [341]. There is no evidence that it was linked to *RHD*.

An unusual form of *D––* produced Tar (RH40) [235], a low frequency antigen usually associated with DVII (Section 5.6.4.6). The D antigen produced by this *D––* haplotype was stronger than that usually associated with Tar, but weaker than normal D.

Like Rh_{null} cells, D–– cells have a substantial reduction in CD47 content (Section 5.5.7 and see Table 5.13), but only slight reduction in RhAG [83].

5.15.2 *D··* and Evans (RH37)

Similarity between *D––* and the Rh haplotype producing the low frequency antigen Evans, recognised during studies on the first two Evans+ propositi, led to the Evans haplotype being denoted *D··* [472]. Inheritance of the haplotype was straightforward. Testing of apparent D–– red cell samples with anti-Evans led to discovery of the first person homozygous for *D··* (HD) [473] and positive reactions of apparent D–– red cells with immune sera from D–– individuals disclosed a second *D··* homozygote [474]. Families with *D··* have been mostly white British.

The antigens produced by *D··* are D, G, Evans, and the high frequency antigens Rh29 and Dav (RH47) [430], although over three decades after the first report of D··

weak e antigen was detected in one case (JD, see below) [475]. The D antigen is elevated, but less so than that produced by D–– [473]. The number of D antigen sites per red cell were estimated to be 56 000 for *D··/D··*, compared with 110 000–202 000 for *D––/D––* and 21 000 for *DcE/DcE* [473].

Molecular analyses on a Scottish family (AT) with five Evans+ members in three generations demonstrated that *D··* comprised *RHCE–D–CE*, with exons 2 (or 3) to 6 derived from *RHCE*, linked to *RHD* [476] (Figure 5.10). A very similar haplotype, but with exon 1 and the 5′ untranslated region of the hybrid gene also derived from *RHD*, was present in a *D··* homozygote (HD) [470] (though a different result was obtained on the same individual in another study [467]). In another family study, in which the propositus (JD) was heterozygous for *D··* and *D––*, the haplotype producing Evans comprised *RHCE–D*, with only exon 1 derived from *RHCE*, plus *RHD–CE*, with the 3′ end of exon 6 (encoding Cys311) and exons 7–10 derived from *RHCE* [448]. In an Evans+ African American, *RHD(1-6)–CE* appeared to be linked to *RHCE*ceMO* (Section 5.14.2.5) [475].

The amino acid sequence encoded by the junction of the 5′ end of *RHD* exon 6 and the 3′ end of *RHCE* exon 7 creates a unique amino acid sequence in the fifth cytoplasmic domain of the protein (Table 5.11). Conformational changes resulting from this sequence are probably responsible for Evans expression. *RHD*DIVb* also has this exon 6–7 junction and also encodes Evans (Section 5.6.4.3 and Figure 5.8) [221].

5.15.3 *Dc–*

The first homozygous *Dc–* propositus was found in an inbred white American family of French extraction [477]. His parents were double first cousins and two of his four siblings were also *Dc–/Dc–*. Other propositi with the Dc– phenotype have been Japanese [478], French [479], Argentinean [480,481], and Chinese (*D––/Dc–*) [440]. All Dc– propositi were ascertained through the presence anti-Hr_o in their serum.

Dc– produces D, G, c, Rh29, and, sometimes, ce. The strength of the D antigen is usually elevated and that of c is depressed; the c antigen may also differ qualitatively from normal c [477].

Transcript analysis on the French *Dc–* homozygote revealed *RHD* plus *RHCE–D(4–9)–CE* [482] (Figure 5.10). Exon 2 encoded Pro103, explaining the c expression. Other *Dc–* haplotypes contain *RHCE(1–3)–D(4–10)* [440] and *RHCE–D(5–7/8)–CE* [481].

Some haplotypes dubbed *Dc–* should, more accurately, be called Dc(e) or Dc((e)), as e can be detected by adsorption/elution tests or even by direct testing [483,484]. These haplotypes, mostly found in black people, have normal c and either normal or only slightly enhanced D.

5.15.4 *DC^w–*

A propositus identified as homozygous for *DC^w–* was a member of a large Canadian family [485]. Her parents were second cousins and four of her eight siblings were also *DC^w–/DC^w–*. *DC^w–* produces D, G, C^w, and Rh29; the D antigen is elevated in strength [485] and the C^w is depressed [486]. Unlike other haplotypes producing C^w, *DC^w–* makes neither C nor c. One other apparent *DC^w–* homozygote has been reported [449].

The original *DC^w–/DC^w–* propositus had transcripts representing *RHD* and *RHCE–D(2 or 3–9)–CE* [482] (Figure 5.10). The second propositus had *RHCE(1)–D*

Table 5.11 Amino acid sequences Leu303 to Cys316 encoded by the 3′ end of exon 6 and the 5′ end of exon 7 of *RHD*, *RHCE*, and two hybrid genes encoding Evans. The important residues in Evans expression appear to be Val/Ile306 and Gly/Val314, but not Tyr/Cys310.

Gene	Exon 6														Exon 7		
RHD						306					310				314		
	. . .	L	I	S	V	G	G	A	K	Y	L	P			G	C	C . . .
RHCE	. . .	–	–	–	I	–	–	–	–	C	–	–			V	–	– . . .
RHD-CE Evans (AT & HD)	. . .	–	–	–	–	–	–	–	–	–	–	–			V	–	– . . .
RHD-CE Evans (JD)	. . .	–	–	–	–	–	–	–	–	C	–	–			V	–	– . . .

–, identical residue to that encoded by *RHD*.

linked (or possibly *in trans*) with *RHD–CE(10)* [449] (Figure 5.10). In both individuals, the *RHCE*-derived exon 1 encoded Arg41, characteristic of C^w (Section 5.12).

5.15.5 *DIV(C)*– and Riv (RH45)

Homozygosity for a haplotype denoted *DIV(C)*– was proposed to explain the reactions of red cells of a woman from the Ivory Coast, whose third child had fatal HDFN [487]. Her two children had the same weak expression of C as their mother. The following antigens are produced by *DIV(C)*–: partial D giving the reactions of a strong DIV antigen; apparently normal G; very depressed C; Go^a (a low frequency antigen always associated with DIVa, Section 5.6.4.3); three other low frequency antigens, Rh33, Riv, and FPTT [188,488]; and three high frequency antigens, Rh29, Nou (RH44), and Dav (RH47) [430,489,490].

No other *DIV(C)*– homozygote has been found, but the association with low frequency antigens has led to the identification of three propositi heterozygous for *DIV(C)*–, all of African ancestry [488]. Transcript and DNA analysis on three heterozygotes revealed that the *DIV(C)*– haplotype comprises *RHD*DIVa.2* linked to *RHCE–DIVa.2(2,3)–CE–D(5)–CE* (Figure 5.10) [491]. Exon 2 of the hybrid gene, derived from *RHD*DIVa.2* is probably responsible for the weak C, the junction of exon 3 from *RHD*DIVa.2* and exon 4 from *RHCE* for Riv, the junction of exon 4 from *RHCE* and exon 5 from *RHD* (or *RHD*DIVa.*2) for FPTT, and the junction of exon 5 from *RHD* and exon 6 from *RHCE* for Rh33 [491].

5.15.6 Anti-Hr_o (-RH17) and related antibodies

People with D–– and related phenotypes, who have been exposed to red cells of common phenotype by transfusion or pregnancy, usually have antibodies to high frequency Rh antigens.

Red cells and immune sera of D––, Dc–, and DC^w– people are mutually compatible, showing that there is no anti-C^w or -c component. All such sera are non-reactive with Rh_{null} cells. In tests with red cells of common Rh phenotypes, these sera appear to contain an antibody to a single high frequency determinant, Hr_o (RH17). In some of the sera, separable anti-e has also been identified [430,461,492].

Heterogeneity within anti-Hr_o specificity is demonstrated by the variable reactions of different anti-Hr_o with red cells of phenotypes representing homozygosity for certain rare Rh haplotypes, *DIV(C)*–, *D··*, r^G, R^{oHar}, and also with Rh_{mod} cells [430,451]. Adsorption and elution tests showed that those D–– sera reactive with DIV(C)– cells contained at least two antibodies to high frequency antigens, one that reacted with the DIV(C)– cells, named anti-Nou (anti-RH44), and one that did not [489,490]. Similar tests with D·· cells revealed an antibody, named anti-Dav (anti-RH47), in some D–– sera [430]. Anti-Dav reacted with cells of common Rh phenotype and with D·· cells. Other antibodies to high frequency antigens, non-reactive with Rh_{null} and D–– cells, have been identified in individuals with unusual Rh phenotypes, such as those homozygous for R^N (anti-Rh46), or for genes producing partial e antigens (anti-Hr, -Hr^B) [335,442]. As polyclonal anti-D represents a mixture of antibodies directed at numerous epitopes on different regions of the RhD protein (Section 5.6.3), so anti-Hr_o represents antibodies to epitopes on different regions of the RhCE protein. D–– cells lack the whole Hr_o mosaic and, when immunised, D–– people can make antibodies to different parts of the mosaic, collectively referred to as anti-Hr_o and including anti-Nou, -Dav, -Hr, and -Hr^B.

Anti-Hr_o in the sera of D––, Dc–, DC^w–, and DIV(C)– mothers has been responsible for severe, and often fatal, HDFN [461,477,485,487,493,494] (reviewed in [493,494]).

Monoclonal antibodies that behave as anti-Hr_o were produced in a mouse immunised with human red cells [444] and in a crab-eating macaque immunised with gorilla and human red cells [495]. These antibodies detect non-polymorphic epitopes on the RhCcEe protein, but not on RhD.

5.16 Rh-deficiency phenotypes: Rh_{null} and Rh_{mod}

The Rh_{null} phenotype, in which no Rh antigens can be detected on the red cells, was first described by Vos *et al.* [496] in 1961. Rh_{null} is very rare, as reflected by the high consanguinity rate among parents of Rh_{null} propositi. Two types of Rh_{null}, with an identical Rh phenotype, are distinguished on the basis of their inheritance and molecular genetics.

1 The amorph type, with apparent homozygosity for silent genes at *RHD* and *RHCE* loci, results from inactivating mutations in *RHCE* and a deletion of *RHD.*

2 The regulator type, in which the Rh genes are normal, but there is homozygosity (or compound heterozygosity) for inactivating mutations in *RHAG*, the gene encoding the Rh-associated glycoprotein (RhAG), without which Rh antigens are not expressed (Section 5.5.6).

Some mutations in *RHAG* give rise to low level expression of Rh antigens, a phenotype called Rh_{mod}.

5.16.1 Rh_{null} of the amorph type

The amorph type of Rh_{null} is extremely rare, with only five propositi reported: Japanese [497,498], German [499–501], Norwegian Lapp, Spanish [500,502] and Caucasian Brazilian [503]. Family analyses suggest that their unusual phenotype resulted from homozygosity for silent or amorph Rh genes and the symbol −−−/−−− has been used for the genotype. The parents and children of amorph Rh_{null} individuals are obligate heterozygotes for the amorph gene and, consequently, always appear, from serological results, to be homozygous for Rh.

Titration of anti-c and -e with red cells of 1803 German donors revealed four apparent heterozygotes for the amorph haplotype, verified by family studies [499]. A frequency of 0.0001–0.0002 was estimated for the amorph haplotype in Sweden [464].

The pertinent mutations have been resolved for three of the amorph Rh_{null} individuals (Table 5.12). In each there is no *RHD*, but homozygosity for a grossly intact *RHCE* gene containing an inactivating mutation. In three propositi, single nucleotide (exon 7) [503], dinucleotide

Table 5.12 Some mutations associated with Rh-deficiency phenotypes.

Rh_{null} amorph				
Name	Population	Mutation		References
DR	German	*RHCE*Ce*	TCA→C, frameshift after Ile322	[499,501]
	Japanese	*RHCE*ce*	Deletion TCTTC, frameshift after Leu26	[497,498]
DAA	Spanish	*RHCE*ce*	Intron 4 5′ splice site	[500,502]
	Brazilian	*RHCE*ce*	GGGG→GGG, frameshift after Gly321	[503]
Rh_{null} regulator				
Name	Population	Mutation		References
SF, JL	White South African	*RHAG*	CCTC→GA, frameshift after Tyr51	[83]
TB	Swiss	*RHAG*	Heterozygous A deletion, frameshift after Ala362 + no detectable transcript	[83]
HT	Japanese	*RHAG*	Val270Ile, Gly280Arg	[504]
WO	Japanese	*RHAG*	Gly380Val (partial exon 9 skipping)	[504]
YT	Australian	*RHAG*	Heterozygous Gly279Glu + intron 1 5′ splice site	[505–507]
TT	Japanese	*RHAG*	Intron 7 5′ splice site, frameshift after Thr315	[79]
AL	White American	*RHAG*	Intron 1 5′ splice site	[83,508]
AC	Spanish, Japanese	*RHAG*	Intron 6 3′ splice site, frameshift after Thr315	[508,509]
	Chinese	*RHAG*	Ser224Arg	[510]
KS		*RHAG*	Gly178Arg	[511]
Rh_{mod}				
Name	Population	Mutation		References
SM	Russian Jewish	*RHAG*	Met1Ile	[512]
VL	White American	*RHAG*	Ser79Asn	[83,513]
CB	French	*RHAG*	Heterozygous Asp399Tyr + ?	[514]
		RHAG	Heterozygous: Gly90Val; Gly187Asp	[515]
JRM	Japanese	*RHAG*	Ser227Leu	[76]
	Japanese	*RHAG*	AAC→AC, frameshift after Asn395	[516]

(exon 7) [500,501], and pentanucleotide (exon 1) [500] deletions cause shifts in the reading frame and premature termination of translation. If translated, the gene with the exon 1 mutation would produce a protein of only 31 amino acids. The genes with the exon 7 mutations would give rise to a 357- or 398-residue protein (compared with 417 residues in the normal protein), with completely changed C-terminal amino acid sequences, which, if produced, would be abnormally folded and unlikely to be inserted in the membrane. Alternatively, the protein might be inserted in the membrane, but not accessible to antibodies. The Spanish propositus had a mutation in the intron 4 donor splice site of *RHCE*, giving rise to aberrant transcripts [500].

5.16.2 Rh_{null} of the regulator type

The first Rh_{null} propositus, an Australian aboriginal woman, was found during an anthropological survey [496,518]. No close relative was available for testing. The second Rh_{null} propositus was a member of a large family [519]. The Rh groups showed that her rare phenotype resulted from inhibition of her Rh genes: her husband was dce/dce, yet their daughter was DCe/dce and must have received D and C from her Rh_{null} mother. Race and Sanger coined the term 'regulator' for this type of Rh_{null}. The regulator type of Rh_{null} can be recognised when a parent or child of the Rh_{null} propositus has both C and c, and/or both E and e. Family studies showed that the regulator locus is not part of the Rh locus.

Although Rh_{null} remains very rare, many propositi with the regulator type have been found in people of white European or Eastern Asian origin. No Rh_{null} of black African origin has been reported.

In 1996, Chérif-Zahar *et al.* [83] showed that Rh_{null} of the regulator type was associated with inactivating mutations in *RHAG*, making *RHAG* a prime candidate for the regulator locus. Other *RHAG* inactivating mutations in Rh_{null} individuals have confirmed the association (Table 5.12). Some Rh_{null} individuals are homozygous, others doubly heterozygous, for *RHCE*-inactivating mutations. In all cases except one, normal *RHD* and *RHCE* were present, the exception having no *RHD* [514]. As with the Rh proteins, it is possible that the predicted proteins, if translated, are not transported to the membrane or cannot be inserted into the membrane.

Some cases of Rh_{null} result from single or double missense mutations in *RHAG* (Table 5.12). The encoded amino acid substitutions are predicted to be in the seventh (Ser224Arg), ninth (Val270 Ile, Gly279Glu, and Gly280Val), and twelfth (Gly380Val) membrane-spanning domains. Why these apparently minor changes to the protein prevent expression of the Rh antigens is unclear. The missense mutation encoding Gly380Val is in the first nucleotide of exon 9 and also causes partial splicing of exon 9 [504].

In some families, heterozygosity for the regulator allele resulted in weakened expression of some Rh antigens [518–524].

5.16.3 Rh_{mod}

A phenotype associated with modified expression of antigens produced by both Rh haplotypes was called Rh_{mod} by Chown *et al.* [513]. Red cells with this phenotype would easily be mistaken for Rh_{null} if only limited testing were done. The rare phenotype was attributed to homozygosity for a modifier gene at a locus separate from Rh [513], subsequently shown by molecular analyses to be *RHAG* (Table 5.12) [83,514]. Parents of most Rh_{mod} propositi are consanguineous.

Rh_{mod} is a heterogeneous phenotype. Apart from G, the Rh antigens of the first propositus were only detected by adsorption/elution tests with selected sera [513]. The Rh antigens of the second propositus were much stronger and C, c, and G could be detected by direct testing [451,525]. The third propositus was originally called Rh_{null}, although D was revealed by adsorption and elution, the only Rh antigen to be detected [526]. Rh_{mod} cells are most easily distinguished from Rh_{null} by immune sera from some people with D−− and related phenotypes (anti-Hr_o) and by some anti-Rh29 [451]. Relatives of Rh_{mod} individuals, heterozygous for the modifier gene, may have reduced expression of Rh antigens [76,451,516,527]. Rh_{mod} cells of one Japanese individual were positive for Ol^a (RHAG1), an antigen of very low frequency also found in members of one Norwegian family with reduced expression of some Rh antigens and heterozygosity for the same mutation [76] (Section 5.20).

Five Rh_{mod} propositi have missense mutations in *RHAG*; three are homozygous [76,83,512], one doubly heterozygous [515], the other heterozygous with no mutation in the *trans* gene detected [514]. One mutation converts the translation-initiating methionine codon to isoleucine. It is probable that the small quantity of RhAG produced is translated from the ATG triplet that normally encodes Met8 [512]. Other amino acid substitutions are located on RhAG in the third membrane-spanning domain (Ser79Asn, Gly90Val) [83,515], the third cytoplasmic loop (Gly187Asp) [515], the extracellular vestibule area around the fourth external domain (Ser227Leu) [76], and the carboxy-terminal tail (Asp399Tyr) [514].

One Japanese Rh_{mod} was homozygous for a single nucleotide deletion in exon 9 of *RHAG* resulting in a new stop codon at positions 1384–1386 and a protein of 461 amino acids instead of the usual 409 [516]. All propositi had low quantities of RhAG in their red cell membranes.

5.16.4 Antibodies in the sera of Rh_{null} people

Anti-Rh29 (anti-'total Rh'), an antibody found in the serum of some immunised Rh_{null} individuals, reacts with red cells of all Rh phenotypes apart from Rh_{null} [499,521,524,528–530]. Anti-Rh29 has been made by people with Rh_{null} of both types, though not all Rh_{null} individuals make anti-Rh29 when immunised; two Rh_{null} sisters had no Rh antibody although they had a total of nine children. Other immunised Rh_{null} individuals are reported to have made anti-e [519] or -Hr_o [502]. Antibodies in the sera of two Rh_{null} donors who had received no known immunising stimulus behaved as anti-Hr_o, reacting with all red cell samples save those of Rh_{null} and D–– phenotypes [530,531].

Anti-Rh29 has been responsible for HDFN, managed successfully by repeated exchange transfusions with red cells of common Rh phenotypes [529,532] or with Rh_{null} red cells from family members [533]. Anti-Hr_o in an Rh_{null} woman was also responsible for HDFN [502]. An HTR caused by anti-Rh29 may have contributed to the death of an elderly Rh_{null} patient transfused with D– blood of common phenotype [534].

Many human autoantibodies are considered Rh-related because they do not react with Rh_{null} cells. Weiner and Vos [535] called these antibodies anti-pdl (anti-partially deleted), to distinguish them from anti-dl (anti-deleted), which react with all red cells, and anti-nl (anti-normal), which react with all red cells except for Rh_{null} and D–– cells.

5.16.5 Other antigens affected in Rh deficiency phenotypes

Affects of the Rh deficiency phenotypes extend beyond the Rh blood group system as a result of the complete or partial deficiency of the Rh proteins and RhAG from the band 3/Rh macrocomplex and possibly from the junctional complex (Section 5.5.7). Several red cell antigens are lacking, or at least show reduced expression, on Rh_{null} and Rh_{mod} red cells.

RhAG is not detected on Rh_{null} red cells of the regulator type and is expressed in reduced quantity on Rh_{mod} cells (Table 5.13). Indeed, as described above, it is the absence or altered conformation of RhAG that is primarily responsible for these phenotypes. RhAG is present on Rh_{null} cells of the amorph type, but in reduced quantity [83]. Duclos and DSLK antigen, located on RhAG [76] (Section 5.20), are also absent from most Rh_{null} cells.

Table 5.13 Estimated numbers of RhAG and CD47 molecules on Rh-deficiency and D–– cells [83,500,508].

	Number of molecules per cell (range)	
Phenotype	RhAG	CD47
Rh_{null} regulator	0	2900–3900
Rh_{null} amorph	44 000–58 000	2000–3000
Rh_{mod}	43 000	6600
D––	180 000–206 000	8000–12 000
D+	220 000–280 000	35 000–50 000

The high frequency antigens of the LW system, LW^a and LW^{ab}, are absent from all Rh_{null} cells and weakly expressed on Rh_{mod} cells (Chapter 16). Anti-Fy5 of the Duffy system behaves like anti-Fy3, except that it does not react with Rh-deficiency cells, none of which are Fy:–3 (Chapter 8). Rh-deficiency cells have reduced expression of glycophorin B and are often U– (Section 3.20). CD47, a glycoprotein on red cells with no blood group activity, is also present in reduced quantity on Rh-deficiency cells (Table 5.13).

Glycophorin A antigens, M, N, and En^a, are reported to be slightly enhanced in the regulator type of Rh_{null} (amorph type not mentioned) [536]. Elevation of i antigen on Rh-deficiency phenotype cells probably results from bone marrow stress caused by the associated anaemia [537]. Antibodies that reacted with Rh_{null} cells and with cells of other 'null' phenotypes, but not with cells of 'normal' phenotypes unless they had been papain-treated, were found in three patients with anaemia [538,539].

Knockout mice for the Rh gene (*Rhd*) had a complete loss of ICAM-4 (LW), but only moderate reduction in RhAG; *Rhag* knockout mice had a complete loss of Rh and ICAM-4, but no effect on CD47 [85].

5.16.6 Rh-deficiency syndrome

Rh_{null} red cells are morphologically and functionally abnormal. Most Rh_{null} and Rh_{mod} individuals have some degree of haemolytic anaemia, the severity of which varies from severe enough to merit splenectomy to a fully

compensated state requiring sophisticated tests to demonstrate shortened red cell survival. Typical symptoms of Rh-deficiency syndrome are the presence of stomatocytes (cup-shaped red cells) and some spherocytes, reduced survival of autologous red cells, increased red cell osmotic fragility, increased reticulocyte counts, increased fetal haemoglobin, enhanced i antigen strength, and reduced haemoglobin and haptoglobin levels (reviews in [514,540]).

Rh_{null} red cells have an abnormal organization of their membrane phospholipids [541], increased cation permeability, partially compensated by an increase in the number of K^+Na^+ pumps [542], reduced cation and water contents, and reduced membrane cholesterol content [528]. It is not known whether any of these defects accounts directly for the autohaemolyis in Rh-deficiency syndrome, but the haemolytic anaemia is alleviated by splenectomy, so whatever the ultimate cause of stomatocytosis in Rh-deficiency syndrome, it is the early sequestration of these abnormally shaped cells that is responsible for the anaemia [543]. Binding of CD47 on red cells to SIRPα on macrophages generates a negative signal that protects against phagocytosis of the red cells (Section 5.5.7), so reduced CD47 levels on Rh_{null} cells could be involved in their elimination, although enhanced phagocytosis of Rh_{null} red cells was not detected by a monocyte monolayer assay [544].

5.17 Low frequency Rh antigens and the antibodies that define them

5.17.1 Low frequency antigens

Twenty-four antigens of low frequency belong to the Rh system (Table 5.14). Presence of these antigens is associated with abnormal (usually partial or depressed) expression of one or more of the DCcEe antigens.

Table 5.14 Antibodies to low frequency Rh antigens.

Antigen		Red cell immune	'Naturally occurring'	Other antibodies present	HDFN	No. of examples	References
C^w	RH8	Yes	Yes	Yes	Yes	Many	[390,391,397,399–402]
C^x	RH9	Yes	Yes	Yes	Mild	Many	[404,405,407]
V	RH10	Yes	No	Yes	No	Many	[410,414,416]
E^w	RH11	Yes	No	No	Yes	Several	[329,330]
VS	RH20		Yes	Yes	No	Many	[411,414]
D^w	RH23		Yes	Yes*	No	Several	[223,545]
Go^a	RH30	Yes	Yes	Yes*	Yes	Several	[219,546]
Rh32	RH32	Yes	Yes	Yes*	Yes	Several	[443,547]
Rh33	RH33		Yes	Yes	No	Few	[191,548]
Rh35	RH35		Yes	Yes	No	1	[441]
Be^a	RH36	Yes	No	No	Yes	Several	[432,433,455–457]
Evans	RH37	Yes	Yes	Yes*	Yes	Several	[472]
Tar	RH40	Yes	Yes	Yes	Yes	Few	[235,236]
Rh42	RH42	Yes	No	No	Mild	2	[412]
Riv	RH45	Yes	No	Yes	Mild	1	[488]
JAL	RH48	Yes	Yes	Yes	Mild	Few	[426]
STEM	RH49	Yes	No	Yes	Mild	Several	[350]
FPTT	RH50		Yes	Yes	No	1	[188]
BARC	RH52			Yes	No	1	[227]
JAHK	RH53		Yes	Yes	No	Several	[377]
DAK	RH54	Yes		Yes	No	Several	[160]
LOCR	RH55	Yes		No	Mild	Several	[454]
CENR	RH56		Yes	Yes	No	Several	[431]
PARG	RH60	Yes		Yes	No	1	[701]

*Not always separable from other Rh specificities, see text.

