Case Report

Molecular analysis of patients with weak D and serologic analysis of those with anti-D (excluding type 1 and type 2)

B.-N. Pham, M. Roussel, D. Gien, C. Andre-Botte, M. Ripaux, C. Auxerre, and P.-Y. Le Pennec

Whether or not patients whose red blood cells (RBCs) carry certain weak D types produce anti-D, and if they do whether it is allo- or autoanti-D, remains controversial. The aim of this study was to determine the serologic features of anti-D in individuals expressing a weak D other than type 1 or type 2 and to assess whether the anti-D was an allo- or autoantibody. Serologic D typing and molecular analyses were performed on 748 individuals. Serologic characterization of anti-D included autologous controls, direct antiglobulin test, elution, and titration of anti-D before and after adsorption of serum onto autologous RBCs. From molecular analyses, 459 individuals exhibited a weak D type. We described seven novel *RHD* variant alleles. The most frequent types of weak D were type 1 (30.1%), type 2 (23.7%), type 4.0 (10.2%), type 4.2.2 (20.3%), type 11 (3.9%), and type 15 (3.7%). Anti-D was identified in the sera of 9 of 47 individuals with weak D type 4.0, in 14 of 93 with weak D type 4.2.2, in 1 of 18 with weak D type 11, in 1 of 17 with weak D type 15, and in 1 weak D type 33 individual. Anti-D was demonstrated to be an alloantibody in weak D type 4.0, type 4.2.2, and type 15 individuals, but an autoantibody in weak D type 11 and type 33 individuals. In conclusion, only a complete serologic investigation of individuals with a given weak D type identified by molecular analysis allows concluding on the nature of the antibody. Transfusing weak D type 4.2.2 and type 15 patients with D– RBC units and proposing anti-D immunoprophylaxis to women with these weak D types should be considered. ***Immunohematology* 2013;29:55–62.**

Key Words: anti-D, weak D, partial D, D variant, *RHD* molecular analysis

The D (RH1) antigen is of clinical importance with regard to hemolytic disease of the fetus and newborn (HDFN) and transfusion medicine.[1] This antigen is still the leading cause of HDFN. Furthermore, anti-D has the potential to cause severe hemolytic transfusion reactions. The D antigen carried by the RhD protein is the most immunogenic of the Rh antigens. It has been described as a mosaic composed of multiple epitopes thought to be highly conformational.[2,3] The high immunogenicity of the D antigen is related to the fact that the entire RhD protein is absent from the red blood cell (RBC) membrane of individuals expressing a D– phenotype. Issitt and Anstee reported that approximately 80 percent of D– healthy volunteers transfused with one or more D+ blood units produce anti-D.[4] However, more recent data show that only 20 to 30 percent of D– patients transfused with one or more D+ units produce anti-D.[5–7]

The RhD protein is exclusively expressed on RBCs. From structural models, this protein has been predicted to consist of 12 transmembranous helixes with six extracellular regions. The Rh proteins are encoded by two homologous genes, *RHD* and *RHCE*. *RHD* encodes the RhD protein, whereas *RHCE* encodes the RhCE protein, carrying the C/c and E/e polymorphisms. Each gene consists of ten exons. The opposite orientation of the *RHD* and *RHCE* genes on chromosome 1 favors great diversity of these genes as a result of genomic rearrangements.[8] A large number of *RHD* alleles that result in D variants have been identified. The term *D variant* refers to RhD proteins associated with a quantitative or qualitative change of D expression. Classically, weak D, related to a quantitative change of D expression, has been defined as RBCs giving a weaker reaction than RBCs of the same Rh phenotype as reference, according to a defined anti-D reagent and a defined technique. Weak D differs from partial D, as the latter is associated with a qualitative change of D expression. This difference is of clinical importance because patients with a partial D phenotype have the potential to produce alloanti-D against the part of D they lack. More recently, D variants have been classified at the molecular level. Based on *RHD* sequence variations, genetic variants changing the amino acid sequence predicted to be in the membrane-spanning or intracellular regions of the RhD protein were considered to be a feature of weak D, whereas genetic variants changing the amino acid sequence predicted to be in the extracellular regions were considered to be a feature of partial D.[9] Until now, assignment of variant D type resulting from molecular analysis has reported more than 70 different weak D types.[10]