Consequently, the low frequency antigens make useful markers for rare haplotypes. Although an oversimplification, as reference to the appropriate sections of this chapter reveals, the low frequency antigens can be loosely classified as follows:

- D^w, Go^a, Rh32, Rh33, Evans, Tar, FPTT, BARC, DAK – associated with partial D (Section 5.6.4);
- C^w, C^x – associated with abnormal C (Section 5.12);
- E^w – associated with abnormal E (Section 5.9.4);
- V, VS, Rh42, STEM – associated with abnormal e (Sections 5.9.5, 5.13);
- Rh32, Rh35, JAL, FPTT, DAK – associated with abnormal *DCe* (Section 5.14.1);
- CENR – associated with abnormal *DCC^{w}e* (Section 5.14.1.4);
- Rh33, JAL – associated with abnormal *Dce* (Section 5.14.2);
- Be^a, LOCR – associated with abnormal *dce* (Sections 5.14.2.3, 5.14.2.2);
- JAHK – associated with abnormal *dCe* (Section 5.11);
- Evans – associated with abnormal *D*–– (Section 5.15.2);
- Go^a, Rh33, Riv, FPTT – associated with *DIV(C)*– (Section 5.15.5).

It is not safe to assume that an antigen belongs to the Rh system simply because it is associated with abnormal expression of Rh antigens. The Ol(a+) members of a large family had weak expression of some Rh antigens while the Rh antigens of the Ol(a–) members were normal, yet the family showed that Ol^a is not inherited at the Rh locus [549] and Ol^a was subsequently shown to be encoded by *RHAG* [76] (Section 5.20.2). The low frequency antigen HOFM (700050) is associated with depressed C antigen in the only family in which it has been detected, but there is insufficient evidence for its elevation to the Rh system [550].

Most of the antigens listed in Table 5.14 are of low incidence in all populations tested, but the frequencies of some vary in different populations. C^w and VS would not be considered private antigens in white and black populations, respectively.

5.17.2 Speculation on the molecular basis of Rh32 and FPTT

The molecular backgrounds to some of the Rh low frequency antigens can be anticipated from the changes to the *RHD* and *RHCE* genes associated with their expression. These are described in other sections of this Chapter. Two antigens, Rh32 and FPTT, are each associated with two serologically very different phenotypes and these will be described in more detail here.

Rh32 is associated with the partial D antigen DBT, usually together with C and e (Section 5.6.4.8). R^N produces Rh32 together with slightly elevated D, weak C, and weak e (Section 5.14.1.1). DBT arises from an *RHD-CE-D* gene in which exons 5–7 (or 5–9) are *RHCE*-derived (Figure 5.11). R^N consists of *RHD* and *RHCE–D(4)–CE*. From comparison of the abnormal hybrid genes in the *DBT* and in the R^N haplotypes it appears that Rh32 could result from the conformation of an Rh polypeptide with the product of *RHD* exon 4 fused to the product of *RHCE* exon 5. Rh32 does not, however, simply arise from a unique linear amino acid sequence resulting from the gene rearrangements. A sequence of 21 amino acids spanning the junction of the products of exons 4 of *RHD* and exon 5 of *RHCE* is identical in the polypeptides encoded by the two genes. Rh32 probably results from interactions between *RHD*- and *RHCE*-specified amino acids within the extracellular vestibule: Met169, Met170, Ile172 from *RHD* and Gln233 from *RHCE* (Figure 5.11).

FPTT is associated with the rare R^{oHar} gene, which also produces a partial D antigen (DHAR), c, very weak e, and another low frequency antigen, Rh33 (Section 5.6.4.12). FPTT is also associated with DFR, another partial D antigen (Section 5.6.4.7). R^{oHar} is an *RHCE–D(5)–CE* gene, whereas DFR is produced by *RHD–CE–D* in which part of exon 4 is *RHCE*-derived. FPTT appears to be the opposite to Rh32, resulting from interactions within the extracellular vestibule: Leu169, Arg170, and Phe172 from exon 4 of *RHCE* and Glu233 from exon 5 of *RHD* (Figure 5.11).

5.17.3 Antibodies to low frequency antigens

Table 5.14 summarises published and unpublished information available to the author; other examples of these antibodies may well exist. Some antibodies have caused severe HDFN, but where antibodies are shown to have caused mild HDFN, in a few cases this may only represent a positive DAT on the baby's red cells. Anti-Go^a is implicated in a delayed HTR [551].

Although some specificities, such as anti-Go^a and anti-Rh32, are found as the sole antibody in some sera and may have caused HDFN, in other sera they occur together and are 'naturally occurring'. When these two specificities are found together they are generally not separable by adsorption. To take a specific example, the Tillett serum contained antibodies to many low incidence antigens, although Mrs Tillett had not been exposed to these antigens. Anti-Go^a, -Evans, and, sometimes, anti-Rh32 were present in Tillett serum, but those specificities could not

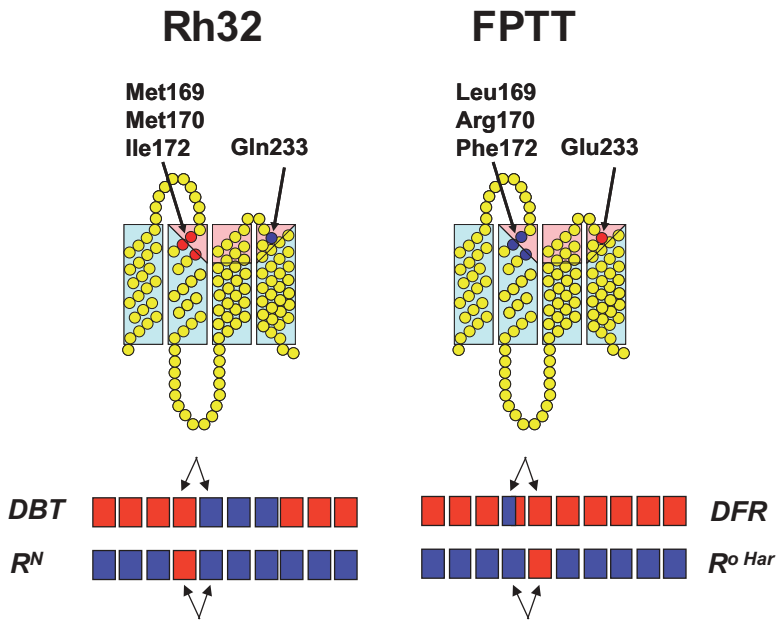

Figure 5.11 Molecular bases of the low frequency Rh antigens Rh32 and FPTT. Both low frequency antigens probably result from the interactions within the extracellular vestibule region involving amino acids encoded by *RHD* exon 4 and *RHCE* exon 5 for Rh32 and by *RHCE* exon 4 and *RHD* exon 5 for FPTT. Two-dimensional models represent part of the Rh proteins, including the extracellular vestibule region in pink.

be isolated from each other by adsorption and elution: adsorption with Rh:32 Go(a–) Evans– cells removed activity against Go(a+) and Evans+ cells, as well as against Rh:32 cells. Adsorption with Rh:32 cells of a later batch of Tillett serum, which no longer contained anti-Rh32, did not affect the strength of the anti-Go^a and -Evans, but adsorption with either Evans+ or Go(a+) cells removed all activity for those cells [157]. Presumably conformational similarities exist between Rh32, Evans, and Go^a determinants and some antibodies detect a common feature of these determinants whereas others can distinguish between them.

Some anti-D^w are specific for the product of *RHD*DVa*, in which the presence of Gln233 in an RhD protein appears to be the key factor (Section 5.6.4.4). Most anti-D^w, however, also react with Rh:32 cells, some reacting only weakly with the Rh:32 cells and some reacting as strongly with Rh:32 cells as with D^w+ cells [552]. Comparison of *RHD*DVa* genes, which produce D^w, with *RHD*DBT* and R^N genes, which produce Rh32 (Figures 5.8 and 5.10), reveals that all involve exon 5 encoding Gln233 (from *RHCE*) adjacent to exon 4 derived from *RHD*.

5.18 Rh antibodies

Most details on the specificities of Rh antibodies are to be found in the preceding sections, where the antigens they define are described. Provided here are more general comments on Rh antibodies, polyclonal and monoclonal, and on their clinical significance.

Rh antibodies are usually produced in response to red cell immunisation resulting from blood transfusion or pregnancy, although 'naturally occurring' Rh antibodies are occasionally encountered. Rh antibodies generally react optimally at 37°C, their reactivity being enhanced by protease treatment of the cells. Most Rh antibodies, in the absence of an enhancing medium, do not directly agglutinate untreated antigen-positive red cells.

Most Rh antibodies should be considered potential agents of HDFN and acute or delayed HTRs.

5.18.1 Anti-D

5.18.1.1 Alloanti-D

Anti-D are mostly IgG, but some sera contain an IgM component. Sera containing IgM anti-D usually

agglutinate saline suspended D+ cells, as do some sera containing relatively high concentrations of IgG anti-D alone. Most anti-D from hyperimmunised subjects also contain an IgA component [112]. IgG1 and IgG3 are the predominant anti-D subclasses; IgG1 is nearly always present and, in individuals who have received multiple immunisations, both subclasses are generally detected. IgG2 and IgG4 anti-D are also found occasionally [112,553,554]. 'Naturally occurring' IgG anti-D in the sera of untransfused men have been described [555,556], but these are rare.

The failure of almost all anti-D to activate complement is attributed to the distance between antigen sites, which prevents the collaboration between IgG molecules required for C1q binding [112]. D+ red cells that had been very heavily coated with anti-D bound up to 1600 molecules of C1q, yet the classical complement pathway was not activated [557]. One well-investigated anti-C+D (Ripley), however, would haemolyse D+ cells through the activation of complement [558,559] and a complement binding anti-D was found in the serum of a woman with a weak partial D [560].

Human anti-D was produced in mice with severe combined immunodeficiency (SCID) that had been reconstituted with peripheral blood mononuclear cells obtained from D– people who had recently been sensitised with D+ red cells [561]. When mononuclear cells were used from donors many years after sensitisation, no anti-D was produced in the mice even though anti-D was still present in the donor's plasma. It appears that long-lived or memory cells are not present in the peripheral blood of individuals who have not been recently boosted.

5.18.1.2 Monoclonal anti-D

The development of the mouse hybridoma technique in the late 1970s and 1980s led to the production of murine monoclonal antibodies to a host of blood group antigens, yet mouse monoclonal anti-D has not been made. Failure to make anti-D in mice stimulated attempts to produce human monoclonal antibodies, primarily to replace polyclonal antibodies as grouping reagents and for prophylactic use. The hybridoma technique of Köhler and Milstein was not very successful when applied to humans, but an alternative approach, that of immortalising human B lymphocytes by *in vitro* transformation with Epstein-Barr virus (EBV), did bring success.

Crawford *et al.* [562] were the first to clone EBV-transformed lymphoblastoid cells to produce a stable cell line secreting anti-D. Cell lines produced in this way are often unstable and difficult to grow in large-scale culture, so to overcome these problems EBV-transformed lymphoblastoid cells are usually fused with myeloma cells to produce a hybridoma [563]. CD40 activation of lymphocytes can been used to replace EBV transformation [564]. Greatest success in producing monoclonal anti-D has been achieved when lymphocytes from recently reboosted antibody makers were used. For reviews see [565–568].

Of the 52 cell lines secreting anti-D summarised by McCann *et al.* [567], 17 were IgM and 35 IgG; of the 33 IgG subclassed, 24 were IgG1, eight IgG3, and one IgG2. The predominance of IgG1 and IgG3 molecules reflects the antibody profile of the lymphocyte donors. EBV-transformation followed by fusion appeared to increase the probability of making IgM anti-D [567]. IgA monoclonal anti-D has been reported [569].

Reactions of monoclonal anti-D with red cells expressing partial D antigens have identified numerous epitopes on the RhD protein (Table 5.6 and Section 5.6.3). Anti-D monoclonals and blends of monoclonals are now almost exclusively used as Rh grouping reagents. IgM monoclonal anti-D reagents agglutinate all but the weakest of weak D samples, though most do not agglutinate DVI cells. Three monoclonal anti-D for detecting DVI antigen were produced by using DVI red cells for rosetting in the production and maintenance of transformed cell lines [229]. The potential use of monoclonal anti-D in HDFN prophylaxis is discussed in Section 5.18.1.4.

5.18.1.3 Anti-D genetics

Human immunoglobulin V-gene cDNA derived from peripheral blood lymphocytes can been amplified and cloned, and the V_H and V_L gene repertoires linked together at random to encode single chain Fv (scFv) antibody fragments. These synthetic scFv cDNAs are inserted into phage vectors so that they can be incorporated into filamentous phages. By linking the scFv cDNA to a gene for a phage coat protein the encoded scFv molecules are represented at the surface of the phage, which then behaves like an antibody. Phages containing genetic information for variable regions that bind to a specific antigen can then be isolated, by selecting with an appropriate solid phase antigen. Red cells can be used for this purpose. *Escherichia coli* can then be transduced with these genes, cloned, and the single chain variable region fragments secreted [570]. By this technique antibody fragments specific for D and E were produced from non-immunised donors, but these antibodies were of low affinity [571, 572]. Subsequently, similar phage repertoire cloning techniques have led to the production of Fab fragments

with D antigen binding characteristics identical to those of the parental antibodies [573,574]. Genetically engineered IgG anti-D molecules, secreted by insect or mammalian cell lines, have a functional Fc domain and behave normally in immunological functional assays [574,575].

Sequencing of the genes encoding the variable regions of the light and heavy chains (V_L and V_H) of monoclonal IgG anti-D, revealed an extremely restricted use of germline genes [269,576–583]. A list of the genetic characteristics of 113 monoclonal anti-D and of 56 non-D Rh antibodies are listed in a workshop report [584]. The germline V_H segments used in IgG anti-D are among the most cationic available in the human V_H repertoire [577]. This would explain the relatively high isoelectric point of anti-D, compared with that of serum IgG, which may be an important factor in binding to the D antigen, which is located close to the membrane lipids.

The IgM heavy chain variable region gene segment V4-34 is present in cold agglutinins with I and i specificity (Section 25.7.3). About 85% of IgM monoclonal anti-D are encoded by V4-34 and these antibodies agglutinate papain-treated D– red cells; some will agglutinate untreated D– cells at 4°C [585,586]. This cold agglutination is usually i-specific, but can be I-specific. V4-34 IgM monoclonal anti-D also exhibit tissue multireactivity, mostly directed against intracellular components, in particular cytoplasmic intermediate filament proteins [586,587]. Clearly the cold agglutinin characteristics of IgM anti-D must be a consideration in the development of reagents. The same non-polar hydrophobic amino acids in the V4-34 framework-1 sequence are critical for both anti-D and -i activity, so it is not possible to remove the cold agglutinin activity by site-directed mutagenesis, without also reducing anti-D activity [580]. Anti-i activity was also detected in a monoclonal anti-c blood grouping reagent [588].

5.18.1.4 Clinical significance of anti-D

Clinically, D is the most important red cell antigen after A and B. Anti-D has the potential to cause severe HTRs, so D+ red cells must never be transfused to patients with anti-D and red cells of donors and recipients must always be typed for D, except possibly in populations where the D– phenotype is extremely rare [589]. About 20–30% of D– patients who receive large volumes of D+ blood make anti-D [113–115], so ideally D+ red cells should not be transfused to D– patients, except in an emergency, and must never be transfused to D– girls and women of childbearing age. The same criteria should be applied to blood products that may be contaminated with red cells. Immunisation rates, however, are zero or close to zero in D– immunosuppressed patients transfused with D+ red cells [590].

Before the 1970s, HDFN caused by anti-D was a significant cause of fetal and neonatal morbidity and mortality. In 1970, the incidence of infant deaths and stillbirths from HDFN caused by anti-D in England and Wales was 1.2 per thousand births; by 1989 the figure had fallen to 0.02 per thousand births [591]. This remarkable fall in prevalence is predominantly the result of immunoprophylaxis with anti-D immunoglobulin, which prevents the production of maternal anti-D following D-incompatible pregnancies. The mechanism for this antibody-mediated immune suppression still remains unclear. Although red cell clearance may play a significant role, other important factors could be B-cell inhibition resulting from IgG-antigen complexes interacting with the inhibitory IgG receptor, FcγRIIB, and IgG-mediated disruption of antigen processing and presentation leading to reduced T-cell help and B-cell activation (reviewed in [592–594]). All D– women must receive anti-D immunoglobulin within 72 hours of delivery of a D+ baby, the dose of anti-D being related to the size of the transplacental haemorrhage. In addition, one of two injections of D– pregnant women with anti-D immunoglobulin at around 28–34 weeks' gestation reduces the rate of antenatal immunisation [595,596].

The severity of anti-D HDFN is highly variable. The most severely affected fetuses die *in utero* from about the 17th week of gestation onwards. In less severe cases, hydrops fetalis may occur. In severely affected infants who are born alive, jaundice may develop rapidly and lead to kernicterus, which can cause permanent cerebral damage [112]. Despite anti-D immunoglobulin prophylaxis, in England and Wales at least 500 fetuses develop haemolytic disease per year, and about 25–30 babies die from HDFN [596]. There have been three recent non-invasive innovations in the prevention and management of HDFN: routine antenatal anti-D prophylaxis (RAADP) mentioned above; Doppler ultrasonography of the middle cerebral artery, which correlates well with increasing levels of bilirubin in the amniotic fluid; and determination of fetal D genotype by analysis of cell-free fetal DNA obtained from the maternal plasma (Section 5.7.1) [596,597]. Preimplantation genetic diagnosis has been applied in the management of pregnancy in couples where the woman has high levels of anti-D and the man is heterozygous for D-positivity [303]. Following *in vitro* fertilisation, biopsy of single cells from early embryos

permits selection of an embryo lacking *RHD* for implantation, guaranteeing a D-negative pregnancy.

Analysis of the quantity, IgG subclass, and functional activity of anti-D can provide a guide to the potential severity of HDFN. Anti-D can be quantified by comparison with a standard in an AutoAnalyser or by flow cytometry. A recommendation for interpretation of anti-D levels is as follows: <4 IU/ml, HDFN unlikely, but continue to monitor; 4–15 IU/ml and >15 IU/ml, moderate and severe risk, respectively, refer to specialist unit [598]. IgG1 and IgG3 anti-D both cause HDFN, IgM, IgG2, and IgG4 do not [112]. IgG1 appears to be more important than IgG3 in the pathogenesis of fetal anaemia [599]. A variety of cellular functional assays model the *in vivo* destruction of antibody-coated red cells following interaction with Fcγ-receptors of the mononuclear phagocyte system [600]. All of these assays provide useful clinical information, but none is entirely reliable for predicting the severity of HDFN.

Anti-D immunoglobulin for the prevention of immunisation is in short supply. It is produced by immunising volunteers with red cells, creating the ethical dilemma of whether healthy individuals should be injected with blood products. For many years it appeared likely that monoclonal antibody and recombinant antibody technology, in which an almost infinite volume of immunoglobulin can be produced *in vitro* from one immunisation, would provide the answer to this problem, but no such product has become available. The difficulty is that clinical trials have been disappointing, with monoclonal antibodies generally less effective than polyclonal antibodies at clearance of D+ red cells. The reason for this is not clear, but could result from unnatural glycosylation of these antibody molecules [601,602].

Peptide immunotherapy, the use of peptides derived from the RhD protein to render D-specific helper T cells tolerant, is another potential approach to preventing anti-D HDFN. Immunodominant peptides from the RhD protein induce the proliferation from T-helper lymphocytes (Th cells) from alloimmunised donors *in vitro* [603]. In a humanised mouse model, an HLA-DR15 transgene conferred the ability to respond to immunisation with purified RhD protein. Treatment of these mice with the peptides administered to the nasal mucosa prevented antibody responses to RhD protein by inducing tolerance, suppressing both antibody production and T-cell activation [604].

Occasional unexpectedly mild cases of HDFN occur in the D+ fetuses of women with anti-D and a history of severe HDFN in previous pregnancies. This can occur as the result of maternal HLA antibodies blocking Fc receptors on fetal macrophages, protecting sensitised fetal red cells from destruction [605,606]. FcγRI-blocking antibodies could have a potential for the treatment of HDFN.

5.18.2 Anti-C, -c, -E, -e, and -G

The numerous complexities of the specificities of antibodies described as anti-C, -c, -E, and -e are described in Sections 5.8 and 5.9. These antibodies share many of the characteristics of anti-D. They are generally immune, mostly IgG, and predominantly IgG1, although IgG2, IgG3, and IgG4 have all been detected [554]. Antibodies of all these specificities have been involved in HTRs, particularly of the delayed type [112]. Anti-c is clinically the most important Rh antigen after anti-D and causes severe HDFN. In three series of studies, between 14 and 21% of c+ babies born to women with anti-c required exchange transfusion [607–610]. Anti-C, -E, -e, and -G have all caused HDFN, but the occurrence is rare and the outcome seldom severe [112]. The clinical significance of antibodies with compound CcEe specificities is described in Section 5.10. Anti-G may be mistaken for anti-C+D (Section 5.11). It is important that D– pregnant women with anti-G or anti-C+G receive anti-D immunoglobulin, to prevent them making anti-D. Unlike other Rh specificities, apparently 'naturally occurring' anti-E are not uncommon [353,354].

Intravascular HTRs have been associated with specific Rh antigens in the absence of any detectable Rh antibody [611]. Rh association was inferred because transfusion of red cells positive for a particular Rh antigen resulted in haemolysis, whereas transfusion of antigen-negative cells resulted in normal red cell survival. C, c, and e have been implicated in this type of reaction.

Human monoclonal antibodies to C, c, E, e, and G have been produced by cloning of EBV-transformed lymphoblastoid cell lines or by cloning of heterohybridomas produced from a fusion of EBV-transformed cells with mouse myeloma cells (see Section 5.18.1.2) [612–614]. Like anti-D (Section 5.18.1.3), antibodies to C, E, e, and G utilise *IGHV* genes restricted to the *IGHV3* superspecies [578,583,615].

5.18.3 'Enzyme-only' antibodies

Some Rh antibodies, often referred to as 'enzyme-only' antibodies, agglutinate red cells treated with protease enzymes, but are not detected by conventional antiglobulin tests with untreated cells. Although these antibodies are most often 'naturally occurring' anti-E [351–354,616], 'enzyme-only' anti-D, -C, -c, -e, and -ce have also been

identified [617–621]. 'Enzyme-only' antibodies are generally clinically insignificant and are not even detected when tests employing protease-treated red cells for antibody screening are avoided. There are rare exceptions, however. Examples of 'enzyme-only' anti-c, -e+ce, -E, and -C (which bound C3) caused HTRs [616–618,621]. One 'enzyme-only' anti-E became active by an indirect antiglobulin test during pregnancy and caused HDFN requiring exchange transfusion [622].

5.18.4 Rh autoantibodies

Rh antigens are the most common targets for warm autoantibodies [623]. The involvement of Rh antibodies in AIHA was first appreciated when autoantibodies with anti-e specificity were recognised [624]. Of the 'simple' Rh-specific autoantibodies, anti-e is the most common, but anti-c, -E, -D, and -C also occur, roughly in that order of prevalence [623,625,626]. These specificities occasionally occur alone, but more often adsorption tests with Rh-phenotyped red cells are necessary to determine the specificities present on the red cells and in the serum of AIHA patients. Some Rh antibodies with apparently simple specificities, dubbed 'mimicking antibodies' by Issitt *et al.* [627], can be totally adsorbed by 'antigen-negative' red cells, demonstrating a broader specificity. Cold type AIHA caused by complement activating IgM autoanti-D has been described [628].

Use of red cells with the rare Rh phenotypes Rh_{null} and D−− showed that antibodies to high incidence Rh antigens often occur as autoantibodies [535]. Rh-related antibodies may also be involved in some cases of drug-induced AIHA [112,623]. Loss or weakness of some Rh antigens has been reported in a few patients with AIHA [629].

Some D+ patients have developed anti-D and a positive DAT after transfusion of D+ blood [630–632]. The anti-D was transient, although an injection of D+ blood in one patient restimulated the antibody [631]. Autoanti-D may occur concurrently with alloanti-D in immunised individuals with partial D antigens [633,634] and, rarely, before the alloanti-D can be detected [635].

5.18.5 Transplant donor-derived Rh antibodies

Anti-D derived from donor lymphocytes in D+ recipients of solid organ transplants (kidney, liver, heart–lung, pancreas) has been responsible for haemolysis, sometimes severe [636–639]. The donor origin of such anti-D has been demonstrated by Gm grouping [640,641]. Anti-c, -E, and -e have also been detected in similar circumstances [636,637,642].

Anti-D has been observed in D+ recipients of D− bone marrow or peripheral blood stem cells [643–646], in one case not appearing until immunosuppression for graft-versus-host disease had been discontinued, 2 years after transplantation [645]. Anti-D, -E, and -G were detected in the serum of a DcE/dce patient 4 months after he received bone marrow from his sister, presumably the result of immunisation of the donor-derived lymphocytes by the patient's D+ E+ red cells [646].

5.19 Rh mosaics and acquired phenotype changes

Abnormal expression of some Rh antigens is occasionally observed in patients with myeloid leukaemias, polycythaemia, and other myeloproliferative disorders. In most cases these patients appear to be mosaics with two populations of red cells of different Rh phenotype [124,647–651], although a few have complete loss of certain Rh antigens [652–656]. One patient with myeloid metaplasia, previously known to be D+, was found to be D− and had made anti-D plus -C [653]. The strength of antigen expression and proportions of the two cell populations can vary over a period of time [647,650, 651,654].

There are also many examples known of apparently healthy people whose blood appears to contain two red cell populations, as judged by tests with Rh antisera, but have no sign of mosaicism in tests for other genetic markers [649,651,657]. The Rh mosaicism is not a transient condition and families of four propositi eliminated chimerism as a possible explanation. Screening with anti-D+C of blood from 552 individuals over 60 years old disclosed one Rh mosaic [657].

Three individuals with Rh mosaicism were also mosaics for another chromosome 1 marker, the Duffy blood group [651,658,659]. Jenkins and Marsh [658] found 30% D+ C+ Fy(a+) and 70% D− C− Fy(a−) red cell populations in a male blood donor. The results of testing his family showed that he could not be dce/dce: his father was DCe/DCe Fy(a+b−), his mother DCe/dce Fy(a−b+), and his sister DCe/dce Fy(a+b+). Extensive serological investigation led to the conclusion that the father had a homozygous dose of C and e. In another case, 30% of red cells were D+ C+ Fy(b+) and 70% D− C− Fy(b−), but no other sign of mosaicism was observed in the many

markers studied. The karyotype determined on lymphocyte and fibroblast cultures was normal [659].

A myelofibrosis patient with a mixture of D+ C+ and D– C– cells (father dce/dce, mother DCe/DCe) had an aberrant karyotype, a cytogenetic mixture with an abnormal population containing a balanced translocation involving chromosomes 1, 4, and 7 [652]. In most cases, however, no abnormality of chromosome 1, which contains the Rh genes, was observed.

A D+ woman became D– over a 3-year period, during which she was diagnosed with chronic myeloid leukaemia [655]. Reticulocyte transcript analysis revealed *RHD* with a deletion of 600G in exon 4, introducing a reading frameshift and premature stop codon, plus *RHCE*Ce* and *RHCE*ce*. The *RHD* mutation, which was present in neutrophils and cultured erythroblasts, but not lymphoid cells, probably resulted from a somatic mutation in a myeloid stem cell.

Körmöczi *et al.* [651] carried out a thorough analysis of individuals with Rh mosaicism or antigen loss (or spontaneous Rh phenotype splitting as they called it), three of whom had haematological diseases. Five individuals presented a stable mixed-field agglutination pattern over a period of time, two exhibited progressively diminishing proportions of D+ red cells, and two showed complete D antigen loss throughout the observation period. One had a mixture of D+ Fy(b+) and D– Fy(b–) red cells. In individuals with mixtures of C or E, the D+ red cells were C+ or E+ and the D– cells C– or E–. Genotyping of erythroid colonies cultured from single erythroid progenitors indicated loss of one complete haplotype (*DCe* or *DcE*) in the D– fraction. Further analyses of microsatellites on different tissues, sorted blood cell subsets, and erythropoietic progenitors indicated myeloid lineage-restricted loss of heterozygosity of variable chromosome 1 stretches encompassing the *RHD* and *RHCE* loci on the short arm and, in one case, even including the Duffy gene on the long arm. In most cases, the loss of heterozygosity probably arose from homologous recombination between chromatids of chromosome 1 prior to mitosis, rather than deletion of substantial segments of the chromosome, explaining the lack of any palpable cytogenetic abnormality.

5.20 The RHAG blood group system

The Rh-associated glycoprotein (RhAG) has long been recognised as a homologue of RhD and RhCE, and member of the Rh protein family closely associated with the Rh proteins in the red cell membrane (Section 5.5.6). In 2010, the recognition that three red cell surface antigens were located on RhAG (Figure 5.7) and encoded by *RHAG* led to the establishment of a new blood group system, RHAG [76] (system 30) (Table 5.15).

5.20.1 Duclos (RHAG1) and DSLK (RHAG3)

Duclos is a high frequency antigen, previously 901013. Apart from the red cells of Mme Duclos, the sole maker of anti-Duclos, the Duclos antigen is lacking only from those Rh_{null} or Rh_{mod} red cells that are also U–. Mme Duclos had an apparently normal DCe/dce phenotype, but a U antigen slightly weaker than normal [660]. A monoclonal antibody, MB-2D10, raised to human red cells, showed a Duclos-like specificity by reacting with all cells except those of Rh_{null} U– and Rh_{mod} U– phenotypes [661]; it differed from the Duclos antibody by reacting

Table 5.15 Antigens of the RHAG blood group system.

Antigen			Molecular basis*		
No.	Name	Frequency	Nucleotides	Exon	Amino acids
RHAG1	Duclos	High	316C (G)	2	Gln106 (Glu)
RHAG2	Ol[a]	Low	680C>T	5	Ser227Leu
RHAG3†	DSLK	High	490A (C)	3	Lys164 (Gln)
RHAG4†		Low	808G>A, 861G>A	6	Val270Ile, Ala280Ala

*Molecular basis of antigen-negative phenotype in parentheses.
†Provisional assignment.

with the red cells of Mme Duclos [662]. Immunoblotting demonstrated that the MB-2D10 epitope is located on RhAG [663]. Sequencing of DNA from archived serum revealed that Mme Duclos was homozygous for an *RHAG* mutation encoding Gln106Glu in the second extracellular loop of RhAG [76] (Figure 5.7). Human embryonic kidney (HEK) cells transfected with *RHAG* reacted with alloanti-Duclos, whereas HEK cells transfected with *RHAG* containing the Duclos mutation, did not.

DSLK is serologically similar to Duclos [76]. Although DSLK– red cells reacted with anti-Duclos and MB-2D10, they had aberrant expression of U and anti-DSLK reacted with all other red cells, including Rh_{null} U+, except Rh_{null} U– cells. The only DSLK– individual was homozygous for a mutation encoding Lys164Gln in the third external loop of RhAG (Figure 5.7).

Although Duclos-negative and DSLK-negative red cells have apparently normal expression of Rh antigens and RhAG, they have a profound weakening of U, in common with most Rh_{null} and Rh_{mod} phenotype red cells. The precise nature of the U antigen is not known, but it is considered to be dependent on an interaction between glycophorin B (GPB) and RhAG because the main causes for absence or reduced expression of U are either absence or gross alteration of GPB or absence or reduced expression of RhAG (Section 3.6). The Duclos- and DSLK-associated substitutions suggest that the interaction of RhAG with GPB involves the second and third extracellular loops of RhAG, with the increase in negative electrostatic potential on the RhAG surface disrupting the interaction with GPB [76].

5.20.2 Ol^a (RHAG2)

Ol^a (previously 700043) is an antigen of very low frequency described in a three-generation Norwegian family. Ol(a+) red cells in this family have weakened D, C, and E antigens, but recombination between the genes governing Rh and Ol^a expression demonstrated that Ol^a could not belong to the Rh system [549]. Two Ol(a+) members of the family were heterozygous for an *RHAG* mutation encoding Ser227Leu in the proposed extracellular vestibule and a Japanese Rh_{mod} individual with strong expression of Ol^a was homozygous for the same mutation [76].

In a three-dimensional model of Ol(a+) RhAG in a trimer with two molecules of RhD, the substituted residue is within 5 Å of the interface between the RhAG and RhD subunits (Figure 5.7). It is possible that the Ser227Leu substitution disrupts the formation of the RhAG-Rh complex, resulting in less Rh protein in the membrane or in an alteration of the molecular surface that forms the Rh epitopes [76].

5.20.3 An antigen provisionally numbered RHAG4

An antibody to an antigen of very low frequency, which caused severe HDFN, reacted with the red cells of the affected baby's father and two of his half-siblings, with evidence of weakened Rh antigen expression. The father was homozygous for two *RHAG* mutations: one silent; the other encoding Val270Ile in the ninth membrane-spanning domain of RhAG. The baby and the two of his half-siblings of the father were heterozygous for those mutations [664]. Val270Ile combined with Gly280Arg were previously described in one Rh_{null} individual [504]. It is possible that the Val270Ile mutation causes the expression of a novel epitope, possibly through improper folding of RhAG or improper quaternary association of RhAG with RhD/RhCE proteins.

5.21 Development and distribution of Rh antigens and RhAG

Rh antigens are readily detected on cord red cells; no surprise considering the part they play in HDFN. D, C, c, E, and e antigens have been detected on fetal red cells at the eighth week of gestation [665].

Rh antigens appear to be erythroid-specific. No D, C, c, E, or e antigen could be detected on granulocytes, lymphocytes, monocytes, or platelets by radioimmunoassay and fluorescent flow cytometry [666,667]. There is no evidence of Rh antigens on cells of other tissues.

Although RhAG is also generally considered erythroid-specific, *RHAG* transcripts have been detected in oesophageal squamous epithelium [668]. RhBG and RhCG, homologues of RhAG, are present in kidney, liver, skin, and testis [80,92,93] and probably in most types of epithelia [668,669], with RhBG and RhCG mainly located on the basolateral and apical membranes, respectively [669,670].

5.22 Functional aspects of the Rh and RhAG proteins

The topology of the Rh proteins in the cell membrane – polytopic, with cytoplasmic N- and C-termini – is characteristic of membrane transporters. RhAG bears even closer resemblance to red cell membrane transporters, as

it has a single *N*-glycan on one of its extracellular loops. There is substantial evidence that RhAG functions as a gas transporter, although the prime substrates – ammonia (NH_3), ammonium (NH_4^-), nitric oxide (NO), carbon dioxide (CO_2), and/or oxygen (O_2) – remain controversial. On the other hand, the RhD and RhCE proteins almost certainly are not transporters and their functions remain obscure.

5.22.1 RhAG: NH_3/NH_4^+ transporter?

NH_4^+ may be friend or foe. It is utilised by prokaryotes and lower eukaryotes as a source of nitrogen, whereas in mammals it is toxic and must be metabolised and excreted. RhAG shares between 20 and 27% sequence identity with proteins of a family of ammonium transporters, the Amt proteins, ubiquitous in lower organisms, including bacteria and yeast, and in plants [671]. These proteins are polytopic and generally have 11 membrane-spanning domains and an extracellular N-terminus [672].

Yeast (*Saccharomyces cerevisae*) cells have three membrane NH_4^+transporters, Mep1, Mep2, Mep3. Yeast cells lacking all three Mep proteins (triple-*mepΔ*) fail to grow in low levels (5 m*M*) of NH_4^+, though any one of the Mep proteins can restore growth. The growth defect in triple-*mepΔ* yeast cells was repaired by transfection with cDNA representing *RHAG* or *RHCG* [93]. Furthermore, transfection of yeast cells with *RHAG* or *RHCG* cDNA conferred resistance to a toxic concentration of methylammonium (CH_3NH_2), suggesting that the human proteins are involved in the export of the NH_4^+analogue.

Similar expression studies in yeast [673], *Xenopus* oocytes [674], and human HeLa cells [675] also indicated that RhAG facilitated the transfer of NH_3 though the membrane, either directly or as charged NH_4^+ (possibly in exchange for H^+), or both. Experiments on human normal or Rh_{null} red cells or resealed ghosts, that is, with or without RhAG, suggested that RhAG can act as a transporter of NH_3/NH_4^+ in its native cell [676,677].

Three-dimensional structural models based on the crystal structures of bacterial *E. coli* AmtB and *Nitrosomas europaea* NeRh50 suggest that human RhAG, RhBG, and RhCG lack the NH_4^+ binding site of AmtB. They have conserved the 'phenylalanine gate', a pair of phenylalanine residues (Phe120 and Phe225 in RhAG) that would only permit small molecules such as NH_3, CO_2, H_2O, O_2, or NO, to enter and pass through the conductance pore. Also conserved is the twin-histidine motif in the pore (His175 and His334 in RhAG), which is essential for substrate conductance. RhAG, therefore, appears to have structural characteristics compatible with conductance of neutral gases, including NH_3, but possibly not of the charged NH_4^+ ion [50–53,678].

NH_3 and CH_3NH_2 transport was severely impaired in red cells of *Rhag*$^{-/-}$ (knockout) mice, but only slightly impaired in those of mice lacking the only Rh gene, *Rhd* [85].

Why would red cells require an NH_3 or NH_4^+ transporter? NH_4^+ concentration is three times higher in red cells than in plasma and it has been suggested that RhAG promotes retention of NH_4^+ in red cells for transport to the liver or kidney and subsequent removal from the body, thus protecting against NH_4^+ toxicity in the brain and other organs [93,679]. Alternatively, it could function to minimise red cell volume changes that could occur when the red cell passes in and out of the high NH_3 concentration of the renal medulla [675].

5.22.2 RhAG: CO_2/O_2/NO channel?

Although there can be little doubt that RhAG can transport NH_3, its function in human red cells remains in question. CO_2 and NH_3 are both gases that are readily hydrated: CO_2 to HCO_3^-; NH_3 to NH_4^+. Results of experiments on the green alga *Chlamydomonas reinhardtii*, which is dependent on CO_2 for photosynthesis, indicated that proteins of the Rh family might function as channels for CO_2. Expression of the algal gene *RH1* was upregulated and downregulated by increased and decreased ambient CO_2 concentration, respectively [680]. *C. reinhardtii* lines lacking *RH1* mRNA and Rh1 protein as a result of RNAi interference, grow slowly even in high CO_2 environments, presumably because they fail to equilibrate CO_2 rapidly [681]. Rh_{null} human red cells have significantly reduced CO_2 permeability compared with normal cells. This reduction is similar to that observed with AQP1-null [Co(a–b–)] red cells, suggesting that RhAG and AQP1 share responsibility for passage of CO_2 through the red cell membrane, together being responsible for at least 50% of CO_2 permeability of the red cell membrane [682] (for further discussion on AQP1, see Section 15.8).

The primary function of the red cell is the transport of respiratory gases, so it is logical that some of the most abundant proteins in the red cell membrane are involved in the rapid transfer of these gases in and out of the cell. From the results of experiments on band 3-deficient red cells, Bruce *et al.* [95] developed the concept of the band 3 metabolon, involving a macrocomplex of membrane proteins, including the anion exchanger band 3, the Rh proteins, and RhAG (Section 10.7), plus various cytosolic proteins, including carbonic anhydrase II (CAII) and

deoxyhaemoglobin. It is proposed that RhAG transfers CO_2 through the membrane into the red cell, where it is hydrated to HCO_3^- by CAII. The highly soluble HCO_3^- is then exported from the cell by band 3 in exchange for Cl^-. If, as appears most likely, RhAG were a relatively non-specific channel for neutral small molecules, it could also function as a channel for O_2 to and from haemoglobin in the area around the metabolon. Another function of red cells is storage of nitric oxide and regulation of vasodilation through its release. Consequently, transport of NO though the membrane represents another potential function of RhAG [52,95]. The discussion in the section above on the suitability of the structure of RhAG as a gas channel for NH_3 would apply equally to CO_2, O_2, and NO.

From a thorough analysis of the evolution of Amt and Rh genes and proteins, Huang and Peng [94] propose that Amt proteins, ancient ancestors of the Rh proteins, evolved as NH_3/NH_4^+ transporters in primitive organisms, but that subsequent duplication and divergence of the genes driven in response to new selection pressures, led to the development of the Rh family of proteins as CO_2 channels, which expanded in vertebrates as the Amt proteins disappeared. Thus, the ability of RhAG to transport NH_3/NH_4^+ could reflect an ancestral characteristic, as opposed to the physiological function of rapid transfer of CO_2 through the red cell membrane.

5.22.3 RhAG and cation transport

Evidence for RhAG as a pore for monovalent cations comes from the association of heterozygosity for *RHAG* mutations with overhydrated stomatocytosis, a disorder characterised by abnormally shaped red cells that leak cations (Na^+ and K^+) at a rate 20–40 times greater than normal [75]. The mutations responsible encode Ile61Arg and Phe65Ser, which are likely to cause opening of the RhAG pore.

5.22.4 RhD and RhCE

The roles of RhD and RhCE are not known, but it is fairly certain that they do not function as transporters. They lack the amino acids conserved in other Rh-family proteins, including RhAG, that are considered essential to gas transport. The pair of Phe residues of the 'phenylalanine gate' are replaced by Met118 and Phe223 in RhD and Met118 and Val223 in RhCE; the twin-His motif is replaced by Tyr173 and Phe332 in both proteins. They could, however, be involved in gas movement by increasing the surface area-to-volume ratio of red cells [681]. It is feasible that RhD and RhCE play a role in facilitating the assembly of the band 3 macrocomplex [52]. RhD, RhCE, and RhAG might serve as independent attachments to the cytoskeletal network through interaction between their C-terminal tails and the adapter protein ankyrin-R and play a part in maintaining the stability of the Rh complex in the membrane [100]. Red cells lacking RhD, RhCE, and RhAG (Rh_{null}) or lacking RhD and RhCE, but 33–38% of normal RhAG levels (Rh_{null} of the amorph type) have abnormally shaped red cells (Section 5.16.6), whereas those lacking just RhD (D–) or RhCE (D––) are of normal shape.

5.23 Evolutionary aspects

Rh genes probably evolved from Amt genes in prokaryotes. They are widely distributed in vertebrates and invertebrates, but are not present in plants. In vertebrates Rh genes diversified and the Rh family expanded to four common paralogous clusters, represented by *RH* (*RHCE* and *RHD*), *RHAG*, *RHBG*, and *RHCG* in humans [94]. The degree of homology between *RHAG* and homologous human and some non-human genes is shown in Table 5.16. *RHAG* has a higher level of homology with its slime mould homologue, than with the *RH* genes [683].

The ancestral *RH* genes were formed, almost certainly, by duplication of an ancestral *RHAG* (or *RH50*) gene. Analyses of the numbers of synonymous and

Table 5.16 Percentage of identity between human *RHAG* and other genes of the human Rh family and *RHAG* homologues in other species [80].

Species	Gene	% identity to *RHAG*
Homo sapiens (man)	*RHAG*	100.0
	RHCG	50.9
	RHBG	49.9
	RHCE	33.0
	RHD	32.8
Mus musculus (mouse)	*Rhag*	76.5
Danio rerio (zebrafish)	*Rhg*	43.0
Drosophila melanogaster (fruit fly)	*Rhp*	41.4
Caenorhabditis elegans (nematode)	*Rhp-1*	35.9
	Rhp-2	43.0
Geodia cydonium (marine sponge)	*Rhg*	41.8
Dictyostelium discoideum (slime mould)	*RhgA*	34.5

non-synonymous substitutions in the *RH* and *RHAG* genes in man, macaque, mouse, and rat suggested that Darwinian selection had acted on both genes, but that *RHAG* is more conserved than the *RH* genes, having evolved 2–3 times more slowly [684,685]. This suggests that *RHAG* has greater functional significance than *RH*. Duplication of an *RHAG*-like gene to form an ancestral *RH* gene is estimated to have occurred around 510 million years ago, before divergence of jawless fish and jawed vertebrates [686]. *RH* gene homologues have been detected in all mammals studied [687].

Rh-related mRNA transcripts were isolated from the bone marrow of chimpanzee, gorilla, gibbon, crab-eating macaque (*Macaca fascicularis*), and rhesus monkey (*M. mulatta*), by reverse-transcriptase PCR using primers designed from the sequence of human *RH* genes [319,688]. The cDNA sequences demonstrated a high degree of homology to the human sequence and predicted proteins of 417 amino acids. Like most humans, chimpanzees and gorillas have at least two *RH* genes, although some chimpanzees have three or four genes; other primates, including orangutans and gibbons, old world and new world monkeys, and prosimians, have only one *RH* gene per haploid genome [689,690]. Consequently, the duplication of the ancestral *RH* gene that led to the evolution of *RHCE* and *RHD* in man must have occurred in the common ancestor of humans, chimpanzees, and gorillas, between 8 and 11 million years ago [691,692]. Based on the gene positions and orientation, *RHCE* appears to represent the ancestral gene [693].

A series of events are predicted to have generated the present Rh haplotypes [65,691]. Duplication of an ancestral *RHCE*, followed by divergence resulting from mutations and complex recombination events, generated genes resembling *RHD* and *RHCE*ce*. *Dce*, therefore, is the root of the human Rh system (Figure 5.12). Deletion or inactivation of *RHD* then created *dce*, non-reciprocal recombination of *RHD* sequences in the exon 2 region into *RHCE*ce* could have produced *DCe*, and a point mutation in the *RHCE*ce* would have produced *DcE*. In harmony with the thesis of Fisher and Race [694] from 1946, Carritt *et al*. [691] proposed that *dCe* arose from recombination between *DCe* and *dce*, *dcE* from recombination between *DcE* and *dce*, and *DCE* from recombination between *DCe* and *DcE*. The very rare haplotype *dCE* must have arisen from recombination between the uncommon haplotypes, *dCE* and *dcE*.

Deletion of *RHD*, to produce the D– haplotype common in Europe, must have occurred after duplication and diversification of the *RH* genes [70]. Selection in favour of a population in which both D+ and D– phenotypes are common is difficult to understand, considering the part they play in HDFN. Analysis of genomic data provided no evidence to support any effects of selection on the *RHD* deletion [695]. Anstee [696] has suggested that genetic drift and migration, rather than natural selection, could provide an explanation, with mixing of two populations, one the essentially D– Palaeolithic people from the Basque region and the other D+ Neolithic migrants [14]. The effect of earlier selective pressures in Africa cannot be dismissed, however, considering that three relatively common genetic mechanisms exist in Africa for deletion or inactivation of *RHD* (Section 5.6.1).

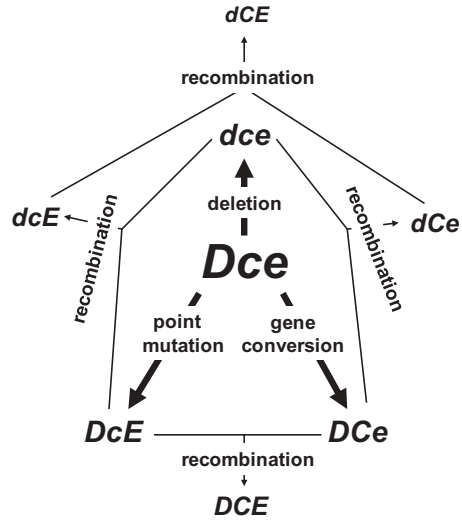

Figure 5.12 Scheme to show the proposed derivation of the eight Rh haplotypes from the ancestral haplotype, *Dce* (after [691]). The haplotype *dce* resulted from deletion of *RHD*, *DcE* from a point mutation within *RHCE*, and *DCe* from a gene conversion event between *RHD* and *RHCE*. The less common haplotypes *dcE*, *dCe*, and *DCE*, then arose from recombination events involving *dce*, *DcE*, and *DCe*. The very rare haplotype *dCE* resulted from recombination between *dcE* and *dCe*.

References

1 Levine P, Stetson RE. An unusual case of intra-group agglutination. *J Am Med Ass* 1939;113:126–127.
2 Landsteiner K, Wiener AS. An agglutinable factor in human blood recognized by immune sera for Rhesus blood. *Proc Soc Exp Biol NY* 1940;43:223.

3 Landsteiner K, Wiener AS. Studies on an agglutinogen (Rh) in human blood reacting with anti-rhesus sera and with human isoantibodies. *J Exp Med* 1941;74:309–320.
4 Wiener AS, Peters HR. Hemolytic reactions following transfusions of blood of the homologous group, with three cases in which the same agglutinogen was responsible. *Ann Int Med* 1940;13:2306–2322.
5 Levine P, Burnham L, Katzin EM, Vogel P. The role of isoimmunization in the pathogenesis of erythroblastosis fetalis. *Am J Obst Gynecol* 1941;42:925–937.
6 Fisk RT, Foord AG. Observations on the Rh agglutinogen of human blood. *Am J Clin Path* 1942;12:545.
7 Levine P, Celano MJ, Wallace J, Sanger R. A human 'D-like' antibody. *Nature* 1963;198:596–597.
8 Race RR, Taylor GL, Cappell DF, McFarlane MN. Recognition of a further common Rh genotype in man. *Nature* 1944;153:52–53.
9 Wiener AS. Genetic theory of the Rh blood types. *Proc Soc Exp Biol Med* 1943;54:316–319.
10 Levine P, Katzin EM, Burnham L. Isoimmunization in pregnancy. Its possible bearing on the etiology of erythroblastosis foetalis. *J Am Med Ass* 1941;116:825–827.
11 Levine P, Vogel P, Katzin EM, Burnham L. Pathogenesis of erythroblastosis fetalis: statistical evidence. *Science* 1941;94:371–372.
12 Fisher RA. Cited by Race RR. An 'incomplete' antibody in human serum. *Nature* 1944;153:771–772.
13 Race RR, Mourant AE, Lawler SD, Sanger R. The Rh chromosome frequencies in England. *Blood* 1948;3:689–695.
14 Mourant AE, Kopec AC, Domaniewska-Sobczak K. *The Distribution of the Human Blood Groups and Other Polymorphisms*, 2nd edn. London: Oxford University Press, 1976.
15 Mackay JF, Wang JC, Wong KS. The incidence of blood groups in 4648 southern Chinese from Toishan district and its vicinity. *J Hong Kong Med Tech Assoc* 1969;1:11–14.
16 Mourant AE. A new Rhesus antibody. *Nature* 1945;155:542.
17 Van den Bosch C. The very rare Rh genotype *Ryr* (*CdE/cde*) in a case of erythroblastosis fœtalis. *Nature* 1948;162:781.
18 Rosenfield RE, Allen FH, Swisher SN, Kochwa S. A review of Rh serology and presentation of a new terminology. *Transfusion* 1962;2:287–312.
19 Tippett P. A speculative model for the Rh blood groups. *Ann Hum Genet* 1986;50:241–247.
20 Tills D, Kopec AC, Tills RE. *The Distribution of the Human Blood Groups and Other Polymorphisms*, Suppl. 1. Oxford: Oxford University Press, 1983.
21 Green FA. Studies of the Rh(D) antigen. *Vox Sang* 1965;10:32–53.
22 Green FA. Erythrocyte membrane sulfhydryl groups and Rh antigen activity. *Immunochemistry* 1967;4:247–257.
23 Green FA. Erythrocyte membrane lipids and Rh antigen activity. *J Biol Chem* 1972;247:881–887.
24 Lorusso DJ, Green FA. Reconstitution of Rh(D) antigen activity from human erythrocyte membranes solubilized by deoxycholate. *Science* 1975;188:66–67.
25 Lorusso DJ, Binette JP, Green FA. The Rh antigen system and disaggregated human erythrocyte membranes. *Immunochemistry* 1977;14:503–508.
26 Moore S, Woodrow CF, McClelland DBL. Isolation of membrane components associated with human red cell antigens Rh(D), (c), (E) and Fy^a. *Nature* 1982;295:529–531.
27 Gahmberg CG. Molecular identification of the human Rh_o(D) antigen. *FEBS Letts* 1982;140:93–97.
28 Ridgwell K, Roberts J, Tanner MJA, Anstee DJ. Absence of two membrane proteins containing extracellular thiol groups in Rh_{null} human erythrocytes. *Biochem J* 1983;213:267–269.
29 Bloy C, Blanchard D, Lambin P, *et al*. Human monoclonal antibody against Rh(D) antigen: partial characterization of the Rh(D) polypeptide from human erythrocytes. *Blood* 1987;69:1491–1497.
30 Moore S, Green C. The identification of specific Rhesus-polypeptide–blood-group-ABH-active-glycoprotein complexes in the human red-cell membrane. *Biochem J* 1987;244:735–741.
31 Gahmberg CG. Molecular characterization of the human red cell Rh_o(D) antigen. *EMBO J* 1983;2:223–227.
32 de Vetten MP, Agre P. The Rh polypeptide is a major fatty acid-acylated erythrocyte membrane protein. *J Biol Chem* 1988;263:18193–18196.
33 Hartel-Schenk S, Agre P. Mammalian red cell membrane Rh polypeptides are selectively palmitoylated subunits of a macromolecular complex. *J Biol Chem* 1992;267:5569–5574.
34 Bloy C, Blanchard D, Lambin P, *et al*. Characterization of the D, c, E and G antigens of the Rh blood group system with human monoclonal antibodies. *Mol Immunol* 1988;25:925–930.
35 Krahmer M, Prohaska R. Characterization of human red cell Rh (Rhesus-)specific polypeptides by limited proteolysis. *FEBS Letts* 1987;226:105–108.
36 Saboori AM, Smith BL, Agre P. Polymorphism in the M_r 32,000 Rh protein purified from Rh(D)-positive and -negative erythrocytes. *Proc Natl Acad Sci USA* 1988;85:4042–4045.
37 Bloy C, Blanchard D, Dahr W, *et al*. Determination of the N-terminal sequence of human red cell Rh(D) polypeptide and demonstration the the Rh(D), (c), and (E) antigens are carried by distinct polypeptide chains. *Blood* 1988;72:661–666.
38 Blanchard D, Bloy C, Hermand P, *et al*. Two-dimensional iodopeptide mapping demonstrates that erythrocyte Rh D, c, and E polypeptides are structurally homologous but nonidentical. *Blood* 1988;72:1424–1427.
39 Avent ND, Ridgwell K, Mawby WJ, *et al*. Protein-sequence studies on Rh-related polypeptides suggest the presence of