The weak D phenotype has been a subject of controversy since it was described in 1946.[11] Stratton first described RBCs reacting in an atypical manner with anti-D, introducing the term *"D^U" phenotype* corresponding to a weakened form of D. Considering the evolution of the anti-D reagents (mostly

monoclonal antibodies or MoAbs) and techniques over the years, no definitive serologic variation has been established in the majority of weak D types. For some time, it was generally accepted that patients with a weak D phenotype express a weak but normal entire D antigen.[8] Consequently, the possibility of D immunization related to weak D types was disregarded by many. However, some authors showed that patients with weak D phenotype may produce alloanti-D,[12–14] suggesting that most weak D types carry altered D antigen.[12] Recently, by performing a complete serologic investigation, we demonstrated that anti-D in weak D type 1 or weak D type 2 individuals were autoantibodies.[15] The aim of the present study was to determine the serologic features of anti-D in individuals expressing a weak D type, excluding type 1 or type 2, and to assess the clinically relevant potential for anti-D immunization in individuals with some weak D types.

Materials and Methods

Samples

Samples were obtained from 748 individuals referred to the Centre National de Référence pour les Groupes Sanguins (CNRGS) between 2007 and 2010 for different reasons, namely, depressed D phenotype, discordant results between two anti-D reagents, or anti-D in individuals with a D+ phenotype. EDTA blood (15 mL) and serum (15 mL) were obtained from these individuals for serologic D typing and molecular *RHD* testing.

Serology

D antigen status, together with C, E, c, and e status, was evaluated using two commercially available reagents: one monoclonal reagent (Ortho BioVue System, Ortho Clinical Diagnostics, Raritan, NJ), and one polyclonal reagent (DiaMed, Cressier/Morat, Switzerland), with gel testing according to the manufacturers' recommendations. These reagents are "CE marked," and they are licensed according to the European Community Standards.

D antigen reactivity was further analyzed using immunoglobulin (Ig) G MoAbs and an IgM MoAb. The IgG MoAbs were HIRO3 and HIRO7 from the Japanese Red Cross (Dr. Uchikawa), D7 from Dr. Sondag-Thüll (Liège, Belgium), 415-1E4 from Dominion Biologicals Ltd. (Dartmouth, Canada), P3x249, P3x35, and HM16 from Diagast (Loos, France), FEF3 from the International Blood Group Reference Laboratory, United Kingdom (Dr. Anstee), and RD7C2 from Institut Pasteur de Paris, France (Dr. Edelman). The IgM MoAb was P3BROU7 from Etablissement Français du Sang, Rennes, France (Dr. Martin).

The direct antiglobulin test (DAT) using gel method (anti-IgG and anti-C3d separately) was performed using the commercial kit DC-Screening II (DiaMed), according to the manufacturer's recommendations.

Elution was performed on the RBCs of individuals producing anti-D using an acid elution method (Gamma Elu-Kit II, Immucor Gamma, Norcross, GA). The kit was used according to the manufacturer's recommendations. The eluate was tested using an indirect antiglobulin test (IAT) gel method (DiaMed), according to the manufacturer's recommendations, with native and papain-treated RBCs.

Anti-D was identified by testing the serum against a panel of RBCs (Reference National Panel, Paris, France). The IAT gel method was performed according to the manufacturer's instructions (DiaMed), with native and papain-treated RBCs.

Adsorption with autologous RBCs was performed when samples were obtained in sufficient quantity and if there was no history of transfusion in the past 4 months. Adsorption of serum onto an equal volume of washed papain-treated autologous RBCs was performed at 37°C for 40 minutes, followed by centrifugation at 3100*g* for 10 minutes. The serum was adsorbed a minimum of three times. The remaining serum was tested against papain-treated RBCs in an IAT gel test.

Anti-D was titrated against a pool of papain-treated D+C–E+c+e– RBCs by using IAT at 37°C. The titration end point of anti-D was determined before and after adsorptions onto autologous RBCs.

Molecular Testing

Genomic DNA was extracted from whole-blood samples by using the MagNA Pure Compact Instrument (Roche Molecular Biochemicals, Mannheim, Germany) with the MagNA Pure Compact Nucleic Acid Isolation Kit I (Roche Molecular Biochemicals), according to the manufacturer's recommendations.