at least two groups of proteins which associate in the human red-cell membrane. *Biochem J* 1988;256:1043–1046.
40 Avent ND, Ridgwell K, Tanner MJA, Anstee DJ. cDNA cloning of a 30 kDa erythrocyte membrane protein associated with Rh (Rhesus)-blood-group-antigen expression. *Biochem J* 1990;271:821–825.
41 Chérif-Zahar B, Bloy C, Le Van Kim C, *et al*. Molecular cloning and protein structure of a human blood group Rh polypeptide. *Proc Natl Acad Sci USA* 1990;87:6243–6247.
42 Hermand P, Mouro I, Huet M, *et al*. Immunochemical characterization of Rhesus proteins with antibodies raised against synthetic peptides. *Blood* 1993;82:669–676.
43 Mouro I, Colin Y, Chérif-Zahar B, Cartron J-P, Le Van Kim C. Molecular genetic basis of the human Rhesus blood group system. *Nature Genet* 1993;5:62–65.
44 Le Van Kim C, Mouro I, Chérif-Zahar B, *et al*. Molecular cloning and primary structure of the human blood group RhD polypeptide. *Proc Natl Acad Sci USA* 1992;89:10925–10929.
45 Kajii E, Umenishi F, Iwamoto S, Ikemoto S. Isolation of a new cDNA clone encoding an Rh polypeptide associated with the Rh blood group system. *Hum Genet* 1993;91:157–162.
46 Arce MA, Thompson ES, Wagner S, *et al*. Molecular cloning of RhD cDNA derived from a gene present in RhD-positive, but not RhD-negative individuals. *Blood* 1993;82:651–655.
47 Simsek S, de Jong CAM, Cuijpers HTM, *et al*. Sequence analysis of cDNA derived from reticulocyte mRNAs coding for Rh polypeptides and demonstration of E/e and C/c polymorphisms. *Vox Sang* 1994;67:203–209.
48 Avent ND, Butcher SK, Liu W, *et al*. Localization of the C termini of the Rh (Rhesus) polypeptides to the cytoplasmic face of the human erythrocyte membrane. *J Biol Chem* 1992;267:15134–15139.
49 Eyers SAC, Ridgwell K, Mawby WJ, Tanner MJA. Topology and organization of human Rh (Rhesus) blood group-related polypeptides. *J Biol Chem* 1994;269:6417–6423.
50 Conroy MJ, Bullough PA, Merrick M, Avent ND. Modelling the human rhesus proteins: implications for structure and function. *Br J Haematol* 2005;131:543–551.
51 Callebaut I, Dulin F, Bertrand O, *et al*. Hydrophobic cluster analysis and modeling of the human Rh protein three-dimensional structures. *Transfus Clin Biol* 2006;13:70–84.
52 Burton NM, Anstee DJ. Nature, function and significance of Rh proteins in red cells. *Curr Opin Hematol* 2008;15:625–630.
53 Gruswitz F, Chaudhary S, Ho JD, *et al*. Function of human Rh based on structure of RhCG at 2.1 Å. *Proc Natl Acad Sci USA* 2010;107:9638–9643.
54 Burton NM, Daniels G. Structural modelling of red cell surface proteins. *Vox Sang* 2011;100:129–139.
55 Flegel WA. Molecular genetics and clinical applications for RH. *Transfus Apher Sci* 2011;44:81–91.
56 Avent ND. New insight into the Rh system: structure and function. *ISBT Sci Ser* 2007;2:35–43.
57 Smythe JS, Anstee DJ. The role of palmitoylation in RhD expression. *Transfus Med* 2000;10(Suppl. 1):30 [Abstract].
58 Conboy J, Mohandas N, Tchernia G, Kan YW. Molecular basis of hereditary elliptocytosis due to protein 4.1 deficiency. *New Engl J Med* 1986;315:680–685.
59 McGuire M, Smith BL, Agre P. Distinct variants of erythrocyte protein 4.1 inherited in linkage with elliptocytosis and Rh type in three white families. *Blood* 1988;72:287–293.
60 Ruddle F, Ricciuti F, McMorris FA, *et al*. Somatic cell genetic assignment of peptidase C and the Rh linkage group to chromosome A-1 in man. *Science* 1972;176:1429–1431.
61 Chérif-Zahar B, Mattéi MG, Le Van Kim C, *et al*. Localization of the human Rh blood group gene structure to chromosome region 1p34.3-1p36.1 by in situ hybridization. *Hum Genet* 1991;86:398–400.
62 Suto Y, Ishikawa Y, Hyodo H, Uchikawa M, Juji T. Gene organization and rearrangements at the human Rhesus blood group locus revealed by fiber-FISH analysis. *Hum Genet* 2000;106:164–171.
63 Chérif-Zahar B, Le Van Kim C, Rouillac C, *et al*. Organization of the gene (*RHCE*) encoding the human blood group RhCcEe antigens and characterization of the promoter region. *Genomics* 1994;19:68–74.
64 Chérif-Zahar B, Raynal V, Cartron J-P. RH gene structure: reassignment of two exon-exon junctions. *Blood* 1997;89:4661–4662.
65 Okuda H, Suganuma H, Kamesaki T, *et al*. The analysis of nucleotide substitutions, gaps, and recombination events between *RHD* and *RHCE* through complete sequencing. *Biochem Biophys Res Comm* 2000;274:670–683.
66 Avent ND, Martin PG, Armstrong-Fisher S, *et al*. Evidence of genetic diversity underlying Rh D^{-}, weak D (D^{u}), and partial D phenotypes as determined by multiplex polymerase chain reaction analysis of the *RHD* gene. *Blood* 1997;89:2568–2577.
67 Okuda H, Kawano M, Iwamoto S, *et al*. The *RHD* gene is highly detectable in RhD-negative Japanese donors. *J Clin Invest* 1997;100:373–379.
68 Suganuma H, Kumada M, Omi T, *et al*. Aly/REF, a factor for mRNA transport, activates *RH* gene promoter function. *FEBS J* 2005;272:2696–2704.
69 Denomme GA, Wang D, Matheson KA, Titolo D. The proximal cis-regulatory region of the *RHD/RHCE* promoter is 105 bp and contains a 55-bp core devoid of known binding motifs but necessary for transcription. *Transfusion* 2009;49:1361–1369.
70 Wagner FF, Flegel WA. *RHD* gene deletion occurred in the *Rhesus box*. *Blood* 2000;95:3662–3668.
71 Matassi G, Chérif-Zahar B, Mouro I, Cartron J-P. Characterization of the recombination hot spot involved in the genomic rearrangement leading to the hybrid *D–CE–D*

gene in the D^{VI} phneotype. *Am J Hum Genet* 1997;60: 808–817.
72 Avent N, Judson PA, Parsons SF, *et al*. Monoclonal antibodies that recognize different membrane proteins that are deficient in Rh_{null} human erythrocytes. *Biochem J* 1988;251:499–505.
73 Ridgwell K, Eyers SAC, Mawby WJ, Anstee DJ, Tanner MJA. Studies on the glycoprotein associated with Rh (Rhesus) blood group antigen expression in the human red blood cell membrane. *J Biol Chem* 1994;269:6410–6416.
74 Ridgwell K, Spurr NK, Laguda B, *et al*. Isolation of cDNA clones for a 50 kDa glycoprotein of the human erythrocyte membrane associated with Rh (Rhesus) blood-group antigen expression. *Biochem J* 1992;287:223–228.
75 Bruce LJ, Guizouarn H, Burton NM, *et al*. The monovalent cation leak in overhydrated stomatocytic red blood cells results from amino acid substitutions in the Rh-associated glycoprotein. *Blood* 2009;113:1350–1357.
76 Tilley L, Green C, Poole J, *et al*. A new blood group system, RHAG: three antigens resulting from amino acid substitutions in the Rh-associated glycoprotein. *Vox Sang* 2010;98: 151–159.
77 Silvy M, Chapel-Fernandes S, Callebaut I, *et al*. Characterization of novel *RHD* alleles: relationship between phenotype, genotype, and trimeric architecture. *Transfusion* 2012; 52:2020–2069.
78 Matassi G, Chérif-Zahar B, Raynal V, Rouger P, Cartron JP. Organization of the human *RH50A* gene (RHAG) and evolution of base composition of the RH gene family. *Genomics* 1998;47:286–293.
79 Huang C-H. The human Rh50 glycoprotein gene. *J Biol Chem* 1998;273:2207–2213.
80 Liu Z, Chen Y, Mo R, *et al*. Characterization of human RhCG and mouse Rhcg as novel nonerythroid Rh glycoprotein homologues predominantly expressed in kidney and testis. *J Biol Chem* 2000;275:25641–25651.
81 Iwamoto S, Omi T, Yamasaki M, *et al*. Identification of 5′ flanking sequence of *RH50* gene and the core region for erythroid-specific expression. *Biochem Biophys Res Comm* 1998;243:233–240.
82 Iwamoto S, Suganuma H, Kamesaki T, *et al*. Cloning and characterization of erythroid-specific DNase I-hypersensitive site in human Rhesus-associated glycoprotein gene. *J Biol Chem* 2000;275:27324–27331.
83 Chérif-Zahar B, Raynal V, Gane P, *et al*. Candidate gene acting as a suppressor of the *RH* locus in most cases of Rh-deficiency. *Nature Genet* 1996;12:168–173.
84 Satchwell TJ, Bell AJ, Pellegrin S, *et al*. Critical band 3 multiprotein complex interactions establish early during human erythropoiesis. *Blood* 2011;118:182–191.
85 Goossens D, Trinh-Trang-Tan M-M, Debbia M, *et al*. Generation and characterisation of *Rhd* and *Rhag* null mice. *Br J Haematol* 2010;148:161–172
86 Smythe JS, Avent ND, Judson PA, *et al*. Expression of *RHD* and *RHCE* gene products using retroviral transduction of K562 cells establishes the molecular basis of Rh blood group antigens. *Blood* 1996;87:2968–2973.
87 Iwamoto S, Yamasaki M, Kawano M, *et al*. Expression analysis of human rhesus blood group antigens by gene transduction into erythroid and non-erythroid cells. *Int J Hematol* 1998;68:257–268.
88 Mouro-Chanteloup I, D-Ambrosio AM, Gane P, *et al*. Cell-surface expression of RhD blood group polypeptide is post-transcriptionally regulated by the RhAG glycoprotein. *Blood* 2002;100:1038–1047.
89 Southcott MJG, Tanner MJA, Anstee DJ. The expression of human blood group antigens during erythropoiesis in a cell culture system. *Blood* 1999;93:4425–4435.
90 Bony V, Gane P, Bailly P, Cartron J-P. Time-course expression of polypeptides carrying blood group antigens during human erythroid differentiation. *Br J Haematol* 1999;107: 263–274.
91 Daniels G, Green C. Expression of red cell surface antigens during erythropoiesis. *Vox Sang* 2000;78(Suppl. 2): 149–153.
92 Liu Z, Peng J, Mo R, Hui C, Huang C-H. Rh type B glycoprotein is a new member of the Rh superfamily and a putative ammonia transporter in mammals. *J Biol Chem* 2001;276:1424–1433.
93 Marini A-M, Matassi G, Raynal V, *et al*. The human Rhesus-associated RhAG protein and a kidney homologue promote ammonium transport in yeast. *Nature Genet* 2000;26: 341–344.
94 Huang C-H, Peng J. Evolutionary conservation and diversification of Rh family genes and protein. *Proc Natl Acad Sci USA* 2005;102:15512–15517.
95 Bruce LJ, Beckmann R, Ribeiro ML, *et al*. A band 3-based macrocomplex of integral and peripheral proteins in the RBC membrane. *Blood* 2003;101:4180–4188.
96 Beckman R, Smythe JS, Anstee DJ, Tanner MJA. Functional cell surface expression of band 3, the human red blood cell anion exchange protein (AE1), in K562 erythroleukemia cells: band 3 enhances the cell surface reactivity of Rh antigens. *Blood* 1998;92:4428–4438.
97 Beckmann R, Smythe JS, Anstee DJ, Tanner MJA. Coexpression of band 3 mutants and Rh polypeptides: differential effects of band 3 on the expression of the Rh complex containing D polypeptide and the Rh complex containing CcEe polypeptide. *Blood* 2001;97:2496–2505.
98 Mouro-Chanteloup I, Delaunay J, Gane P, *et al*. Evidence that the red cell skeleton protein 4.2 interacts with the Rh membrane complex member CD47. *Blood* 2003;101: 338–344.
99 Bruce LJ, Ghosh S, King MJ, *et al*. Absence of CD47 in protein 4.2-deficient hereditary spherocytosis in man: an interaction between the Rh complex and the band 3 complex. *Blood* 2002;100:1878–1885.

100 Nicolas V, Mouro-Chanteloup I, Lopez C, *et al*. Functional interactions between Rh proteins and the spectrin-based skeleton in erythroid and epithelial cells. *Transfus Clin Biol* 2006;13:23–28.

101 Salomao M, Zhang X, Yang Y, *et al*. Protein 4.1R-dependent multiprotein complex: new insights into the structural organization of the red blood cell membrane. *Proc Natl Acad Sci USA* 2008;105:8026–8031.

102 Bloy C, Blanchard D, Hermand P, *et al*. Properties of the blood group LW glycoprotein and preliminary comparison with Rh proteins. *Mol Immunol* 1989;26:1013–1019.

103 Dahr W, Kordowicz M, Moulds J, *et al*. Characterization of the Ss sialoglycoprotein and its antigens in Rh_{null} erythrocytes. *Blut* 1987;54:13–24.

104 Hsu K, Lee T-Y, Chao H-P, *et al*. The expression of the Rh/RhAG complex is reduced in Mi.III erythrocytes. *Vox Sang* 2012;102:221–227.

105 Lindberg FP, Gresham HD, Schwarz E, Brown EJ. Molecular cloning of integrin-associated protein: an immunoglobulin family member with multiple membrane-spanning domains implicated in $\alpha_v\beta_3$-dependent ligand binding. *J Cell Biol* 1993;123:485–496.

106 Lindberg FP, Lublin DM, Telen MJ, *et al*. Rh-related antigen CD47 is the signal-transducer integrin-associated protein. *J Biol Chem* 1994;269:1567–1570.

107 Miller YE, Daniels GL, Jones C, Palmer DK. Identification of a cell-surface antigen produced by a gene on human chromosome 3(cen-q22) and not expressed by Rh_{null} cells. *Am J Hum Genet* 1987;41:1061–1070.

108 Jeremy KP, Plummer ZE, Head DJ, *et al*. 4.1R-deficient human red blood cells have altered phosphatidylserine exposure pathways and are deficient in CD44 and CD47 glycoproteins. *Haematologica* 2009;94:1354–1361.

109 Olsson M, Nilsson A, Oldenborg PA. Target cell CD47 regulates macrophage activation and erythrophagocytosis. *Transfus Clin Biol* 2006;13:39–43.

110 Stewart A, Urbaniak S, Turner M, Bessos H. The application of a new quantitative assay for the monitoring of integrin-associated protein CD47 on red blood cells during storage and comparison with the expression of CD47 and phosphatidylserine with flow cytometry. *Transfusion* 2005;45:1496–1503.

111 Burger P, Hilarius-Stokman P, de Korte D, van den Berg TK, van Bruggen R. CD47 functions as a molecular switch for erythrocyte phagocytosis. *Blood* 2012;119:5512–5521.

112 Klein HK, Anstee DJ. *Mollison's Blood Transfusion in Clinical Medicine*, 11th edn. Oxford: Blackwell Publishing, 2005.

113 Frohn C, Dümbgen L, Brand J-M, *et al*. Probability of anti-D development in D– patients receiving D+ RBCs. *Transfusion* 2003;43:893–898.

114 Yazer MH, Triulzi DJ. Detection of anti-D in D– recipients transfused with D+ red blood cells. *Transfusion* 2007;47:2197–2201.

115 Gonzalez-Porras JR, Graciani IF, Perez-Simon JA, *et al*. Prospective evaluation of a transfusion policy of D+ red blood cells into D– patients. *Transfusion* 2008;48:1318–1324.

116 Mak KH, Yan KF, Cheng SS, Yuen MY. Rh phenotypes of Chinese blood donors in Hong Kong, with special reference to weak D antigens. *Transfusion* 1993;33:348–351.

117 Levine P, Celano M, Lange S, Stroup M. The influence of gene interaction on dosage effects with complete anti-D sera. *Vox Sang* 1959;4:33–39.

118 Masouredis SP, Chi CA, Ferguson E. Relationship between $Rho_{(}D)$ genotype and quantity of I^{131} anti-$Rh_{o}(D)$ bound to red cells. *J Clin Invest* 1960;39:1450–1462.

119 Silber R, Gibbs MB, Jahn EF, Akeroyd JH. Quantitative hemagglutination studies in the Rh blood group system. II. A study of the D (Rh_o) agglutinogen. *Blood* 1961;17:291–301.

120 Gibbs MB, Rosenfield RE. Immunochemical studies of the Rh system. IV. Hemagglutination assay of antigenic expression regulated by interaction between paired Rh genes. *Transfusion* 1966;6:462–474.

121 Araszkiewicz P, Szymanski IO. Quantitative studies on the Rh-antigen D. Effect of the C gene. *Transfusion* 1987;27:257–261.

122 McGann PT, Despotovic JM, Howard TA, Ware RE. A novel laboratory technique demonstrating the influences of *RHD* zygosity and the RhCcEe phenotype on erythrocyte D antigen expression. *Am J Hematol* 2012;87:266–271.

123 Hughes-Jones NC, Bloy C, Gorick B, *et al*. Evidence that the c, D and E epitopes of the human Rh blood group system are on separate polypeptide molecules. *Molec Immunol* 1988;25:931–936.

124 van Bockstaele DR, Berneman ZN, Muylle L, Cole-Dergent J, Peetermans ME. Flow cytometric analysis of erythrocytic D antigen density profile. *Vox Sang* 1986;51:40–46.

125 Nicholson G, Lawrence A, Ala FA, Bird GWG. Semi-quantitative assay of D antigen site density by flow cytometric analysis. *Transfus Med* 1991;1:87–90.

126 The Rhesus web site. http://www.uni-ulm.de/~fwagner/RH/RB (last accessed 14 October 2012).

127 Colin Y, Chérif-Zahar B, Le Van Kim C, *et al*. Genetic basis of the RhD-positive and RhD-negative blood group polymorphism as determined by Southern analysis. *Blood* 1991;78:2747–2752.

128 Singleton BK, Green CA, Avent ND, *et al*. The presence of an *RHD* pseudogene containing a 37 base pair duplication and a nonsense mutation in most Africans with the Rh D-negative blood group phenotype. *Blood* 2000;95:12–18.

129 Blunt T, Daniels G, Carritt B. Serotype switching in a partially deleted RHD gene. *Vox Sang* 1994;67:397–401.

130 Faas BHW, Beckers EAM, Wildoer P, *et al*. Molecular background of VS and weak C expression in blacks. *Transfusion* 1997;37:38–44.

131 Daniels GL, Faas BHW, Green CA, *et al*. The Rh VS and V blood group polymorphisms in Africans: a serological and molecular analysis. *Transfusion* 1998;38:951–958.
132 Pham B-N, Peyrard T, Juszczak G, *et al*. Heterogeneous molecular background of the weak C, VS+, hr^{B}–, Hr^{B}– phenotype in black persons. *Transfusion* 2009;49:495–504.
133 Wagner FF, Moulds JM, Tounkara A, Kouriba B, Flegel WA. *RHD* allele distribution in Africans of Mali. *BMC Genet* 2003;4:14.
134 Fukumori Y, Hori Y, Ohnoki S, *et al*. Further analysis of De_l (D-elute) using polymerase chain reaction (PCR) with *RHD* gene-specific primers. *Transfus Med* 1997;7:227–231.
135 Sun C-F, Chou C-S, Lai N-C, Wang W-T. RHD gene polymorphisms among RhD-negative Chinese in Taiwan. *Vox Sang* 1998;75:52–57.
136 Shao C-P, Maas J-H, Su Y-Q, Köhler M, Legler TJ. Molecular background of Rh D-positive, D-negative, D_{el} and weak D phenotypes in Chinese. *Vox Sang* 2002;83:156–161.
137 Qun X, Grootkerk-Tax MGHM, Maaskant-van Wijk PA, van der Schoot CE. Systemic analysis and zygosity determination of the *RHD* gene in a D-negative Chinese Han population reveals a novel D-negative *RHD* gene. *Vox Sang* 2005;88:35–40.
138 Kim JK, Kim SY, Kim C-A, Yon GS, Park SS. Molecular characterization of D– Korean persons: development of a diagnostic strategy. *Transfusion* 2005;45:345–352.
139 Wagner FF, Frohmajer A, Flegel WA. *RHD* positive haplotypes in D negative Europeans. *BMC Genet* 2001;2:10.
140 Stratton F. A new Rh allelomorph. *Nature* 1946;158: 25–26.
141 Bush M, Sabo B, Stroup M, Masouredis SP. Red cell D antigen sites and titration scores in a family with weak and normal D^{u} phenotypes inherited from a homozygous D^{u} mother. *Transfusion* 1974;14:433–439.
142 Cunningham NA, Zola AP, Hui HL, Taylor LM, Green FA. Binding characteristics of anti $Rho_{(}D)$ antibodies to $Rh_{o}(D)$-positive and D^{u} red cells. *Blood* 1985;66:765–768.
143 Szymanski IO, Araszkiewicz P. Quantitative studies on the D antigen of red cells with the D^{u} phenotype. *Transfusion* 1989;29:103–105.
144 Merry AH, Hodson C, Moore S. Variation in the level of Rh(D) antigen expression. *Transfusion* 1988;28:397–398.
145 Gorick B, McDougall DCJ, Ouwehand WH, *et al*. Quantitation of D sites on selected 'weak D' and 'partial D' red cells. *Vox Sang* 1993;65:136–140.
146 Agre PC, Davies DM, Issitt PD, *et al*. A proposal to standardize terminology for weak D antigen. *Transfusion* 1992;32:86–87.
147 Contreras M, Knight RC. Controversies in transfusion medicine. Testing for D^{u}: Con. *Transfusion* 1991;31: 270–272.
148 Argall CI, Ball JM, Trentelman E. Presence of anti-D antibody in the serum of a D^{u} patient. *J Lab Clin Med* 1953;41:895–898.
149 Wagner FF, Gassner C, Müller TH, *et al*. Molecular basis of weak D phenotypes. *Blood* 1999;93:385–393.
150 Wagner FF, Frohmajer A, Ladewig B, *et al*. Weak D alleles express distinct phenotypes. *Blood* 2000;95:2699–2708.
151 McGann H, Wenk RE. Alloimmunization to the D antigen by a patient with weak D type 21. *Immunohematology* 2010;26:27–29.
152 Yu X, Wagner FF, Witter B, Flegel WA. Outliers in RhD membrane integration are explained by variant *RH* haplotypes. *Transfusion* 2006;46:1343–1351.
153 Hemker MB, Ligthart PC, Berger L, *et al*. *DAR,* a new RhD variant involving exons 4, 5, and 7, often in linkage with *ceAR,* a new Rhce variant frequently found in African blacks. *Blood* 1999;94:4337–4342.
154 Daniels G, Poole G, Poole J. Partial D and weak D: can they be distinguished? *Transfus Med* 2007;17:145–146.
155 Tippett P, Sanger R. Observations on subdivisions of the Rh antigen D. *Vox Sang* 1962;7:9–13.
156 Tippett P, Sanger R. Further observations on subdivisions of the Rh antigen D. *Ärztl Lab* 1977;23:476–480.
157 Tippett P. Rh blood group system: the D antigen and high- and low-frequency Rh antigens. In: Vengelen-Tyler V, Pierce S, eds. *Blood Group Systems: Rh*. Arlington: American Association of Blood Banks, 1987:25–53.
158 Tippett P, Lomas-Francis C, Wallace M. The Rh antigen D: partial D antigens and associated low incidence antigens. *Vox Sang* 1996;70:123–131.
159 Avent ND, Jones JW, Liu W, *et al*. Molecular basis of the D variant phenotypes DNU and D^{II} allows localization of critical amino acids required for expression of Rh D epitopes epD3, 4 and 9 to the sixth external domain of the Rh D protein. *Br J Haematol* 1997;97:366–371.
160 Reid ME, Storry JR, Sausais L, *et al*. DAK, a new low-incidence antigen in the Rh blood group system. *Transfusion* 2003;43:1394–1397.
161 Huang C-H, Chen Y, Reid M. Human D^{IIIa} erythrocytes: RhD protein is associated with multiple dispersed amino acid variations. *Am J Hematol* 1997;55:139–145.
162 Rouillac C, Le Van Kim C, Blancher A, *et al*. Lack of G blood group antigen in D^{IIIb} erythrocytes is associated with segmental DNA exchange between *RH* genes. *Br J Haematol* 1995;89:424–426.
163 Grootkerk-Tax MGHM, van Wintershoven JD, Ligthart PC, *et al*. *RHD*(T201R, F223V) cluster analysis in five different ethnic groups and serological characterization of a new Ethiopean variant *DARE,* the *DIII type 6,* and the *RHD*(F223V). *Transfusion* 2006;46:606–615.
164 Lomas-Francis C, Halter Hipsky C, Velliquette RW, Reid ME. DIII Type 7 is likely the orininal serologically defined DIIIb. *Transfusion* 2012;52:39–42.
165 Beckers EAM, Faas BHW, Ligthart P, *et al*. Characterization of the hybrid *RHD* gene leading to the partial D category IIIc phenotype. *Transfusion* 1996;36:567–574.

166 Tilley L, Mathlouthi R, Need M, *et al*. Five novel RHD alleles resulting in D variant phenotypes. *Transfus Med* 2009;19(Suppl. 1):23 [Abstract].
167 Rouillac C, Colin Y, Hughes-Jones NC, *et al*. Transcript analysis of D category phenotypes predicts hybrid Rh D-CE-D proteins associated with alteration of D epitopes. *Blood* 1995;85:2937–2944.
168 Wagner FF, Eicher NI, Jørgensen JR, Lonicer CB, Flegel WA. DNB: a partial D with anti-D frequent in Central Europe. *Blood* 2002;100:2253–2256.
169 Hyodo H, Ishikawa Y, Tsuneyama H, *et al*. New RhD^{IVb} identified in Japanese. *Vox Sang* 2000;79:116–117.
170 Omi T, Okuda H, Iwamoto S, *et al*. Detection of Rh23 in the partial D phenotype associated with the D^{Va} category. *Transfusion* 2000;40:256–258.
171 Omi T, Takahashi J, Tsudo N, *et al*. The genomic organization of the partial D category D^{Va}: the presence of a new partial D associated with the D^{Va} phenotype. *Biochem Biophys Res Comm* 1999;254:786–794.
172 Hyodo H, Ishikawa Y, Kashiwase K, *et al*. Polymorphisms of RhD^{Va} and a new RhD^{Va}-like variant found in Japanese individuals. *Vox Sang* 2000;78:122–125.
173 Avent ND, Liu W, Jones JW, *et al*. Molecular analysis of Rh transcripts and polypeptides from individuals expressing the D^{VI} variant phenotype: and *RHD* gene deletion event does not generate all D^{VI}ccEe phenotypes. *Blood* 1997;89:1779–1786.
174 Mouro I, Le Van Kim C, Rouillac C, *et al*. Rearrangements of the blood group RhD gene associated with the D^{VI} category phenotype. *Blood* 1994;83:1129–1135.
175 Wagner FF, Gassner C, Müller TH, *et al*. Three molecular structures cause Rhesus D category VI phenotypes with distinct immunohematologic features. *Blood* 1998;91:2157–2168.
176 Ye L, Wang P, Gao H, *et al*. Partial D phenotypes and genotypes in the Chinese population. *Transfusion* 2012;52:241–246.
177 Esteban R, Montero R, Flegel WA, *et al*. The *D category VI type 4* allele is prevalent in the Spanish population. *Transfusion* 2006;46:616–623.
178 Rouillac C, Le Van Kim C, Beolet M, Cartron J-P, Colin Y. Leu110Pro substitution in the RhD polypeptide is responsible for the D^{VII} category blood group phenotype. *Am J Hematol* 1995;49:87–88.
179 Wagner FF, Ladewig B, Angert KS, *et al*. The *DAU* allele cluster of the *RHD* gene. *Blood* 2002;100:306–311.
180 Chen Q, Flegel WA. Random survey for *RHD* alleles among D+ European populations. *Transfusion* 2005;45:1183–1191.
181 Denomme GA, Wagner FF, Fernandes BJ, Li W, Flegel WA. Partial D, weak D types, and novel *RHD* alleles among 33 864 multiethnic patients: implications for anti-D alloimmunization and prevention. *Transfusion* 2005;45:1554–1560.
182 Flegel WA, von Zabern I, Doescher A, *et al*. DCS-1, DCS-2, and DFV share amino acid substitutions at the extracellular RhD protein vestibule. *Transfusion* 2008;48:25–33.
183 Touinssi M, Chapel-Fernandes S, Granier T, *et al*. Molecular analysis of inactive and active *RHD* alleles in native Congolese cohorts. *Transfusion* 2009;49:1353–1360.
184 Wallace M, Lomas-Francis C, Beckers E, *et al*. DBT: a partial D phenotype associated with the low-incidence antigen Rh32. *Transfus Med* 1997;7:233–238.
185 Beckers EAM, Faas BHW, Simsek S, *et al*. The genetic basis of a new partial D antigen: D^{DBT}. *Br J Haematol* 1996;93:720–727.
186 Huang CH, Chen Y, Reid ME, Okubo Y. Evidence for a separate genetic origin of the partial D phenotype DBT in a Japanese family. *Transfusion* 1999;39:1259–1265.
187 Lomas C, Grässmann W, Ford D, *et al*. FPTT is a low-incidence Rh antigen associated with a 'new' partial Rh D phenotype, DFR. *Transfusion* 1994;34:612–616.
188 Bizot M, Lomas C, Rubio F, Tippett P. An antiserum identifying a red cell determinant expressed by Rh:33 and by some 'new' depressed Rh phenotypes. *Transfusion* 1988;28:342–345.
189 von Zabern I, Flegel WA. IVS5-38del4 deletion in the *RHD* gene does not cause a DEL phenotype: relevance for *RHD* alleles including *DFR-3*. *Transfusion* 2007;47:1552–1555.
190 Flegel WA, von Zabern I, Doescher A, *et al*. D variants at the RhD vestibule in the weak D type 4 and Eurasian clusters. *Transfusion* 2009;49:1059–1069.
191 Giles CM, Crossland JD, Haggas WK, Longster G. An Rh gene complex which results in a 'new' antigen detectable by a specific antibody, anti-Rh33. *Vox Sang* 1971;21:289–301.
192 Wallace M, Lomas-Francis C, Tippett P. The D antigen characteristic of R_o^{Har} is a partial D antigen. *Vox Sang* 1996;70:169–172.
193 Beckers EAM, Porcelijn L, Ligthart P, *et al*. The R_o^{Har} antigen complex is associated with a limited number of D epitopes and alloanti-D production: a study of three unrelated persons and their families. *Transfusion* 1996;36:104–108.
194 Beckers EAM, Faas BHW, von dem Borne AEGK, *et al*. The R_0^{Har} Rh:33 phenotype results from substitution of exon 5 of the *RHCE* gene by the corresponding exon of the *RHD* gene. *Br J Haematol* 1996;92:751–757.
195 Jones J. Identification of two new D variants, D^{HMi} and D^{HMii} using monoclonal anti-D. *Vox Sang* 1995;69:236–241.
196 Liu W, Jones JW, Scott ML, Voak D, Avent ND. Molecular analysis of two D-variants, D^{HMi} and D^{HMii}. *Transfus Med* 1996;6(Suppl. 2):21 [Abstract].
197 Müller TH, Wagner FF, Trockenbacher A, *et al*. PCR screening for common weak D types shows different distributions in three Central European populations. *Transfusion* 2001;41:45–52.

198 Jones JW, Finning K, Mattock R, *et al*. The serological profile and molecular basis of a new partial D phenotype, DHR. *Vox Sang* 1997;73:252–256.
199 Avent ND, Poole J, Singleton B, *et al*. Studies of two partial Ds: DMH and DOL. *Transfus Med* 1999;9(Suppl. 1):33 [Abstract].
200 Flegel WA, Eicher NI, Doescher A, *et al*. In-frame triplet deletions in *RHD* alter the D antigen phenotype. *Transfusion* 2006;46:2156–2161.
201 Körmöczi GF, Legler TJ, Daniels GL, *et al*. Molecular and serologic characterization of DWI, a novel 'high-grade' partial D. *Transfusion* 2004;4:575–580.
202 Doescher A, Flegel WA, Petershofen EK, Bauerfeind U, Wagner FF. Weak D type 1.1 exemplifies another complexity in weak D genotyping. *Transfusion* 2005;45:1568–1573.
203 Körmöczi GF, Gassner C, Shao C-P, Uchikawa M, Legler TJ. A comprehensive analysis of DEL types: partial DEL individuals are prone to anti-D alloimmunization. *Transfusion* 2005;45:1561–1567.
204 Gassner C, Doescher A, Drnovsek TD, *et al*. Presence of *RHD* in serologically D–, C/E+ individuals: a European mulitcenter study. *Transfusion* 2005;45:527–538.
205 Richard M, Perreault J, Constanzo-Yanez J, Khalifé S, St-Louis M. A new *DEL* variant caused by exon 8 deletion. *Transfusion* 2007;47:852–857.
206 Lomas C, Tippett P, Thompson KM, Melamed MD, Hughes-Jones NC. Demonstration of seven epitopes on the Rh antigen D using human monoclonal anti-D antibodies and red cells from D categories. *Vox Sang* 1989;57:261–264.
207 Tippett P, Moore S. Monoclonal antibodies against Rh and Rh related antigens. *J Immunogenet* 1990;17:309–319.
208 Tippett P. Serologically defined Rh determinants. *J Immunogenet* 1990;17:247–257.
209 Lomas C, McColl K, Tippett P. Further complexities of the Rh antigen D disclosed by testing category D^{II} cells with monoclonal anti-D. *Transfus Med* 1993;3:67–69.
210 Gorick BD, Thompson KM, Melamed MD, Hughes-Jones NC. Three epitopes on the human Rh antigen D recognized by ^{125}I-labelled human monoclonal IgG antibodies. *Vox Sang* 1988;55:165–170.
211 Scott M. Section 1A. Rh serology. Coordinators report. *Transfus Clin Biol* 2002;9:23–29.
212 Rios M, Storry JR, Hue-Roye K, *et al*. Incidence of partial D, DIIIa, in black donors as determined by PCR-RFLP analysis. *Transfusion* 1998;38(Suppl.):63S [Abstract].
213 Westhoff CM, Vege S, Halter-Hipsky C, *et al.* DIIIa and DIII Type 5 are encoded by the same allele and are associated with altered RHCE*ce alleles: clinical implications. *Transfusion* 2010;50:1303–1311.
214 Rossi U, Lomas C, Tippett P, *et al*. Inheritance of a D^{IIIc} antigen: study of an Italian family. *Trasfusione Sangue* 1991;36:566–570.
215 Rosenfield RE, Haber G, Gibbel N. A new Rh variant. *Proc 6th Congr Int Soc Blood Transfus* 1956:90–95.
216 Lewis M, Chown B, Kaita H, *et al*. Blood group antigen Go^a and the Rh system. *Transfusion* 1967;7:440–441.
217 Chown B, Lewis M, Kaita H, *et al*. On the antigen Go^a and the Rh system. *Vox Sang* 1968;15:264–271.
218 Lassiter GR, Issitt PD, Garris ML, Welborn R. Further studies on the D^{Cor} (Go^a) antigen of the Rh system. *Transfusion* 1969;9:282 [Abstract].
219 Alter AA, Gelb AG, Chown B, Rosenfield RE, Cleghorn TE. Gonzales (Go^a), a new blood group character. *Transfusion* 1967;7:88–91
220 Lovett DA, Crawford MN. Js^b and Go^a screening of Negro donors. *Transfusion* 1967;7:442.
221 Lomas-Francis C, Reid ME. The Rh blood group system: the first 60 years of discovery. *Immunohematology* 2000;16:7–17.
222 Okubo Y, Seno T, Yamano H, *et al.* Partial D antigens disclosed by a monoclonal anti-D in Japanese blood donors. *Transfusion* 1991;31:782.
223 Chown B, Lewis M, Kaita H. A new Rh antigen and antibody. *Transfusion* 1962;2:150–154.
224 Legler TJ, Wiemann V, Ohto H, *et al*. D^{Va} category phenotype and genotype in Japanese families. *Vox Sang* 2000;78:194–197.
225 Avent ND, Finning KM, Liu W, Scott ML. Molecular biology of partial D phenotypes. *Transfus Clin Biol* 1996;6:511–516.
226 Sacchi R, Tippett P, Reali G, Sermasi G. Su un nuovo caso di D anti-D (D^{Vb}). *Trasfusione Sangue* 1974;19:699–702.
227 Green CA, Lomas-Francis C, Wallace M, Gooch A, Simonsen AC. Family evidence confirms that the low frequency antigen BARC is an Rh antigen. *Transfus Med* 1996;6(Suppl. 2):26 [Abstract].
228 Maaskant-van Wijk PA, Beckers EAM, van Rhenen DJ, *et al*. Evidence that the RHD^{VI} deletion genotype does not exist. *Blood* 1997;90:1709–1720.
229 Leader KA, Kumpel BM, Poole GD, *et al*. Human monoclonal anti-D with reactivity against category D^{VI} Cells used in blood grouping and determination of the incidence of the category D^{VI} phenotype in the D^{U} population. *Vox Sang* 1990;58:106–111.
230 van Rhenen DJ, Thijssen PMHJ, Overbeeke MAM. Serological characteristics of partial D antigen category VI in 8 unrelated blood donors. *Vox Sang* 1994;66:133–136.
231 Beck ML, Hardman JT. Incidence of D category VI among D^{U} donors in the USA. *Transfusion* 1991;31(Suppl.):25S [Abstract].
232 Watt J. The incidence of category VI amongst weak Rh(D) positive Sydney blood donors. *Transfus Med* 1993;3:72 [Abstract].
233 Flegel WA, Wagner FF. The frequency of RHD protein variants in Caucasians. *Transfus Clin Biol* 1996;3(spécial):10s [Abstract].

234 Lewis M, Kaita H, Allerdice PW, *et al*. Assignment of the red cell antigen, Targett (Rh40), to the Rh blood group system. *Am J Hum Genet* 1979;31:630–633.
235 Humphreys J, Stout TD, Kaita H, Lewis M. A family in which the Targett (Rh:40) antigen is carried with R^o (–D–). *Vox Sang* 1980;39:277–281.
236 Levene C, Sela R, Grunberg L, *et al.* The Rh antigen Tar (Rh40) causing haemolytic disease of the newborn. *Clin Lab Haematol* 1983;5:303–305.
237 Lomas C, Bruce M, Watt A, *et al*. Tar+ individuals with anti-D, a new category D^{VII}. *Transfusion* 1986;26:560 [Abstract].
238 Flegel WA, Hillesheim B, Kerowgan M, Kasulke D, Wagner FF. Lack of heterogeneity in the molecular structure of RHD category VII. *Transfusion* 1996;36(Suppl.):50S [Abstract].
239 Faas BHW, Beuling EA, Ligthart PC, van Rhenen DJ, van der Schoot CE. Partial expression of RHc from the RHD polypeptide. *Transfusion* 2001;41:1136–1142.
240 Cowley NM, Saul A, Hyland CA. *RHD* gene mutations and the weak D phenotype: an Australian blood donor study. *Vox Sang* 2000;79:251–252.
241 Rodrigues MJ, Rodrigues F, Tilley L, *et al*. Several new examples of weak D type 38 in the Portuguese population. *Transfusion* 2006;46(Suppl.):141A–142A [Abstract].
242 St-Louis M, Richard M, Côté M, Éthier C, Long A. *Weak D* type 42 cases found in individuals of European descent. *Immunohematology* 2011;27:20–24.
243 Dogic V, Bingulac-Popovic J, Babic I, *et al*. Distribution of weak D types in Croatian population. *Transfus Med* 2011;21:278–279.
244 Polin H, Graszner W, Hackl C, *et al*. On the trail of anti-CDE to unexpected highlights of the RHD*weak 4.3 allele in the Upper Austrian population. *Vox Sang* 2012;103:130–136.
245 Beckers EAM, Ligthart PC, Overbeke MAM, Maaskant PA, van Rhenen DJ. A patient with weak D type 1 and anti-D: auto or allo? *Vox Sang* 2004;87(Suppl. 3):75 [Abstract].
246 Roxby D, Coloma M, Flegel WA, *et al*. Observation of anti-D after D-positive transfusion in an individual with weak D type-1 phenotype. *Vox Sang* 2004;87(Suppl. 3):77–78 [Abstract].
247 Pelc-Klopotowska M, Guz K, Walaszcyk A, Orzinska A, Michalewska B. Weak D type two patient with allo anti-D. *Vox Sang* 2010;99(Suppl. 1):367 [Abstract].
248 Vege S, Fong C, Lomas-Francis C, *et al*. Weak D type 2 and production of anti-D. *Transfusion* 2011;51(Suppl.):41A [Abstract].
249 Pham B-N, Roussel M, Peyrard T, *et al*. Anti-D investigations in individuals expressing weak D type 1 or weak D type 2: allo- or autoantibodies? *Transfusion* 2011;51:2679–2685.
250 Bruce D, Rounding L, Barnes S, Grimsley S, Poole J. Immune alloanti-D in a patient with weak D type 33 genotype. *Transfus Med* 2011;21(Suppl.1):15 [Abstract].
251 Martinez Badas MP, Garcia Sanchez F, Moreno Jimenez G, *et al*. Anti-D immunization in a D-positive mother. *Vox Sang* 2007;87(Suppl. 1):187 [Abstract].
252 Hazenberg CAM, Beckers EAM, Overbeeke MAM. Hemolytic disease of the newborn caused by alloanti-D from an R_o^{Har}r Rh:33 mother. *Transfusion* 1996;36:478–479.
253 Flegel WA, Wagner FF, Chen Q, *et al*. The *RHCE* allele *ceCF*: the molecular basis of Crawford (RH43). *Transfusion* 2006;46:1334–1342.
254 Hipsky CH, Lomas-Francis C, Fuchisawa A, *et al*. *RHCE*ceCF* encodes partial c and partial e but not CELO, an antigen antithetical to Crawford. *Transfusion* 2011;51:25–31.
255 Wagner FF, Ladewig B, Flegel WA. The *RHCE* allele ceRT: D epitope 6 expression does not require D-specific amino acids. *Transfusion* 2003;43:1248–1253.
256 Chen Q, Hustinx H, Flegel WA. The *RHCE* allele *ceSL*: the second example for D antigen expression without D-specific amino acids. *Transfusion* 2006;46:766–772.
257 Nogues N, Boto N, Ibanez M, *et al*. RhD antigen expression from the ceAR allele: an RhD positive blood donor not carrying the DAR variant usually associated. *Vox Sang* 2009;96(Suppl. 1):27 [Abstract].
258 Okubo Y, Yamaguchi H, Tomita T, Nagao N. A D variant, D_{el}? *Transfusion* 1984;24:542.
259 Shao C-P, Wen X, Zhou Y-Y. Multiple isoforms excluding normal RhD mRNA detected in Rh blood group Del phenotype with RHD 1227A allele. *Transfus Apher Sci* 2006;34:142–152.
260 Liu H-C, Eng H-L, Yang Y-F, *et al*. Aberrant RNA splicing in *RHD* 7-9 exons of DEL individuals in Taiwan: a mechanism study. *Biochim Biophys Acta* 2010;1800:565–573.
261 Li Q, Hou L, Ye L-Y, Yue D-Q, Zhu Z-Y. Molecular basis of the *RHD* gene in blood donors with DEL phenotypes in Shanghai. *Vox Sang* 2009;97:139–146.
262 Shao C-P. Transfusion of RhD-positive blood in 'Asia type' DEL recipients. *New Engl J Med* 2010;362:472–473.
263 Flegel WA, von Zabern I, Wagner FE. Six years' experience performing *RHD* genotyping to confirm D– red blood cell units in Germany for preventing anti-D immunizations. *Transfusion* 2009;49:465–471.
264 Gardener GJ, Leger TJ, Hyett JA, *et al*. Anti-D in pregnant women with the *RHD*(IVS3+1G>A)-associated DEL phenotype. *Transfusion* 2012;52:2016–2019.
265 Wagner T, Körmöczi GF, Buchta C, *et al*. Anti-D immunization by DEL red blood cells. *Transfusion* 2005;45:520–526.
266 Liu W, Smythe JS, Scott ML, *et al*. Site-directed mutagenesis of the human D antigen: definition of D epitopes on the sixth external domain of the D protein expressed on K562 cells. *Transfusion* 1999;39:17–25.
267 Liu W, Avent ND, Jones JW, Scott ML, Voak D. Molecular configuration of Rh D epitopes as defined by site-directed mutagenesis and expression of mutant Rh constructs in

K562 erythroleukemia cells. *Blood* 1999;94:3986–3996. See also *Blood* 2000;96:1197–1199.

268 Siegel DL, Chang TY, Russell SL, Bunya VY. Isolation of cell surface-specific human monoclonal antibodies using phage display and magnetically-activated cell sorting: applications in immunohematology. *J Immunol Methods* 1997;206: 73–85.

269 Chang TY, Siegel DL. Genetic and immunological properties of phage-displayed human anti-Rh(D) antibodies: implications for Rh(D) epitope topology. *Blood* 1998; 91:3066–3078. See also *Blood* 2000;96:1196–1197.

270 Ceppellini R, Dunn LC, Turri M. An interaction between alleles at the Rh locus in man which weakens the reactivity of the Rh_o factor (D^u). *Proc Natl Acad Sci USA* 1955; 41:283–288.

271 McGee R, Levine P, Celano M. First example of genotype r^yr^y: a family study. *Science* 1957;125:1043.

272 Hasekura H, Ota M, Ito S, *et al*. Flow cytometric studies of the D antigen of various Rh phenotypes with particular reference to D^u and D_{el}. *Transfusion* 1990;30: 236–238.

273 Jones JW, Lloyd-Evans P, Kumpel BM. Quantitation of Rh D antigen sites on weak D and D variant red cells by flow cytometry. *Vox Sang* 1996;71:176–183.

274 Rochna E, Hughes-Jones NC. The use of purified ^{125}I-labelled anti-γ globulin in the determination of the number of D antigen sites on red cells of different phenotypes. *Vox Sang* 1965;10:675–686.

275 Masouredis SP, Dupuy ME, Elliot M. Relationship between Rh_o(D) zygosity and red cell Rh_o(D) antigen content in family members. *J Clin Invest* 1967;46:681–694.

276 Hughes-Jones NC, Gardner B, Lincoln PJ. Observations of the number of available c, D, and E antigen sites on red cells. *Vox Sang* 1971;21:210–216.

277 Working Party of the British Committee for Standards in Haematology Blood Transfusion Task Force. Guidelines for compatibility procedures in blood transfusion laboratories. *Transfus Med* 2004;14:59–73.

278 AABB. *Standards for blood banks and transfusion services*, 27th edn. Bethesda, MD: AABB 2011.

279 Chen W, Li L, Tsai SL, Lin K. Anti-D immunization by D_{el} red blood cells in Taiwan: two case reports. *Transfusion* 2006;46(Suppl.):129A [Abstract].

280 Flegel WA, Khull SR, Wagner FF. Primary anti-D immunization by weak D type 2 RBCs.*Transfusion* 2000;40: 428–434.

281 Mota M, Fonseca NL, Rodrigues Á, Kutner JM, Castilho L. Anti-D alloimmunization by weak D type 1 red blood cells with a very low antigen density. *Vox Sang* 2005;88: 130–135.

282 Engelfriet CP, Reesink HW, eds. International forum. Testing for weak D. *Vox Sang* 2006;90:140–153.

283 Krog GR, Clausen FB, Berkowicz A, *et al*. Is current serologic RhD typing of blood donors sufficient for avoiding immunization of recipients? *Transfusion* 2011;51:2278–2285.

284 Mayne K, Bowell P, Woodward T, *et al*. Rh immunization by the partial D antigen of category D^{Va}. *Br J Haematol* 1990;76:537–539.

285 Needs M, Poole J, Tilley L, *et al*. Stimulation of anti-D in pregnancy by a weak D type 3 foetus. *Transfus Med* 2007;17(Suppl. 1):40 [Abstract].

286 Lowes BCR. Hemolytic disease of the newborn due to anti-Rh14 (Rh^B). *Vox Sang* 1969;16:231–232.

287 Hill Z, Vacl J, Kalasová E, Calábková M, Pintera J. Haemolytic disease of new-born due to anti-D antibodies in a D^u-positive mother. *Vox Sang* 1974;27:92–94.

288 Lacey PA, Caskey CR, Werner DJ, Moulds JJ. Fatal hemolytic disease of a newborn due to anti-D in an Rh-positive D^u variant mother. *Transfusion* 1983;23:91–94.

289 White CA, Stedman CM, Frank S. Anti-D antibodies in D– and D^u-positive women: a cause of hemolytic disease of the newborn. *Am J Obstet Gynecol* 1983;145: 1069–1075.

290 Østgård P, Fevang F, Kornstad L. Anti-D in a 'D positive' mother giving rise to severe haemolytic disease of the newborn. *Acta Paediat Scand* 1986;75:175–178.

291 Lubenko A, Contreras M, Habash J. Should anti-Rh immunoglobulin be given to D variant women? *Br J Haematol* 1989;72:429–433.

292 Flegel WA, Wagner FF, Müller TH, Gassner C. Rh phenotype prediction by DNA typing and its application to practice. *Transfus Med* 1998;8:281–302.

293 Lo YMD, Corbetta N, Chamberlain PF, *et al*. Presence of fetal DNA in maternal plasma and serum. *Lancet* 1997;350:485–487.