Allele-Specific Polymerase Chain Reactions

RHD allele-specific primer amplification assays, primer sequences, and polymerase chain reaction (PCR) conditions to detect the *RHD*weak D type 1* (*RHD*01W.1*) and the *RHD*weak D type 2* (*RHD*01W.2*) alleles were previously described.[16]

Exon Amplification for DNA Sequence Analysis

PCR exon amplification was performed on genomic DNA for sequence analysis. *RHD* primer sequences were previously described.[17,18] The ten *RHD* exon PCRs were performed in a thermal cycler on 100 ng of genomic DNA in a total reaction volume of 50 μL. Reaction mixtures contained 10 μM of each primer, 200 μM of each deoxyribonucleoside triphosphate (Amersham Biosciences, Piscataway, NJ), and 2.5 U of *Taq* DNA polymerase (Gold, Applied Biosystems, Carlsbad, CA) or 1 μL of *Taq* DNA polymerase for exon 5 (Advantage 2 polymerase mix, Clontech Laboratories, Mountain View, CA) in the appropriate buffer.

PCR products were purified (Exosap-it, Affymetrix UK Ltd., High Wycombe, UK), and cycle sequenced by using BigDye terminator chemistry (ABI-PRISM BigDye Terminator v1.1 Cycle Sequencing Kits, Applied Biosystems). Sequences were analyzed on an automated fluorescence-based ABI Prism 3130 (Applied Biosystems).

Results

Molecular Data

From the 748 individuals systematically tested using molecular *RHD* analysis, a total of 459 individuals (61.3%) exhibited a D variant that could be named weak D according to the RhesusBase classification.[10] The different weak D types found in this study and their prevalence are reported in Table 1. The *RHD* allele characterized by the 602C>G, 667T>G, 744C>T, 957G>A, and 1025T>C polymorphisms in *RHD* exons 4, 5, and 7 was named *RHD*weak D type 4.2.2* according to its first molecular description.[12,19] This *RHD* allele may be dubbed *DAR,* an allele which shares the 602C>G, 667T>G, and 1025T>C nucleotide polymorphisms found in weak D type 4.2.2 but lacks the silent 744C>T and 957G>A polymorphisms.[20] Weak D type 4.2.2 is currently listed as a "partial D" on the RhesusBase; International Society of Blood Transfusion (ISBT) allele terminology describes this allele as carrying the 602C>G, 667T>G, 744C>T, and 1025T>C polymorphisms. We described seven novel variant *RHD* alleles (Table 1). The N1, N2, N3, N4, N5, N6, and N7 novel variant alleles were derived from a *DCe* (R^1), *DCe, DcE* (R^2), *Dce* (R^0), *DcE, DCe,* and *DCe* haplotype, respectively.

Of the 748 individuals, 138 (18.5%) exhibited a partial D. As the focus of this study is weak D types, no further analysis or discussion of these samples is included here. Genomic DNA sequencing of all exons in 65 cases (8.7%) did not identify known variant *RHD* alleles. No exonic variants were found in most cases. cDNA analysis would be necessary to rule out other variants; these samples were not pursued further. An additional 86 samples (11.5%) were not pursued further; these included samples with two variant *RHD* alleles or incomplete or pending analysis.

Serologic Data

Anti-D was identified in 7 weak D type 1 individuals (5.1%), 6 weak D type 2 individuals (5.5%), 9 weak D type 4.0 individuals (19.1%), 14 weak D type 4.2.2 individuals (15.1%), 1 weak D type 11 individual (5.5%), 1 weak D type 15 individual (5.9%), and 1 weak D type 33 individual (Table 1).

Weak D Type 4.0

All 47 individuals expressing a weak D type 4.0 exhibited a D+C−E−c+e+ phenotype. The reactivity of weak D type 4.0 RBCs with selected D MoAbs is detailed in Table 2.

When using the panel of anti-D MoAbs, the strength of the positive reaction obtained varied when using IgG MoAbs; RD7C2 gave a negative reaction (data not shown).

Anti-D was detected in 9 of the 47 individuals (19.1%) exhibiting a weak D type 4.0. Serologic data from five cases (C-1 to C-5) are reported in Table 3. Transfusion history and pregnancies related to these individuals are listed in Table 3. No data were reported for the four other cases as a result of recent transfusion. To sum up, anti-D was demonstrated to be an alloantibody in one case (C-1). In this case, the anti-D reactivity was not significantly reduced after autologous adsorptions. Autologous controls, DAT, and eluate were negative. In contrast, anti-D was demonstrated to be an autoantibody in two other cases (C-4 and C-5). In these cases, the titer and score were significantly reduced (at least 2 dilutions for the titer and 16 for the score) after autologous adsorptions. In C-4, the autologous controls and the DAT were positive, and anti-D was present in the eluate. In C-5, autologous controls and DAT were negative. Finally, no conclusion about the nature (alloantibody or autoantibody) of anti-D could be reached from the incomplete serologic data in the last two cases (C-2 and C-3). No alloantibody against other antigens of blood group systems was detected in C-1 to C-5.