294 Lo YM, Tein MSC, Lau TK, *et al*. Quantitative analysis of fetal DNA in maternal plasma and serum: implications for noninvasive prenatal diagnosis. *Am J Hum Genet* 1998; 62:768–775.

295 Geifman-Holtzman O, Grotegut CA, Gaughan JP. Diagnostic accuracy of noninvasive fetal genotyping from maternal blood: a meta analysis. *Am J Obstet Gynecol* 2006;195: 1163–1173.

296 Daniels G, Finning K, Martin P, Massey E. Non-invasive prenatal diagnosis of fetal blood group phenotypes: current practice and future prospects. *Prenat Diagn* 2009;29: 101–107.

297 Minon J-M, Semterre J-M, Schaaps J-P, Foidart J-P. An unusual false positive fetal *RHD* typing result using DNA derived from maternal plasma from a solid organ recipient. *Transfusion* 2006;46:1454.

298 van der Schoot CE, Ait Soussan A, Koelewijn J, *et al*. Non-invasive antenatal RHD typing. *Transfus Clin Biol* 2006;13: 53–57.

299 Finning K, Martin P, Summers J, *et al*. Effect of high throughput RHD typing of fetal DNA in maternal plasma on use of anti-RhD immunoglobulin in RhD negative preg-

nant women: prospective feasibility study. *Br Med J* 2008;336:816–818
300 Wikman AT, Tiblad E, Karlsson A, Olsson ML, Westgren M, Reilly M. Noninvasive single-exon fetal *RHD* determination in a routine screening program in early pregnancy. *Obstet Gynecol* 2012;120:227–234.
301 Daniels G, Finning K, Wade A, *et al*. Implementation of fetal *RHD* typing in all RhD-negative pregnant women: timing, costs, and efficiency. *Vox Sang* 2012;103(Suppl.1)34 [Abstract].
302 Clausen FB, Christiansen M, Steffensen R, *et al*. Report of the first nationally implemented clinical routine screening for fetal *RHD* in D– pregnant women to ascertain the requirement for antenantal RhD prophylaxis. *Transfusion* 2012;52:752–758.
303 Seeho SKM, Burton G, Leigh D, *et al*. The role of preimplantation genetic diagnosis in the management of severe rhesus alloimmunization: first unaffected pregnancy: case report. *Hum Reprod* 2005:20(3):697–701.
304 Chiu RW, Murphy MF, Fidler C, *et al*. Determination of RhD zygosity: comparison of a double amplification refractory mutation system approach and a multiplex real-time quantitative PCR approach. *Clin Chem* 2001;47:667–672.
305 Perco P, Shao C-P, Mayr WR, Panzer S, Legler TJ. Testing for the D zygosity with three different methods revealed altered *Rhesus boxes* and a new weak D type. *Transfusion* 2003;43:335–339.
306 Matheson KA, Denomme GA. Novel 3′ *Rhesus box* sequences confound *RHD* zygosity assignment. *Transfusion* 2002;42: 645–650.
307 Grootkerk-Tax MGHM, Maaskant-van Wijk PA, an Drunen J, C van der Schoot CE. The highly variable *RH* locus in nonwhite persons hampers RHD zygosity determination but yields more insight into *RH*-related evolutionary events. *Transfusion* 2005;45:327–337.
308 Wagner FF, Moulds JM, Flegel WA. Genetic mechanisms of *Rhesus* box variation. *Transfusion* 2005;45:338–334.
309 Pertl B, Pieber D, Panzitt T, *et al*. RhD genotyping by quantitative fluorescent polymerase chain reaction: a new approach. *BJOG* 2000;107:1498–1502.
310 Skov F, Hughes-Jones NC. Observations of the number of available C antigen sites on red cells. *Vox Sang* 1977;33: 170–174.
311 Smythe JS, Anstee DJ. Expression of C antigen in transduced K562 cells. *Transfusion* 2001;41:24–30.
312 Tournamille C, Meunier-Costes N, Costes B, *et al*. Partial C antigen in sickle cell disease patients: clinical relevance and prevention of alloimmunization. *Transfusion* 2010;50: 13–19.
313 Mouro I, Colin Y, Gane P, *et al*. Molecular analysis of blood group Rh transcripts from a r^Gr variant. *Br J Haematol* 1996;93:472–474.
314 Levine P, Rosenfield RE, White J. The first example of the Rh phenotype r^Gr^G. *Am J Hum Genet* 1961;13:299–305.
315 Rosenfield RE, Levine P, Heller C. Quantitative Rh typing of r^Gr^G with observations on the nature of G (Rh12) and anti-G. *Vox Sang* 1975;28:293–304.
316 Avent ND, Daniels GL, Martin PG, *et al*. Molecular investigation of the Rh C/c polymorphism. *Transfus Med* 1997;7(Suppl. 1):18 [Abstract].
317 Westhoff CM, Silberstein LE, Wylie DE, Skavdahl M, Reif ME. 16Cys encoded by the *RHce* gene is associated with altered expression of the e antigen and is frequent in the R_o haplotype. *Br J Haematol* 2001;113:666–671.
318 Gassner C, Schmarda A, Kilga-Nogler S, *et al*. *RHD/CE* typing by polymerase chain reaction using sequence-specific primers. *Transfusion* 1997;37:1020–1026.
319 Salvignol I, Calvas P, Socha WW, *et al*. Structural analysis of the RH-like blood group gene products in nonhuman primates. *Immunogenet* 1995;41:271–281.
320 Westhoff CM, Silberstein LE, Wylie DE. Evidence supporting the requirement for two proline residues for expression of c. *Transfusion* 2000;40:321–324.
321 Tax MGHM, van der Schoot E, van Doorn R, *et al*. *RHC* and *RHc* genotyping in different ethnic groups. *Transfusion* 2002;42:634–644.
322 Poulter M, Kemp TJ, Carritt B. DNA-based Rhesus typing: simultaneous determination of RHC and RHD status using the polymerase chain reaction. *Vox Sang* 1996;70: 164–168.
323 Rozman P, Dovc T, Gassner C. Differentiation of autologous *ABO*, *RHD*, *RHCE*, *KEL*, *JK*, and *FY* blood group genotypes by analysis of peripheral blood samples of patients who have recently received multiple transfusions. *Transfusion* 2000;40:936–942.
324 Issitt PD, Tessel JA. On the incidence of antibodies to the Rh antigens G, rh_i(Ce), C, and C^G in sera containing anti-CD or anti-C. *Transfusion* 1981;21:412–418.
325 Masouredis SP, Sudora EJ, Mahan L, Victoria EJ. Antigen site densities and ultrastructural distribution patterns of red cell Rh antigens. *Transfusion* 1976;16:94–106.
326 Steers F, Wallace M, Johnson P, Carritt B, Daniels G. Denaturing gradient gel electrophoresis: a novel method for determining Rh phenotype from genomic DNA. *Br J Haematol* 1996;94:417–421.
327 Faas BHW, Simsek S, Bleeker PMM, *et al*. Rh E/e genotyping by allele-specific primer amplification. *Blood* 1995; 85:829–832.
328 Strobel E, Noizat-Pirenne France, Hofmann S, Cartron JP, Bauer MF. The molecular basis of the Rhesus antigen E^w. *Transfusion* 2004;44:407–409.
329 Greenwalt TJ, Sanger R. The Rh antigen E^w. *Br J Haematol* 1955;1:52–54.
330 Grobel RK, Cardy JD. Hemolytic disease of the newborn due to anti-E^w. A fourth example of the Rh antigen, E^w. *Transfusion* 1971;11:77–78.
331 Noizat-Pirenne F, Mouro I, Gane P, *et al*. Heterogeneity of blood group RhE variants revealed by serological analysis

and molecular alteration of the *RHCE* gene and transcript. *Br J Haematol* 1998;103:429–436.
332 Noizat-Pirenne F, Mouro I, Le Pennec P, *et al*. Molecular basis of category EIV variant phenotype. *Transfusion* 1999;39(Suppl.):103S [Abstract].
333 Kashiwase K, Ishikawa Y, Hyodo H, *et al*. E variants found in Japanese and c Antigenicity alteration without substitution in the second extracellular loop. *Transfusion* 2001;41: 1408–1412.
334 Green C, Bugert P, Hustinx H, Daniels G, Karamatic Crew V. A silent mutation 939g>a in RHCE (Pro313) causes los of a splice site and complete skipping of exon 6 resulting in expression of a novel E antigen. *Transfus Med* 2010;20(Suppl. 1):33 [Abstract].
335 Issitt PD. An invited review: the Rh antigen e, its variants, and some closely related serological observations. *Immunohematology* 1991;7:29–36.
336 Shapiro M. Serology and genetics of a new blood factor: hr^S. *J Forens Med* 1960;7:96–105.
337 Moores P. Rh18 and hr^S blood group and antibodies. *Vox Sang* 1994;66:225–230.
338 Noizat-Pirenne F, Lee K, Le Pennec P-Y, *et al*. Rare RHCE phenotypes in black individuals of Afro-Caribbean origin: identification and transfusion safety. *Blood* 2002;100: 4223–4231.
339 Smart EA, Fogg P, Nadesan S, Joseph I. A review of the clinical significance of anti-RH19 and anti-RH34 antibodies detected in South African patients. *Vox Sang* 2006; 91(Suppl. 3):132 [Abstract].
340 Reid ME, Halter Hipsky C, Hue-Roye K, Coghlan G, Olsen C, Lomas-Francis C. The low-prevalence Rh antigen STEM (RH49) is encoded by two different RHCE*ce818T alleles that are often in cis to *RHD*DOL*. *Transfusion* 2012, in press.
341 Noizat-Pirenne F, Mouro I, Le Pennec P-Y, *et al*. Two new alleles of the *RHCE* gene in Black individuals: the *RHce* allele *ceMO* and the *RHcE* allele *cEMI*. *Br J Haematol* 2001;113:672–679.
342 Shapiro M, le Roux M, Brink S. Serology and genetics of a new blood factor: hr^B. *Haematologia* 1972;6:121–128.
343 Moores P, Smart E. Serology and genetics of the red blood cell factor Rh34. *Vox Sang* 1991;61:122–129.
344 Pham B-N, Peyrard T, Tourret S, *et al*. Anti-Hr^B and anti-hr^B revisited. *Transfusion* 2009;49:2400–2405.
345 Halter Hipsky C, Larimore KS, Velliquette RW, Lomas-Francis C, Reid ME. Molecular background of *RHCE* in Bastiaan, the RH:–31,–34 index case. *Transfusion* 2011; 51(Suppl.):35A [Abstract].
346 Vengelen-Tyler V, Mogck N. Two cases of 'hrB-like' autoantibodies appearing as alloantibodies. *Transfusion* 1991;31: 254–256.
347 Lomas-Francis C, Yomtovian R, McGrath C, Walker PS, Reid ME. A confusion in antibody identification: anti-D production after anti-hr^B. *Immunohematology* 2007;23: 158–160.
348 Chou ST, Westhoff CM. Molecular biology of the Rh system: clinical considerations for transfusion in sickle cell disease. *Am Soc Hematol* 2009:178–184.
349 Silvy M, Di Cristofaro J, Beley S, *et al*. Identification of *RHCE* and *KEL* alleles in large cohorts of Afro-Caribbean and Comorian donors by multiplex SNaPshot and fragment assays: a transfusion support for sickle cell disease patients. *Br J Haematol* 2011;154:260–270.
350 Marais I, Moores P, Smart E, Martell R. STEM, a new low-frequency Rh antigen associated with the e-variant phenotypes hr^S– (Rh:–18,–19) and hrB– (Rh:–31,–34). *Transfus Med* 1993;3:35–41.
351 Heistö H. An anti-E antibody reacting strongly with enzyme-treated cells, but showing no signs of agglutination or absorption with untreated cells. *Acta Path Microbiol Scan* 1955;26:381–384.
352 Vogt E, Krystad E, Heistö H, Myhre K. A second example of a strong anti-E reacting *in vitro* almost exclusively with enzyme treated E positive cells. *Vox Sang* 1958;3:118–123.
353 Dybkjaer E. Anti-E antibodies disclosed in the period 1960–1966. *Vox Sang* 1967;13:446–448.
354 Harrison J. The 'naturally occurring' anti-E. *Vox Sang* 1970;19:123–131.
355 Sanger R, Race RR, Rosenfield RE, Vogel P, Gibbel N. Anti-f and the 'new' Rh antigen it defines. *Proc Natl Acad Sci USA* 1953;39:824–834.
356 Grundorfer J, Kopchik W, Tippett P, Sanger R. Anti-f in the serum of a *CDe/cDE* person: the second example. *Vox Sang* 1961;6:618–619.
357 Jones AR, Steinberg AG, Allen FH, Diamond LK, Kriete B. Observations on the new Rh agglutinin anti-f. *Blood* 1954;9:117–122.
358 Levine P, White J, Stroup M, Zmijewski CM, Mohn JF. Hæmolytic disease of the newborn probably due to anti-f. *Nature* 1960;185:188–189.
359 Freda VJ, D'Esopo DA, Rosenfield RE, Haber GV. Erythroblastosis due to anti-Rh6. *Transfusion* 1963;3:281–282.
360 Spielmann W, Seidl S, von Pawel J. Anti-ce (anti-f) in a *CDe/cD*– mother, as a cause of haemolytic disease of the newborn. *Vox Sang* 1974;27:473–477.
361 O'Reilly RA, Lombard CM, Azzi RL. Delayed hemolytic transfusion reaction associated with Rh antibody anti-f: first reported case. *Vox Sang* 1985;49:336–339.
362 Lucia SP, Wild GM, Hunt ML. Anti-f sensitization detected by an acidified indirect Coombs test. *Vox Sang* 1960;5: 377–382.
363 Rosenfield RE, Haber GV. An Rh blood factor, rh_i (Ce), and its relationship to hr (ce). *Am J Hum Genet* 1958;10: 474–480.
364 Race RR, Sanger R, Levine P, *et al*. A position effect of the *Rh* blood group genes. *Nature* 1954;174:460–461.
365 Wagner T, Resch B, Legler TJ, *et al*. Severe HDN due to anti-Ce that required exchange transfusion. *Transfusion* 2000;40:571–574.

366 Malde R, Stanworth S, Patel S, Knight R. Haemolytic disease of the newborn due to anti-Ce. *Transfus Med* 2000;10: 305–306.
367 Ranasinghe E, Goodyear E, Burgess G. Anti-Ce complicating two consecutive pregnancies with increasing severity of haemolytic disease of the newborn. *Transfus Med* 2003; 13:53–55.
368 Molthan L, Matulewicz TJ, Bansal-Carver B, Benz EJ. An immediate hemolytic transfusion reaction due to anti-C and a delayed hemolytic transfusion reaction due to anti-Ce+e: hemoglobinemia, hemoglobinuria and transient impaired renal function. *Vox Sang* 1984;47:348–353.
369 Lee E, Knight RC. A case of autoimmune haemolytic anaemia with an IgA anti-Ce autoantibody. *Vox Sang* 2000;78(Suppl. 1):abstract P130.
370 Svoboda RK, Van West B, Grumet FC. Anti-Rh41, a new Rh antibody found in association with an abnormal expression of chromosome 1 genetic markers. *Transfusion* 1981;21: 150–156.
371 Dunsford I. A new Rh antibody: anti-CE. *Proc 8th Congr Europ Soc Haemat* 1961:491.
372 Keith P, Corcoran PA, Caspersen K, Allen FH. A new antibody; anti-Rh[27] (cE) in the Rh blood group system. *Vox Sang* 1965;10:528–535.
373 Kline WE, Sullivan CM, Pope M, Bowman RJ. An example of a naturally occurring anti-cE (Rh27) that binds complement. *Vox Sang* 1982;43:335–339.
374 Stout TD, Moore BPL, Allen FH, Corcoran P. A new phenotype – D+G– (Rh:1,–12). *Vox Sang* 1963;8:262–268.
375 Faas BHW, Beckers EAM, Simsek S, *et al*. Involvement of Ser103 of the Rh polypeptides in G epitope formation. *Transfusion* 1996;36:506–511.
376 Case J. Quantitative variation in the G antigen of the Rh blood group system. *Vox Sang* 1973;25:529–539.
377 Green C, Coghlan G, Bizot M, *et al*. JAHK: a low frequency antigen associated with the r^G complex of the Rh blood group system. *Transfus Med* 2002;12:55–61.
378 Poole J, Warke N, Shepherd D, Hall C. A C typing problem and observations on the r''^G phenotype. Transfus Med 2001;11(Suppl. 1):32–33 [Abstract].
379 Allen FH, Tippett PA. A new Rh blood type which reveals the Rh antigen G. *Vox Sang* 1958;3:321–330.
380 Scharberg EA, Green C, Daniels G, *et al*. Molecular basis of the JAHK (RH53) antigen. *Transfusion* 2005;45:1314–1318.
381 Skov F. Observations of the number of available G (rh^G, Rh12) antigen sites on red cells. *Vox Sang* 1976;31:124–130.
382 Allen FH, Tippett PA. Blocking tests with the Rh antibody anti-G. *Vox Sang* 1961;6:429–434.
383 Jakobowicz R, Simmons RT. Iso-immunization in a mother which demonstrates the 'new' Rh blood antigen G (rh^G) and anti-G (rh^G). *Med J Aust* 1959;ii:357–358.
384 Jakobowicz R, Whittingham S, Barrie JU, Simmons RT. A further investigation of polyvalent anti-C (rh′) and anti-G (rh^G) antibodies produced by iso-immunization in pregnancy. *Med J Aust* 1962;i:895–896.
385 Vos GH. The evaluation of specific anti-G (CD) eluate obtained by a double absorption and elution procedure. *Vox Sang* 1960;5:472–478.
386 Palfi M, Gunnarsson C. The frequency of anti-C + anti-G in the absence of anti-D in alloimmunized pregnancies. *Transfus Med* 2001;11:207–210.
387 Smith TR, Sherman SP, Nelson CA, Taswell HF. Formation of anti-G by the transfusion of D-negative blood. *Transfusion* 1978;18:388 [Abstract].
388 Sistonen P, Sareneva H, Pirkola A, Eklund J. MAR, a novel high-incidence Rh antigen revealing the existence of an allelic sub-system including C^W (Rh8) and C^x (Rh9) with exceptional distribution in the Finnish population. *Vox Sang* 1994;66:287–292.
389 Mouro I, Colin Y, Sistonen P, *et al*. Molecular basis of the RhC^w (Rh8) and RhC^x (Rh9) blood group specificities. *Blood* 1995;86:1196–1201.
390 Callender ST, Race RR. A serological and genetical study of multiple antibodies formed in response to blood transfusion by a patient with lupus erythematosus diffusus. *Ann Eugen* 1946;13:102–117.
391 Leonard GL, Ellisor SS, Reid ME, Sanchez PD, Tippett P. An unusual Rh immunization. *Vox Sang* 1976;31:275–276.
392 Watt J, Pepper R. Anti-C in an Rl^wRl^w patient. *Joint Congr Int Soc Blood Transfus and Am Ass Blood Banks*, 1990:80 [Abstracts].
393 Govoni M, Battistini I, Menini C. A rare case of Rh immunization. *Vox Sang* 1990;58:137.
394 Thorpe SJ, Boult CE, Thompson KM. Immunochemical characterization of the Rh C^w antigen using human monoclonal antibodies. *Vox Sang* 1997;73:174–181.
395 Sachs HW, Reuter W, Tippett P, Gavin J. An Rh gene complex producing both C^w and c antigen. *Vox Sang* 1978;35:272–274.
396 Giannetti M, Stadler E, Rittner C, Lomas C, Tippett P. A rare Rh haplotype producing C^w and c, and D and e in a German family. *Vox Sang* 1983;44:319–321.
397 Bowman JM, Pollock J. Maternal C^w alloimmunization. *Vox Sang* 1993;64:226–230.
398 Lawler SD, van Loghem JJ. The Rhesus antigen C^w causing hæmolytic disease of the newborn. *Lancet* 1947;ii:545–546.
399 Kollamparambil TG, Jani BR, Aldouri M, Soe A, Ducker DA. Anti-C^w alloimmunization presenting as hydrops fetalis. *Acta Pediatrica* 2005;94:499–507.
400 Byers BD, Gordon MC, Higby K. Severe hemolytic disease of the newborn due to anti-C^w. *Obstet Gynecol* 2005;106: 1180–11822.
401 May-Wewers J, Kaiser JR, Moore EK, Blackall DP. Severe neonatal hemolysis due to a maternal antibody to the low frequency Rh antigen C^w. *Am J Perinat* 2006;23:213–217.

402 Chu H-P, Kanagalingam D, Chan D K-L. Severe intrauterine hemolysis due to anti-C^w. *Am J Perinatol* 2007;24: 623–626.

403 Sistonen P, Aden Abdulle O, Sahid M. Evidence for a 'new' Rh gene complex producing the rare C^x (Rh9) antigen in the Somali population of East Africa. *Transfusion* 1987;27:66–68.

404 Stratton F, Renton PH. Haemolytic disease of the newborn caused by a new Rh antibody, anti-C^x. *Br Med J* 1954;i: 962–965.

405 Finney RD, Blue AM, Willoughby MLN. Haemolytic disease of the newborn caused by the rare Rhesus antibody anti-C^x. *Vox Sang* 1973;25:39–42.

406 Mougey R, Martin J, Hackbart C. A new high frequency Rh red cell antigen associated with the Rh antigen C^x. *Transfusion* 1983;23:410 [Abstract].

407 Plaut G, Booth PB, Giles CM, Mourant AE. A new example of the Rh antibody, anti-C^x. *Br Med J* 1958;i:1215–1217.

408 Bradford MF, Porter M, Lacey PA, Moulds JJ. Further complexities of anti-C: an antibody produced by an $R_1{}^x r$ individual. *Transfusion* 1985;25:471 [Abstract].

409 O'Shea KP, Øyen R, Sausais L, *et al*. A MAR-like antibody in a DC^we/DC^we person. *Transfusion* 2001;41:53–55.

410 DeNatale A, Cahan A, Jack JA, Race RR, Sanger R. V, a 'new' Rh antigen, common in Negroes, rare in white people. *J Am Med Ass* 1955;159:247–250.

411 Sanger R, Noades J, Tippett P, *et al*. An Rh antibody specific for V and R'^s. *Nature* 1960;186:171.

412 Moulds JJ, Case J, Thornton S, Pulver VB, Moulds MK. Anti-Ce^s: a previously undescribed Rh antibody. *Transfusion* 1980;20:631–632 [Abstract].

413 Pham B-N, Peyrard T, Juszczak G, *et al*. Alloanti-c (RH4) revealing that the *(C)ce*^{s} haplotype encodes a partial c antigen. *Transfusion* 2009;49:1329–1334.

414 Tregellas WM, Issitt PD. An investigation of the relationship between the V (hr^V), VS, and hr^H antigens. *Transfusion* 1978;18:15–20.

415 Shapiro M. Serology and genetics of a 'new' blood factor: hr^H. *J Forens Med* 1964;11:52–66.

416 Cheng MS. Saline-reactive anti-V. *Transfusion* 1982;22:401.

417 Tilley L, Coghlan G, Zelinski T, Spruell P, Daniels G. An unusual Rh haplotype encoding VS and V together with C, but not c. *Transfus Med* 2007;17:42–43 [Abstract].

418 Byrne PC, Howard JH. The incidence of V (Rh10) and Js^a (K6) in the contemporary African American blood donor. *Immunohematology* 1994;10:136–138.

419 Cotorruelo CM, Munini GM, Borrás SEG, *et al*. The Dc(G48)e^s haplotype is frequent among the Dce haplotypes within a white population. *Transfusion* 2007;47: 486–491.

420 Hue-Roye K, Hipsky CH, Velliquette RW, *et al*. A novel *RHCE*48C, 733G* allele with nucleotide 941C in exon 7 encodes an altered red blood cell e antigen. *Transfusion* 2011;51:32–35.

421 Peyrard T, Pham B-N, Poupel S, *et al*. Alloanti-c/ce in a c+ *ceAR/Ce* patient suggests that the rare *RHCE*ceAR* allele (*ceAR*) encodes a partial c antigen. *Transfusion* 2009;49: 2406–2411.

422 Halter Hipsky CH, Lomas-Francis C, Fuchisawa A, Reid ME. *RHCE*ceAR* encodes a partial c (RH4) antigen. *Immunohematology* 2010;26:57–59.

423 Pham B-N, Peyrard T, Juszczak G, *et al*. Analysis of RhCE variants among 806 individuals in France: consideration for transfusion safety, with emphasis on patients with sickle cell disease. *Transfusion* 2011;51:1249–1260.

424 Rosenfield RE, Haber GV, Schroeder R, Ballard R. Problems in Rh typing as revealed by a single Negro family. *Am J Hum Genet* 1960;12:147–159.

425 Rouillac C, Gane P, Cartron J, *et al*. Molecular basis of the altered antigenic expression of RhD in weak D (D^u) and RhC/c in R^N phenotypes. *Blood* 1996;87:4853–4861.

426 Lomas C, Poole J, Salaru N, *et al*. A low-incidence red cell antigen JAL associated with two unusual Rh gene complexes. *Vox Sang* 1990;59:39–43.

427 Hustinx H, Poole J, Bugert P, *et al*. Molecular basis of the Rh antigen RH48 (JAL). *Vox Sang* 2009;96:234–239.

428 Westhoff CM, Vege S, Wylie D, *et al*. The JAL antigen (RH48) is the result of a change in *RHCE* that encodes Arg114Trp. *Transfusion* 2009;49:725–732.

429 Noizat-Pirenne F, Le Pennec P-Y, Mouro I, *et al*. Molecular background of *D(C)(e)* haplotypes within the white population. *Transfusion* 2002;42:627–633.

430 Daniels GL. An investigation of the immune response of homozygotes for the Rh haplotype –D– and related haplotypes. *Rev Franc Transfus Immuno-Hémat* 1982;25: 185–197.

431 Westhoff CM, Storry JR, Walker P, Lomas-Francis C, Reid ME. A new hybrid RHCE gene (CeNR) is responsible for expression of a novel antigen. *Transfusion* 2004;44: 1047–1051.

432 Davidsohn I, Stern K, Strauser ER, Spurrier W. Be, a new 'private' blood factor. *Blood* 1953;8:747–754.

433 Hue-Roye K, O'Shea K, Gillett R, *et al*. The low prevalence Rh antigen Be^a (Rh36) is associated with RHCE*ce 662C>G in exon 5, which is predicted to encode Rhce 221Arg. *Vox Sang* 2010;98:e263–e268.

434 Huestis DW, Catino ML, Busch S. A 'new' Rh antibody (anti-Rh 26) which detects a factor usually accompanying hr'. *Transfusion* 1964;4:414–418.

435 Faas BHW, Ligthart PC, Lomas-Francis C, *et al*. Involvement of Gly96 in the formation of the Rh26 epitope. *Transfusion* 1997;37:1123–1130.

436 Coghlan G, Moulds M, Nylen E, Zelinski T. Molecular basis of the LOCR (Rh55) antigen. *Transfusion* 2006;46:1689–1692.

437 Noizat-Pirenne F, Tournamille C, Gallon P, *et al*. *ceRA*: an *RH* allele variant producing a new rare blood. *Transfusion* 2006;46:1232–1236.

438 Vege S, Nickle PA, Shirey R, Westhoff C. A novel 254G>C (Ala85Gly) change associated with partial Rhe and alloanti-e. *Transfusion* 2009;49(Suppl.):15A [Abstract].
439 Westhoff CM, Vege S, Nickle P, *et al.* Nucleotide deletion in *RHCE*cE (907delC)* is responsible for a D–– haplotype in Hispanics. *Transfusion* 2011;51:2142–2147.
440 Huang C-H, Peng J, Chen HC, *et al. RH* locus contraction in a novel *Dc-/D––*genotype resulting from separate genetic recombination events. *Transfusion* 2004;44:853–859.
441 Giles CM, Skov F. The *CDe* Rhesus gene complex; some considerations revealed by a study of a Danish family with an antigen of the Rhesus gene complex *(C)D(e)* defined by a 'new' antibody. *Vox Sang* 1971;20:328–334.
442 Le Pennec PY, Rouger P, Klein MT, *et al.* A serologic study of red cells and sera from 18 Rh:32,–46 (R^N/R^N) persons. *Transfusion* 1989;29:798–802.
443 Chown B, Lewis M, Kaita H. The Rh system. An anomaly of inheritance, probably due to mutation. *Vox Sang* 1971;21:385–396.
444 Rouger P, Edelman L. Murine monoclonal antibodies associated with Rh17, Rh29, and Rh46 antigens. *Transfusion* 1988;28:52–55.
445 Le Pennec PY, Buffière F, Gane P, *et al.* Study of a human monoclonal anti-RH46. Transfusion 1996;36(Suppl.):54S [Abstract].
446 Poole J, Hustinx H, Gerber H, *et al.* The red cell antigen JAL in the Swiss population: family studies showing that JAL is an Rh antigen (RH48). *Vox Sang* 1990;59:44–47.
447 Lomas-Francis C, Alcantara G, Westhoff C, *et al.* JAL (RH48) blood group antigen: serologic observations. *Transfusion* 2009;49:719–724.
448 Cheng G-J, Chen Y, Reid ME, Huang C-H. Evans antigen: a new hybrid structure occurring on background of D·· and D–– Rh complexes. *Vox Sang* 2000;78:44–51.
449 Huang C-H. Alteration of *RH* gene structure and expression in human dCCee and DC^w– red blood cells: phenotypic homozygosity versus genotypic heterozygosity. *Blood* 1996;88:2326–2333.
450 Broman B, Heiken A, Tippett PA, Giles CM. The *D(C)(e)* gene complex revealed in the Swedish population. *Vox Sang* 1963;8:588–593.
451 Tippett P. Depressed Rh phenotypes. *Rev Franc Transfus Immuno-Hémat* 1978;21:135–150.
452 Heiken A, Giles CM. On the Rh gene complexes D––, D(C)(e) and d(c)(e). *Hereditas* 1965;53:171–186.
453 Ong J, Walker PS, Schulbach E, *et al.* Alloanti-c in a c-positive, JAL-positive patient. *Vox Sang* 2009;96:240–243.
454 Coghlan G, McCreary J, Underwood V, Zelinski T. A 'new' low-incidence red cell antigen, LOCR, associated with altered expression of Rh antigens. *Transfusion* 1994;34:492–495.
455 Stern K, Davidsohn I, Jensen FG, Muratore R. Immunologic studies on the Be^a factor. *Vox Sang* 1958;3:425–434.
456 Clark J, Yorek H, Schuler S, Milam JD. Hemolytic disease of the newborn due to anti-Berrens antibody: the third reported case. *Joint Congr Int Soc Blood Transfus and Am Ass Blood Banks*, 1990:81 [Abstracts].
457 Amil M, Casais C, Vicente I, *et al.* Severe hemolytic disease of the new born caused by anti-Rh36. *Vox Sang* 2000;78(Suppl.):abstract P122.
458 Schneider W, Tippett P. Rh33, another possible homozygote. *Transfusion* 1978;18:392 [Abstract].
459 Silvy M, Barrault A, Velliquette RW, *et al. RHCE*cE734C* allele encodes an altered c antigen and a suppressed E antigen not detected with standard reagents. *Transfusion* 2012, in press.
460 Race RR, Sanger R, Selwyn JG. A probable deletion in a human Rh chromosome. *Nature* 1950;166:520.
461 Race RR, Sanger R, Selwyn JG. A possible deletion in a human Rh chromosome: a serological and genetical study. *Br J Exp Path* 1951;32:124–135.
462 Mulvihal M, Moores P. The Rh haplotype *D––* identified in five Cape Colored families. *Transfusion* 1991;31:188–189.
463 Kendrick L, Dunstan-Adams C, Humphreys J, *et al.* The rare Rh haplotypes –D– and ––– in a family with a –D–/––– propositus. *J Immunogenet* 1981;8:243–247.
464 Rasmuson M, Heiken A. Frequency of occurrence of the human Rh complexes *D(C)(e)*, *d(c)(e)*, *D––* and –––. *Nature* 1966;212:1377–1379.
465 Ólafsdóttir S, Jensson O, Thordarson G, Sigurdardóttir S. An unusual Rhesus haplotype, –D–, in Iceland. *Forens Sci Int* 1983;22:183–187.
466 Okubo Y, Tomita T, Nagao N, Yamaguchi H, Tanaka M. Mass screening donors for –D– and Jk(a–b–) using the Groupamatic-360. *Transfusion* 1983;23:362–363.
467 Kemp TJ, Poulter M, Carritt B. A recombination hot spot in the Rh genes revealed by analysis of unrelated donors with the rare D–– phenotype. *Am J Hum Genet* 1996;59:1066–1073.
468 Blunt T, Steers F, Daniels G, Carritt B. Lack of RH C/E expression in the Rhesus D–– phenotype is the result of a gene deletion. *Ann Hum Genet* 1994;58:19–24.
469 Huang C-H, Reid ME, Chen Y. Identification of a partial internal deletion in the *RH* locus causing the human erythrocyte D–– phenotype. *Blood* 1995;86:784–790.
470 Cherif-Zahar B, Raynal V, Cartron J-P. Lack of RHCE-encoded proteins in the D–– phenotype may result from homologous recombination between the two *RH* genes. *Blood* 1996;88:1518–1520.
471 Okuda H, Fujiwara H, Omi T, *et al.* A Japanese propositus with D–– phenotype characterized by the deletion of both the *RHCE* gene and D1S80 locus situated in chromosome 1p and the existence of a new *CE–D–CE* hybrid gene. *J Hum Genet* 2000;45:142–153.
472 Contreras M, Stebbing B, Blessing M, Gavin J. The Rh antigen Evans. *Vox Sang* 1978;34:208–211.

473 Contreras M, Armitage S, Daniels GL, Tippett P. Homozygous ·*D*·. *Vox Sang* 1979;36:81–84.

474 Skradski K, Sabo B, Daniels G. A second homozygous ·*D*·. *Transfusion* 1981;21:472.

475 Lomas-Francis C, Velliquette RW, Vege S, *et al*. Surprising finding with RBCs expressing the low prevalence RH antigen Evans. *Transfusion* 2011;51(Suppl.):35A–36A [Abstract].

476 Huang C-H, Chen Y, Reid ME, Ghosh S. Genetic recombination at the human *RH* locus: a family study of the red-cell Evans phenotype reveals a transfer of exons 2–6 from the *RHD* to the *RHCE* gene. *Am J Hum Genet* 1996;59: 825–833.

477 Tate H, Cunningham C, McDade MG, Tippett PA, Sanger R. An Rh gene complex *Dc*–. *Vox Sang* 1960;5:398–402.

478 Yamaguchi H, Okubo Y, Tomita T, Yamano H, Tanaka M. A case of Rh gene complex cD–/cD– found in a Japanese. *Proc Jpn Acad* 1969;45:618–620.

479 Delmas-Marsalet Y, Goudemand M, Tippett P. Un nouvel exemple de génotype rhésus cD–/cD–. *Rev Franc Transfus* 1969;12:233–238.

480 Cotorruelo C, Biondi C, Garcia Rosasco M, *et al*. Deletion of E and e antigens in a pregnant woman. *Transfusion* 1996;36:191.

481 Cotorruelo CM, Biondi CS, Garcia Borrás SE, Di Mónaco RA, Racca A. A Dc– phenotype encoded by an *RHCE–D(5–7/8)–CE* hybrid allele. *Vox Sang* 2003;85:102–108.

482 Chérif-Zahar B, Raynal V, D'Ambrosio A-M, Cartron JP, Colin Y. Molecular analysis of the structure and expression of the RH locus in individuals with *D*––, *Dc*–, and *DC^{w}*– gene complexes. *Blood* 1994;84:4354–4360.

483 Leyshon WC. The Rh gene complex *cD*– segregating in a Negro family. *Vox Sang* 1967;13:354–356.

484 Moores P, Vaaja U, Smart E. *D*–– and *Dc*– gene complexes in the coloureds and blacks of Natal and the Eastern Cape and blood group phenotype and gene frequency studies in the Natal coloured population. *Hum Hered* 1991;41:295–304.

485 Gunson HH, Donohue WL. Multiple examples of the blood genotype C^{w}D–/C^{w}D– in a Canadian family. *Vox Sang* 1957;2:320–331.

486 Tippett P, Gavin J, Sanger R. The antigen C^{W} produced by the gene complex *C^{W}D*–. *Vox Sang* 1962;7:249–250.

487 Salmon C, Gerbal A, Liberge G, *et al*. Le complexe génique D^{IV}(C)–. *Rev Franc Transfus* 1969;12:239–247.

488 Delehanty CL, Wilkinson SL, Issitt PD, *et al*. Riv: a new low incidence Rh antigen. *Transfusion* 1983;23:410 [Abstract].

489 Perrier P, Habibi B, Salmon C. Allo antibodies in partially silent Rh phenotypes. Evidence for a possibly new high frequency Rh antigen. *Rev Franc Transfus Immuno-Hémat* 1980;23:327–339.

490 Habibi B, Perrier P, Salmon C. Antigen Nou. A new high frequency Rh antigen. *Rev Franc Transfus Immuno-Hémat* 1981;24:117–120.

491 Hipsky CH, Hue-Roye K, Lomas-Francis C, Huang C-H, Reid ME. Molecular basis of the rare gene complex, *DIVa(C)*–, which encodes four low-prevalence antigens in the Rh blood group system. *Vox Sang* 2012;102:167–170.

492 Hackel E. Rh antibodies in the serum of two –D–/–D– people. *Vox Sang* 1957;2:331–341.

493 Hirose M, Nakanishi K, Kaku S, *et al*. Fetal hemolytic disease due to anti-Rh17 alloimmunization. *Fetal Diagn Ther* 2004;19:182–186.

494 Li B, Jiang Y, Yuan F, Ye H. Exchange transfusion of least compatible blood for severe hemolytic disease of the newborn due to anti-Rh17. *Transfus Med* 2010;20: 66–69.

495 Blancher A, Roubinet F, Reid ME, *et al*. Characterization of a macaque anti-Rh17-like monoclonal antibody. *Vox Sang* 1998;75:58–62.

496 Vos GH, Vos D, Kirk RL, Sanger R. A sample of blood with no detectable Rh antigens. *Lancet* 1961;i:14–15.

497 Ishimori T, Hasekura H. A Japanese with no detectable Rh blood group antigens due to silent Rh alleles or deleted chromosomes. *Transfusion* 1967;7:84–87.

498 Kato-Yamazaki M, Okuda H, Kawano M, *et al*. Molecular genetic analysis of the Japanese amorph Rh$_{null}$ phenotype. *Transfusion* 2000;40:617–618.

499 Seidl S, Spielmann W, Martin H. Two siblings with Rh$_{null}$ disease. *Vox Sang* 1972;23:182–189.

500 Chérif-Zahar B, Matassi G, Raynal V, *et al*. Molecular defects of the *RHCE* gene in the Rh-deficient individuals of the amorph type. *Blood* 1998;92:639–646.

501 Huang C-H, Chen Y, Reid ME, Seidl C. Rh$_{null}$ disease: the amorph type results from a novel double mutation in RhCe gene on D-negative background. *Blood* 1998;92: 664–671.

502 Pérez-Pérez C, Taliano V, Mouro I, *et al*. Spanish Rh$_{null}$ family caused by a silent Rh gene: hematological, serological, and biochemical studies. *Am J Hematol* 1992;40: 306–312.

503 Rosa KA, Reid ME, Lomas-Francis C, *et al*. Rh$_{null}$ syndrome: identification of a novel mutation in *RHce*. *Transfusion* 2005;45:1796–1798.

504 Huang C-H, Cheng G, Liu Z, *et al*. Molecular basis for Rh$_{null}$ syndrome: identification of three new missense mutations in the Rh50 glycoprotein gene. *Am J Hematol* 1999;62: 25–32.

505 Huang C-H, Liu Z, Cheng G, Chen Y. Rh50 glycoprotein gene and Rh$_{null}$ disease: a silent splice donor is *trans* to a Gly$_{279}$→Glu missense mutation in the conserved transmembrane segment. *Blood* 1998;92:1776–1784.

506 Hyland CA, Chérif-Zahar B, Cowley N, *et al*. A novel single missense mutation identified along the RH50 gene in a composite heterozygous Rh$_{null}$ blood donor of the regulator type. *Blood* 1998;91:1458–1463.

507 Cowley NM, Saul A, Cartron J-P, Hyland CA. A single point mutation at a splice site generates a silent *RH50* gene in a composite heterozygous Rh$_{null}$ blood donor. *Vox Sang* 1999;76:247–248.

508 Chérif-Zahar B, Matassi G, Raynal V, *et al*. Rh-deficiency of the regulator type caused by splicing mutations in the human *RH50* gene. *Blood* 1998;92:2535–2540.

509 Kawano M, Iwamoto S, Okuda H, *et al*. A splicing mutation of the *RHAG* gene associated with the Rh_{null} phenotype. *Ann Hum Genet* 1998;62:107–113.

510 Tian L, Song N, Yao Z-Q, Huang M, Hou L. A family of the Chinese Rh_{null} individual of the regulator type: a novel single missense mutation identified in *RHAG* gene. *Transfusion* 2011;51:2686–2689.

511 Bruce LJ. Red cell membrane transport abnormalities. *Curr Opin Hematol* 2008;15:184–190.

512 Huang C-H, Cheng G-J, Reid ME, Chen Y. Rh_{mod} syndrome: a family study of the translation-initiator mutation in the Rh50 glycoprotein gene. *Am J Hum Genet* 1999;64:108–117.

513 Chown B, Lewis M, Kaita H, Lowen B. An unlinked modifier of Rh blood groups: effects when heterozygous and when homozygous. *Am J Hum Genet* 1972;24:623–637.

514 Cartron J-P. *RH* blood group system and molecular basis of Rh-deficiency. *Baillière's Clin Haemat* 1999;12:655–689.

515 Scharberg A, Tsuneyama H, Ogasawara K, *et al*. Rhmod phenotype caused by double heterozygosity for two alleles of the RHAG gene. *Vox Sang* 2006;91(Suppl. 3):129 [Abstract].

516 Kamesaki T, Iwamoto S, Kajii E, *et al*. A new mutation detected in *RhAG* of a Japanese family with Rh_{mod} syndrome may form a longer RhAG protein. *Transfusion* 2002;42:383–384.

517 Lomas C, Storry JR, Spruell P, Moulds M. Apparent alloanti-C produced by r'^{S} people: five examples. *Transfusion* 1994;34(Suppl.):25S [Abstract].

518 Boettcher B, Hasekura H. Rh blood groups of Australian Aborigines in the tribe containing the original Rh_{null} proposito. *Vox Sang* 1971;21:200–209.

519 Levine P, Celano MJ, Falkowski F, *et al*. A second example of –––/––– or Rh_{null} blood. *Transfusion* 1965;5:492–500.

520 Senhauser DA, Mitchell MW, Gault DB, Owens JH. Another example of phenotype Rh_{null}. *Transfusion* 1970;10:89–92.

521 Gibbs BJ, Moores P. Rh_{null} red cells and pregnancy. *Vox Sang* 1983;45:83–86.

522 Gomez Casal G, Poderos Baeta C, Romero Colas MS, *et al*. Déficit de antigenos eritrocitarios como causa de patologia. II. Enfermedad del Rh-null. *Rev Clin Espanola* 1983;171:19–21.

523 Sistonen P, Palosuo T, Snellman A. Identical twins with the Rh_{null} phenotype of the regulator type in a Finnish Lapp family. *Vox Sang* 1985;48:174–177.

524 Snowden JA, Poole J, Bates AJ, *et al*. The Rh_{null} phenotype in an English individual: haematological, serological and immunochemical studies. *Clin Lab Haematol* 1997;19:143–148.

525 McGuire Mallory D, Rosenfield RE, Wong KY, *et al*. Rh_{mod}, a second kindred (Craig). *Vox Sang* 1976;30:430–440.

526 Stevenson MM, Anido V, Tanner AM, Swoyer J. Rh 'null' is not always null. *Br Med J* 1973;i:417.

527 Saji H, Hosoi T. A Japanese Rh_{mod} family: serological and haematological observations. *Vox Sang* 1979;37:296–304.

528 Ballas SK, Clark MR, Mohandas N, *et al*. Red cell membrane and cation deficiency in Rh null syndrome. *Blood* 1984;63:1046–1055.

529 Lubenko A, Contreras M, Portugal CL, *et al*. Severe haemolytic disease in an infant born to an Rh_{null} proposita. *Vox Sang* 1992;63:43–47.

530 Naoki K, Uda M, Uchiyama E, *et al*. Rh_{null} with naturally occurring antibody. *Transfusion* 1984;24:182–183.

531 Nagel V, Kneiphoff H, Pekker S, *et al*. Unexplained appearance of antibody in an Rh_{null} donor. *Vox Sang* 1972;22:519–523.

532 Harrison CR, Bryan EC, Huff RW. Variability in the severity of hemolytic disease of the newborn in a patient with Rh null syndrome and high titre anti-Rh29. *Vox Sang* 2002;83(Suppl. 2):148–149 [Abstract].

533 Kijkornpan S, Tardtong P, Poole J, *et al*. A successful treatment of Rh hemolytic disease of the newborn from an Rh null mother with anti-Rh 29. *Vox Sang* 2002;83(Suppl. 2):149 [Abstract].

534 Badon SJ, DeLong EN, Cable RG, Jagathambal K. Rh_{null} disease: a case report. *Transfusion* 1998;38(Suppl.):35S [Abstract].

535 Weiner W, Vos GH. Serology of acquired hemolytic anemias. *Blood* 1963;22:606–613.

536 Sturgeon P. Haematological observations on the anemia associated with blood type Rh_{null}. *Blood* 1970;36:310–320.

537 Schmidt PJ, Lostumbo MM, English CT, Hunter OB. Aberrant U blood group accompanying Rh_{null}. *Transfusion* 1967;7:33–34.

538 McGinniss MH, Kaplan HS, Bowen AB, Schmidt PJ. Agglutinins for 'null' red blood cells. *Transfusion* 1969;9:40–42.

539 McGinniss MH, Schmidt PJ, Perry MC. An autoagglutinin for 'null' red blood cells. *26th Ann Mtg Am Ass Blood Banks*, 1973:114–115 [Abstracts].

540 Nash R, Shojania AM. Hematological aspect of Rh deficiency syndrome: a case report and a review of the literature. *Am J Hematol* 1987;24:267–275.

541 Kuypers F, van Linde-Sibenius-Trip M, Roelofsen B, *et al*. Rh_{null} human erythrocytes have an abnormal membrane phospholipid organization. *Biochem J* 1984;221:931–934.

542 Lauf PK, Joiner CH. Increased potassium transport and ouabain binding in human Rh_{null} red blood cells. *Blood* 1976;48:457–468.

543 Issitt PD. Null red blood cell phenotypes: associated biological changes. *Transfus Med Rev* 1993;7:139–155.

544 Arndt PA, Garratty G. Rh_{null} red blood cells with reduced CD47 do not show increased interactions with peripheral blood monocytes. *Br J Haematol* 2004;125:412–414.

545 Spruell P, Lacey P, Bradford MF, *et al*. Incidence of hemolytic disease of the newborn due to anti-D^{w}. *Transfusion* 1997;37(Suppl.):43S [Abstract].
546 Leschek E, Pearlman SA, Boudreaux I, Meek R. Severe hemolytic disease of the newborn caused by anti-Gonzales antibody. *Am J Perinat* 1993;10:362–364.
547 Orlina AR, Unger PJ, Lacey PA. Anti-Rh32 causing severe hemolytic disease of the newborn. *Rev Franc Transfus Immuno-Hémat* 1984;27:613–618.
548 Issitt PD, Wren MR, McDowell MA, Strohm PL, Roberts TM. Anti-Rh33, the second separable example, also made by a person who made anti-D and has C+ red cells. *Transfusion* 1986;26:506–510.
549 Kornstad L. A rare blood group antigen, Ol^{a} (Oldeide), associated with weak Rh antigens. *Vox Sang* 1986;50: 235–239.
550 Hoffman JJML, Overbeeke MAM, Kaita H, Loomans AAH. A new, low incidence red cell antigen (HOFM), associated with depressed C antigen. *Vox Sang* 1990;59:240–243.
551 Larson PJ, Lukas MB, Friedman DF, Manno CS. Delayed hemolytic transfusion reaction due to anti-Go^{a}, an antibody against the low-prevalence Gonzalez antigen. *Am J Hematol* 1996;53:248–250.
552 Reid ME, Sausais L, Zaroulis CG, *et al*. Two examples of an inseparable antibody that reacts equally well with D^{w}+ and Rh32+ red blood cells. *Vox Sang* 1998;75:230–233.
553 Devey ME, Voak D. A critical study of the IgG subclasses of Rh anti-D antibodies formed in pregnancy and in immunized volunteers. *Immunology* 1974;27:1073–1079.
554 Hardman JT, Beck ML. Hemagglutination in capillaries: correlation with blood group specificity and IgG subclass. *Transfusion* 1981;21:343–346.
555 Contreras M, de Silva M, Teesdale P, Mollison PL. The effect of naturally occurring Rh antibodies on the survival of serologically incompatible red cells. *Br J Haematol* 1987; 65:475–478.
556 Algora M, Barbolla L, Contreras M. Naturally occurring anti-D, anti-K, anti-Fy^{a}, and anti-Le^{ab}. *Vox Sang* 1991;61:141.
557 Hughes-Jones N, Ghosh S. Anti-D-coated Rh-positive red cells will bind the first component of the complement pathway, C1q. *FEBS Letts* 1981;128:318–320.
558 Waller M, Lawler SD. A study of the properties of the Rhesus antibody (Ri) diagnostic for the rheumatoid factor and its application to Gm grouping. *Vox Sang* 1962;7: 591–606.
559 Harboe M, Müller-Eberhard HJ, Fudenberg H, Polley MJ, Mollison PL. Identification of the components of complement participating in the antiglobulin reaction. *Immunology* 1963;6:412–420.
560 Ayland J, Horton MA, Tippett P, Waters AH. Complement binding anti-D made in a D^{u} variant woman. *Vox Sang* 1978;34:40–42.
561 Leader KA, Macht LM, Steers F, Kumpel BM, Elson CJ. Antibody responses to the blood group antigen D in SCID mice reconstituted with human blood mononuclear cells. *Immunology* 1992;76:229–234.
562 Crawford DH, Barlow MJ, Harrison JF, Winger L, Huehns ER. Production of human monoclonal antibody to Rhesus D antigen. *Lancet* 1983;i:386–388.
563 Kozbor D, Roder JC, Chang TH, Steplewski Z, Koprowski H. Human anti-tetanus toxoid monoclonal antibody secreted by EBV-transformed human B cells fused with murine myeloma. *Hybridoma* 1982;1:323–328.
564 Thompson JM, Lowe J, McDonald DF. Human monoclonal anti-D secreting heterohybridomas from peripheral B lymphocytes expanded in the CD40 system. *J Immunol Methods* 1994;175:137–140.
565 James K, Bell GT. Human monoclonal antibody production. Current status and future prospects. *J Immunol Methods* 1987;100:5–40.
566 Crawford DH, Azim T, Daniels GL, Huehns ER. Monoclonal antibodies to the Rh D antigen. In: Cash JD, ed. *Progress in Transfusion Medicine*, vol. 3. Edinburgh: Churchill Livingstone, 1988:175–197.
567 McCann MC, James K, Kumpel BM. Production and use of human monoclonal anti-D antibodies. *J Immunol Methods* 1988;115:3–15.
568 Fletcher A, Thomson A. The introduction of human monoclonal anti-D for therapeutic use. *Transfus Med Rev* 1995;9:314–326.
569 Thompson KM, Hough DW, Maddison PJ, Melamed MD, Hughes-Jones N. The efficient production of stable, human monoclonal antibody-secreting hybridomas from EBV-transformed lymphocytes using the mouse myeloma X63-Ag8.653 as a fusion partner. *J Immunol Methods* 1986;94: 7–12.
570 Hoogenboom HR, Marks JD, Griffiths AD, Winter G. Building antibodies from genes. *Immunol Rev* 1992;130: 41–68.
571 Marks JD, Ouwehand WH, Bye JM, *et al*. Human antibody fragments specific for human blood group antigens from a phage display library. *Bio/technology* 1993;11:1145–1149.
572 Hughes-Jones NC, Gorick BD, Bye JM, *et al*. Characterization of human blood group scFv antibodies derived from a V gene phage-display library. *Br J Haematol* 1994;88: 180–186.
573 Dziegiel M, Nielsen LK, Andersen PS, *et al*. Phage display used for gene cloning of human recombinant antibody against the erythrocyte surface antigen, rhesus D. *J Immunol Methods* 1995;182:7–19.
574 Edelman L, Margaritte C, Chaabihi H, *et al*. Obtaining a functional recombinant anti-rhesus (D) antibody using the baculovirus-insect cells expression system. *Immunology* 1997;91:13–19.
575 Miescher S, Zahn-Zabal M, De Jesus M, *et al*. CHO expression of a novel human recombinant IgG1 anti-RhD antibody isolated by phage display. *Br J Haematol* 2000;111: 157–166.

576 Bye JM, Carter C, Cui Y, *et al*. Germline variable region gene segment derivation of human monoclonal anti-Rh (D) antibodies. Evidence for affinity maturation by somatic hupermutation and repertoire shift. *J Clin Invest* 1992;90:2481–2490.
577 Boucher G, Broly H, Lemieux R. Restricted use of cationic germline V_H gene segments in human Rh(D) red cell antibodies. *Blood* 1997;89:3277–3286.
578 Hughes-Jones NC, Bye JM, Gorick BD, Marks JD. Synthesis of Rh Fv phage-antibodies using VH and VL germline genes. *Br J Haematol* 1999;105:811–816.
579 Perera WS, Moss MT, Urbaniak SJ. V(D)J germline gene repertoire analysis of monoclonal D antibodies and the implication for D epitope specificity. *Transfusion* 2000; 40:846–855.
580 Thorpe SJ, Ball C, Fox B, *et al*. Anti-D and anti-I activities are inseparable in $V_{4\text{-}34}$-encoded monoclonal anti-D: the same framework 1 residues are required for both reactivities. *Transfusion* 2008;48:930–940.
581 Proulx C, Boyer L, St-Amour I, Bazin R, Lemieux R. Higher affinity human D MoAb prepared by light-chain shuffling and selected by phage display. *Transfusion* 2002;42:59–65.
582 St-Amour I, Proulx C, Lemieux R, Bazin R. Modulations of anti-D affinity following promiscuous binding of the heavy chain with naïve light chains. *Transfusion* 2003;43:246–253.
583 Dohmen SE, Verhagen OJHM, Muit J, Ligthart PC, van der Schoot CE. The restricted use of IGHV3 superspecies genes in anti-Rh is not limited to hyperimmunized anti-D donors. *Transfusion* 2006;46:2162–2168.
584 Siegel DL, Czerwinski M, Spitalnik SL. Section 5: structural/genetic analysis of mAbs to blood group antigens. Coordinator's report. *Transfus Clin Biol* 2002;9:83–97.
585 Thorpe SJ, Boult CE, Stevenson FK, *et al*. Cold agglutinin activity is common among human monoclonal IgM Rh system antibodies using the $V_{4\text{–}34}$ heavy chain variable segment. *Transfusion* 1997;37:1111–1116.
586 Thorpe SJ, Turner CE, Stevenson FK, *et al*. Human monoclonal antibodies encoded by the V4–34 gene segment show cold agglutinin activity and variable multireactivity which correlates with the predicted charge of the heavy-chain variable region. *Immunology* 1998;93:129–136.
587 Thorpe SJ, Boult CE, Bailey SW, Thompson KM. The basis of unexpected cross-reactions shown by human monoclonal antibodies against blood group antigens as revealed by immunohistochemistry. *Appl Immunohistochem* 1996; 4:190–200.
588 Strobel E, Bauer MF. False-positive reactions of some monoclonal anti-c reagents. *Transfus Med* 2009;19: 141–145.
589 Lin M. Taiwan experience suggests that RhD typing for blood transfusion is unnecessary in southeast Asian populations. *Transfusion* 2006;46:95–98.
590 Yuan S, Davis R, Lu Q, Goldfinger D, Ziman AF. Low risk of alloimmunization to the D antigen in D– orthotopic liver transplant recipients receiving D+ RBCs perioperatively. *Transfusion* 2009;48:2653–2654.
591 Tovey LAD. Towards the conquest of Rh haemolytic disease: Britain's contribution and the role of serendipity. *Transfus Med* 1992;2:99–109.
592 Kumpel BM. On the mechanism of tolerance to the Rh D antigen mediated by passive anti-D (Rh D prophylaxis). *Immunol Letts* 2002;82:67–73.
593 Kumpel B. On the imunologic basis of Rh immune globulin (anti-D) prophylaxis. *Transfusion* 2006;46:1271–1275, corrected version: *Transfusion* 2006;46:1652–1656.
594 Brinc D, Lazarus AH. Mechanisms of anti-D action in prevention of hemolytic disease of the fetus and newborn. *Am Soc Hematol* 2009:185–191.
595 Jones ML, Wray J, Wight J, *et al*. A review of the clinical effectiveness of routine antenatal anti-D prophylaxis for rhesus-negative woman who are pregnant. *BJOG* 2004; 111:892–902.
596 Kumar S, Regan F. Management of pregnancies with RhD alloimmunisation. *BMJ* 2005;330:1255–1258.
597 Moise KJ Jr. Management of Rhesus alloimmunization in pregnancy. *Obstet Gynecol* 2008;112:164–176.
598 Gooch A, Parker J, Wray J, Qureshi H. Guideline for blood grouping and antibody testing in pregnancy. *Transfus Med* 2007;17:252–262.
599 Lambin P, Debbia M, Puillandre P, Brossard Y. IgG1 and IgG3 anti-D in maternal serum and on the RBCs of infants suffering from HDN: relationship with the severity of the disease. *Transfusion* 2002;42:1537–1546.
600 Hadley AG. Laboratory assays for predicting the severity of haemolytic disease of the fetus and newborn. *Transpl Immunol* 2002;10:191–198.
601 Béliard R. Monoclonal anti-D antibodies to prevent alloimmunization: lessons from clinical trials. *Transfus Clin Biol* 2006;13:58–64.
602 Kumpel BM. Efficacy of Rh monoclonal antibodies in clinical trials as replacement therapy for prophylactic anti-D immunoglobulin: more questions than answers. *Vox Sang* 2007;93:99–111.
603 Stott L-M, Barker RN, Urbaniak SJ. Identification of alloreactive T-cell epiotpes on the Rhesus D protein. *Blood* 2000;96:4011–4019.
604 Hall AM, Cairns LS, Altmann DM, Barker RN, Urbaniak SJ. Immune responses and tolerance to the RhD blood group protein in HLA-transgenic mice. *Blood* 2005;105:2175–2179.
605 Dooren MC, Kuijpers RWAM, Joekes EC, *et al*. Protection against immune haemolytic disease of newborn infants by maternal monocyte-reactive IgG alloantibodies (anti-HLA-DR). *Lancet* 1992;339:1067–1070.
606 Shepard SL, Noble AL, Filbey D, Hadley AG. Inhibition of the monocyte chemiluminescent response to anti-D-sensitized

red cells by FcγRI-blocking antibodies which ameliorate the severity of haemolytic disease of the newborn. *Vox Sang* 1996;70:157–163.

607 Astrup J, Kornstad L. Presence of anti-c in the serum of 42 women giving birth to c positive babies: serological and clinical findings. *Acta Obstet Gynecol Scand* 1977;56: 185–188.

608 Hardy J, Napier JAF. Red cell antibodies detected in antenatal tests on Rhesus positive women in south and mid Wales, 1948–1978. *Br J Obstet Gynaecol* 1981;88:91–100.

609 Kozlowski CL, Lee D, Shwe KH, Love EM. Quantitation of anti-c in haemolytic disease of the newborn. *Transfus Med* 1995;5:37–42.

610 Hackney DN, Knudtson EJ, Rossi KQ, Krugh D, O'Shaughnessy RW. Management of pregnancies complicated by anti-c isoimmunization. *Obstet Gynecol* 2004;103: 24–30.

611 Harrison CR, Hayes TC, Trow LL, Benedetto AR. Intravascular hemolytic transfusion reaction without detectable antibodies: a case report and review of the literature. *Vox Sang* 1986:51:96–101.

612 Goossens D, Champomier F, Rouger P, Salmon C. Human monoclonal antibodies against blood group antigens. Preparation of a series of stable EBV immortalized B clones producing high levels of antibody of different isotypes and specificities. *J Immunol Methods* 1987;101:193–200.

613 Foung SKH, Blunt J, Perkins S, Winn L, Grumet FC. A human monoclonal antibody to rh^G. *Vox Sang* 1986;50: 160–163.

614 Thompson K, Barden G, Sutherland J, Beldon I, Melamed M. Human monoclonal antibodies to C, c, E, e and G antigens of the Rh system. *Immunology* 1990;71:323–327.

615 Dohmen SE, Muit J, Ligthart PC, Verhagen OJHM, van der Schoot CE. Anti-e found in a case of hemolytic disease of the fetus and newborn make use of the IGHV3 superspecies genes. *Transfusion* 2008;48:194–195.

616 Michalewska B, Ejduk A, Pniewska K. Acute haemolytic transfusion reaction apparently caused by the 'enzyme-only' anti-E. *Vox Sang* 2005;89:61.

617 Issitt PD, Combs MR, Bredehoeft SJ, *et al*. Lack of clinical significance of 'enzyme-only' red cell alloantibodies. *Transfusion* 1993;33:284–293.

618 Devenish A, Kay LA. Hemolytic transfusion reactions due to anti-e+f detectable only by nonstandard serologic techniques. *Immunohematology* 1994;10:120–123.

619 Jemmolo G, Malferrari F, Tazzari PL, Conte R. Critical review of protease-treated red cell tests for the detection of irregular blood group antibodies during pregnancy. *Vox Sang* 1995;69:144.

620 Hundric-Haspl Z, Jurakovic-Loncar N, Grgicevic D. Enzyme-only blood group alloantibodies in pregnancy. *Transfusion* 1998;38:318.

621 Judd WJ, Davenport RD, Suchi M. An 'enzyme-only' IgG C3-binding alloanti-C causing an acute hemolytic trnasfusion reaction. *Transfusion* 2000;40(Suppl.):119S [Abstract].

622 Garner SF, Devenish A, Barber H, Contreras M. The importance of monitoring 'enzyme-only' red cell antibodies during pregnancy. *Vox Sang* 1991;61:219–220.

623 Petz LD, Garratty G. Immune hemolytic anemias, 2nd edn. Philadelphia: Churchill Livingstone, 2004.

624 Weiner W, Battey DA, Cleghorn TE, Marson FGW, Meynell MJ. Serological findings in a case of haemolytic anaemia with some general observations on the pathogenesis of this syndrome. *Br Med J* 1953;ii:125–128.

625 Bell CA, Zwicker H, Sacks HJ. Autoimmune hemolytic anemia: routine serologic evaluation in a general hospital population. *Am J Clin Pathol* 1973;60:903–911.

626 Issitt PD, Pavone BG, Goldfinger D, *et al*. Anti-Wr^b, and other autoantibodies responsible for positive direct antiglobulin tests in 150 individuals. *Br J Haematol* 1976;34:5–18.

627 Issitt PD, Zellner DC, Rolih SD, Duckett JB. Autoantibodies mimicking alloantibodies. *Transfusion* 1977;17: 531–538.

628 Longster GH, Johnson E. IgM anti-D as auto-antibody in a case of 'cold' auto-immune haemolytic anaemia. *Vox Sang* 1988;54:174–176.

629 Zimring JC, Cadwell CM, Spitalnik SL. Antigen loss from antibody-coated red blood cells. *Transfus Med Rev* 2009;23:189–204.

630 Anderson LD, Race GJ, Owen M. Presence of anti-D antibody in an Rh(D)-positive person. *Am J Clin Path* 1958; 30:228–229.

631 Chown B, Kaita H, Lewis M, Roy RB, Wyatt L. A 'D-positive' man who produced anti-D. *Vox Sang* 1963;8:420–429.

632 Krikler SH, Ferguson DJ, Akabutu JJ, Lomas CG. Transient anti-D in an Rh-positive patient with congenital dyserythropoietic anemia type II. *Transfusion* 1984;24:169–170.

633 Lalezari P, Talleyrand NP, Wenz B, Schoenfeld ME, Tippett P. Development of direct antiglobulin reaction accompanying alloimmunization in a patient with Rh^d (D, Category III) phenotype. *Vox Sang* 1975;28:19–24.

634 Macpherson CR, Stevenson TD, Gayton J. Anti-D in a D-positive man, with positive direct Coombs test and normal red cell survival. *Am J Clin Path* 1966;45:748–750.

635 Church A, Storry J, Jefferies LC. Uncommon presentation of auto anti-D in a D mosaic individual. *Transfusion* 1991;31(Suppl.):28S [Abstract].

636 Ramsey G. Red cell antibodies arising from solid organ transplants. *Transfusion* 1991;31:76–86.

637 Kim BK, Whitsett CF, Hillyer CD. Donor origin Rh antibodies as a cause of significant hemolysis following ABO-identical orthotopic liver transplantation. *Immunohematology* 1992;8:100–101.

638 Cserti-Gazdewich CM, Waddell TK, Singer LG, *et al*. Passenger lymphocyte syndrome with or without immune hemolytic anemia in al Rh-positive recipients of lungs from

rhesus alloimmunized donors: three new cases and a review of the literature. *Transfus Med Rev* 2009;23:134–145.

639 Ainsworth CD, Crowther MA, Treleaven D, *et al*. Severe hemolytic anemia post-renal transplantation produced by donor anti-D passenger lymphocytes: case report and literature review. *Transfus Med Rev* 2009;23: 155–159.

640 Swanson J, Sebring E, Sastamoinen R, Chopek M. Gm allotyping to determine the origin of the anti-D causing hemolytic anemia in a kidney transplant recipient. *Vox Sang* 1987;52:228–230.

641 Knoop C, Andrien M, Antoine M, *et al*. Severe hemolysis due to a donor anti-D antibody after heart-lung transplantation. Association with lung and blood chimerism. *Am Rev Respir Dis* 1993;148:504–506.

642 Larrea L, de la Rubia J, Arriaga F, Sanchez J, Marty ML. Severe hemolytic anemia due to anti-E after renal transplantation. *Transplantation* 1997;64:550–551.

643 Lasky LC, Warkentin PI, Kersey JH, *et al*. Hemotherapy in patients undergoing blood group incompatible bone marrow transplantation. *Transfusion* 1983;23:277–285.

644 Hows J, Beddow K, Gordon-Smith E, *et al*. Donor-derived red blood cell antibodies and immune hemolysis after allogeneic bone marrow transplantation. *Blood* 1986;67: 177–181.

645 Gandini G, Franchini M, de Gironcoli M, *et al*. Detection of an anti-RhD antibody 2 years after sensitization in a patient who had undergone an allogeneic BMT. *Bone Marrow Transplant* 2000;25:457–459.

646 Heim MU, Schleuning M, Eckstein R, *et al*. Rh antibodies against the pretransplant red cells following Rh-incompatible bone marrow transplantation. *Transfusion* 1988;28:272–275.

647 Tovey GH, Lockyer JW, Tierney RBH. Changes in Rh grouping reactions in a case of leukaemia. *Vox Sang* 1961;6:628–631.

648 Callender ST, Kay HEM, Lawler SD, *et al*. Two populations of Rh groups together with chromosmally abnormal cell lines in the bone marrow. *Br Med J* 1971;i:131–133.

649 Habibi B, Lopez M, Salmon C. Two new cases of Rh mosaicism. Selective study of red cell populations. *Vox Sang* 1974;27:232–242.

650 Bracey AW, McGinniss MH, Levine RM, Whang-Peng J. Rh mosaicism and aberrant MNSs antigen expression in a patient with chronic myelogenous leukemia. *Am J Clin Path* 1983;79:397–401.

651 Körmöczi GF, Dauber E-M, Haas OA, *et al*. Mosaicism due to myeloid lineage-restricted loss of heterozygosity as a cause of spontaneous Rh phenotype splitting. *Blood* 2007;110:2148–2157.

652 Marsh WL, Chaganti RSK, Gardner FH, *et al*. Mapping human autosomes: evidence supporting assignment of Rhesus to the short arm of chromosome No. 1. *Science* 1974;183:966–968.

653 Cooper B, Tishler PV, Atkins L, Breg WR. Loss of Rh antigen associated with acquired Rh antibodies and a chromosome translocation in a patient with myeloid metaplasia. *Blood* 1979;54:642–647.

654 Mohandas K, Najfield V, Gilbert H, Azar P, Skerrett D. Loss and reappearance of Rh_o (D) antigen in an individual with acute myelogenous leukemia. *Immunohematology* 1994;10: 134–135.

655 Chérif-Zahar B, Bony V, Steffensen R, *et al*. Shift from Rh-positive to Rh-negative phenotype caused by a somatic mutation within the *RHD* gene in a patient with chronic myelocytic leukaemia. *Br J Haematol* 1998;102:1263–1270.

656 Murdock A, Assip D, Hue-Roye K, *et al*. *RHD* deletion in a patient with chronic myeloid leukemia. *Immunohematology* 2008;24:160–164.

657 Salaru NNR, Lay WH. Rh blood group mosaicism in a healthy elderly woman. *Vox Sang* 1985;48:362–365.

658 Jenkins WJ, Marsh WL. Somatic mutation affecting the Rhesus and Duffy blood group systems. *Transfusion* 1965;5:6–10.

659 Northoff H, Goldmann SF, Lattke H, Steinbach P. A patient, mosaic for Rh and Fy antigens lacking other signs of chimerism or chromosomal disorder. *Vox Sang* 1984;47: 164–169.

660 Habibi B, Fouillade MT, Duedari N, *et al*. The antigen Duclos. A new high frequency red cell antigen related to Rh and U. *Vox Sang* 1978;34:302–309.

661 von dem Borne AEGK, Bos MJE, Lomas C, *et al*. Murine monoclonal antibodies against a unique determinant of erythrocytes, related to Rh and U antigens: expression on normal and malignant erythrocyte precursors and Rh_{null} red cells. *Br J Haematol* 1990;75:254–261.

662 Le Pennec PY, Klein MT, Le Besnerais M, *et al*. Immunological characterization of 18 monoclonal antibodies directed against Rh, G and LW molecules. *Rev Franc Transfus Immuno-Hémat* 1988;31:123–131.

663 Mallinson G, Anstee DJ, Avent ND, *et al*. Murine monoclonal antibody MB-2D10 recognizes Rh-related glycoproteins in the human red cell membrane. *Transfusion* 1990;30:222–225.

664 Poole J, Grimsley S, Ligthart P, *et al*. A novel RHAG blood group antigen associated with severe HDFN. *Vox Sang* 2011;101 (Suppl. 1):70 [Abstract].

665 Gemke RJBJ, Kanhai HHH, Overbeeke MAM, *et al*. ABO and Rhesus phenotyping of fetal erythrocytes in the first trimester of pregnancy. *Br J Haematol* 1986;64:689–697.

666 Dunstan RA, Simpson MB, Rosse WF. Erythrocyte antigens on human platelets. Absence of Rh, Duffy, Kell, Kidd, and Lutheran antigens. *Transfusion* 1984;24:243–246.

667 Dunstan RA. Status of major red cell blood group antigens on neutrophils, lymphocytes and monocytes. *Br J Haematol* 1986;62:301–309.

668 Chen B-S, Xu Z-X, Xu X, *et al*. *RhCG* is downregulated in oesophageal squamous cell carcinomas, but expressed in

multiple squamous epithelia. *Eur J Cancer* 2002;38:1927–1936.
669 Huang C-H, Ye M. The Rh protein family: gene evolution, membrane biology, and disease association. *Cell Mol Life Sci* 2010;67:1203–1218.
670 Quentin F, Eladari D, Cheval L, *et al*. RhBG and RhCG, the putative ammonia transporters, are expressed in the same cells in the distal nephron. *Am Soc Nephrol* 2003;14: 545–554.
671 Marini A-M, Urrestarazu A, Beauwens R, André B. The Rh (Rhesus) blood group polypeptides are related to NH_4^+ transporters. *Trends Biochem Sci* 1997; 22: 460–461.
672 Thomas GH, Mullins JG, Merrick M. Membrane topology of the Mep/Amt family of ammonium transporters. *Mol Microbiol* 2000;37:331–344.
673 Westhoff CM, Siegel DL, Burd CG, Foskett JK. Mechanism of genetic complementation of ammonium transport in yeast by human erythrocyte Rh-associated glycoprotein. *J Biol Chem* 2004;279;17443–17448.
674 Westhoff CM, Ferreri-Jacobia M, Mak DD, Foskett JK. Identification of the erythrocyte Rh blood group glycoprotein as a mammalian ammonium transporter. *J Biol Chem* 2002;277:12499–12502.
675 Benjelloun F, Bakouh N, Fritsch J, *et al*. Expression of human erythroid Rh glycoprotein (RhAG) enhances both NH_3 and NH_4^+ transport in HeLa cells. *Pflugers Arch – Eur J Physiol* 2005;450:155–167.
676 Hemker MB, Cheroutre G, van Zwieten R, *et al*. The Rh complex exports ammonium from human red blood cells. *Br J Haematol* 2003;122:333–340.
677 Ripoche P, Bertrand O, Gane P, *et al*. Human Rhesus-associated glycoprotein mediates facilitated transport of NH_3 into red blood cells. *Proc Natl Acad Sci USA* 2004;101:17222–17227.
678 Cartron J-P. Blood groups: genetics and physiology. *ISBT Sci Ser* 2010;5:27–45.
679 Heitman J, Agre P. A new face of the Rhesus antigen. *Nature Genet* 2000;26:258–259.
680 Soupene E, King N, Field E, *et al*. Rhesus expression in a green alga is regulated by CO_2. *Proc Natl Acad Sci USA* 2002;99:7769–7773.
681 Soupene E, Inwood W, Kustu S. Lack of Rhesus protein Rh1 impairs growth of the green alga *Chlamydomonas reinhardtii* at high CO_2. *Proc Natl Acad Sci USA* 2004;101: 7787–7792.
682 Endeward V, Cartron J-P, Ripoche P, Gros G. RhAG protein of the Rhesus complex is a CO_2 channel in the human red cell membrane. *FASEB J* 2008;22:64–73.
683 Huang C-H, Liu PZ, Cheng JG. Molecular biology and genetics of the Rh blood group system. *Semin Hematol* 2000;37:150–165.
684 Kitano T, Sumiyama K, Shiroishi T, Saitou N. Conserved evolution of the Rh50 gene compared to its homologous Rh blood group gene. *Biochem Biophys Res Comm* 1998;249:78–85.
685 Matassi G, Chérif-Zahar B, Pesole G, Raynal V, Cartron JP. The members of the *RH* gene family (*RH50* and *RH30*) followed different evolutionary pathways. *J Mol Evol* 1999;48:151–159.
686 Kitano Y, Saitou N. Evolutionary history of the *Rh* blood group-related genes in vertebrates. *Immunogenetics* 2000; 51:856–862.
687 Westhoff CM, Wylie DE. Investigation of the human Rh blood group system in nonhuman primates and other species with serologic and Southern blot analysis. *J Mol Evol* 1994;39:87–92.
688 Mouro I, Le Van Kim C, Chérif-Zahar B, *et al*. Molecular characterization of the Rh-like locus and gene transcripts from the Rhesus monkey (*Macaca mulatta*). *J Mol Evol* 1994;38:169–176.
689 Blancher A, Reid ME, Socha WW. Cross-reactivity of antibodies to human and primate red cell antigens. *Transfus Med Rev* 2000;14:161–179.
690 Suto Y, Ishikawa Y, Hyodo H, *et al*. Gene arrangement at the Rhesus blood group locus of chimpanzees detected by fiber-FISH. *Cytogenet Genome Res* 2003;101:161–165.
691 Carritt B, Kemp TJ, Poulter M. Evolution of the human RH (rhesus) blood group genes: a 50-year-old prediction (partially) fulfilled. *Hum Molec Genet* 1997;6:843–850.
692 Blancher A, Socher WW. The Rhesus system. In: Blancher A, Klein J, Socha WW, eds. *Molecular Biology and Evolution of Blood Group and MHC Antigens in Primates*. Berlin: Springer, 1997:147–218.
693 Wagner FF, Flegel WA. *RHCE* represents the ancestral *RH* position, while *RHD* is the duplicated gene. *Blood* 2002;99:2272–2273.
694 Fisher RA, Race RR. Rh gene frequencies in Britain. *Nature* 1946;157:48–49.
695 Perry GH, Xue Y, Smith RS, *et al*. Evolutionary genetics of the human Rh blood group system. *Hum Genet* 2012; 131:1205–1216.
696 Anstee DJ. The relationship between blood groups and disease. *Blood* 2010;115:4635–4643.
697 Chen YX, Peng J, Novaretti M, Reid ME, Huang C-H. Deletion of arginine codon 229 in the Rhce gene alters e and f but not c antigen expression. *Transfusion* 2004; 44:391–398.
698 Westhoff CM, Vege S, Halter Hipsky C, *et al*. *RHCE*ceTI* encodes partial c and partial e and is often in cis to *RHD*DIVa*. *Transfusion* 2012, in press.
699 Roussel M, Poupel S, Nataf J, *et al*. *RHD*DOL1* and *RHD*DOL2* encode a partial D antigen and are in cis with the rare *RHCE*ceBI* allele in people of African descent. *Transfusion* 2012, in press.
700 de Haas M, van der Ploeg CPB, Scheffer PG, *et al*. A nationwide fetal *RHD* screening programme for targeted antenatal and postnatal anti-D. *ISBT Sci Ser* 2012;7:164–167.
701 Scharberg A, Kanbur N, Baudendistel R, *et al*. PARG: a new low prevalence Rh blood group antigen. *Vox Sang* 2012;103(Suppl.1):57 [abstract].