Weak D Type 4.2.2

All of the 93 individuals expressing a weak D type 4.2.2 exhibited a D+C−E−c+e+ phenotype. The reactivity of weak D type 4.2.2 RBCs with selected D MoAbs is detailed in Table 2.

When using the panel of anti-D MoAbs, the strength of reaction obtained varied when using HIRO3, HIRO7, D7, 415-1E4, P3x249, P3x35, and HM16 IgG MoAbs (data not shown).

Table 1. Weak D type and antibody status in individuals exhibiting a weak D phenotype identified in this study

Trivial names*	Molecular changes carried by the corresponding *RHD* allele	N	% of the total weak Ds	Number of individuals with anti-D (%)
Weak D type 1	809T>G (*RHD*01W.1*)	138	30.1%	7 (5.1%)
Weak D type 2	1154G>C (*RHD*01W.2*)	109	23.7%	6 (5.5%)
Weak D type 3	8C>G (*RHD*01W.3*)	6	1.3%	0
Weak D type 4.0	602C>G, 667T>G, 819G>A (*RHD*weak partial 4.0*)	47	10.2%	9 (19.1%)
Weak D type 4.2.2	602C>G, 667T>G, 744C>T, 957G>A, 1025T>C† (*RHD*weak 4.2.2*)	93	20.3%	14 (15.1%)
Weak D type 4.2.3	602C>G, 667T>G, 744C>T, 1025T>C	7	1.5%	0
Weak D type 5	446C>A (*RHD*01W.5*)	1	NA	0
Weak D type 10	1177T>C (*RHD*01W.10*)	2	NA	0
Weak D type 11	885G>T (*RHD*weak partial 11*)	18	3.9%	1 (5.5%)
Weak D type 15	845G>A (*RHD*weak partial 15*)	17	3.7%	1 (5.9%)
Weak D type 17	340C>T (*RHD*01W.17*)	1	NA	0
Weak D type 18	19C>T (*RHD*01W.18*)	4	NA	0
Weak D type 20	1250T>C (*RHD*01W.20*)	1	NA	0
Weak D type 27	661C>T (*RHD*01W.27*)	2	NA	0
Weak D type 33	520G>A (*RHD*01W.33*)	1	NA	1
Weak D type 38	833G>A (*RHD*01W.38*)	1	NA	0
Weak D type 56	65C>A (*RHD*01W.56*)	3	NA	0
Weak D type 59	1148T>C (*RHD*01W.59*)	1	NA	0
Novel weak D = N1	208C>T, 818C>T, 1195G>A	1	NA	0
Novel weak D = N2	542T>C	1	NA	0
Novel weak D = N3	730G>C	1	NA	0
Novel weak D = N4	731C>T	1	NA	0
Novel weak D = N5	751A>C	1	NA	0
Novel weak D = N6	884T>C	1	NA	0
Novel weak D = N7	1107A>C	1	NA	0
		Total = 459		Total = 39 (8.5%)

* Trivial names assigned by RhesusBase.[10]

† The *RHD* allele was named *RHD*weak D type 4.2.2* according to its first molecular description.[12] This *RHD* allele may be dubbed *DAR*, the sequence of which shares the 602C>G, 667T>G, and 1025T>C polymorphisms found in *RHD*weak D type 4.2.2* but lacks the silent 744C>T and 957G>A polymorphisms.[20]

NA = not applicable.

Table 2. Reaction strengths of samples with weak D type 4.0 and weak D type 4.2.2 in tests with MoAb anti-D

	Weak D type 4.0 (N = 47)		Weak D type 4.2.2 (N = 93)	
Strength	Monoclonal anti-D	Polyclonal anti-D	Monoclonal anti-D	Polyclonal anti-D
4+	25	4	20	3
3+	19	27	58	11
2+	1	14	12	44
1+	0	1	3	17
Negative	2	1	0	18

Anti-D was detected in 14 of the 93 individuals (15.1%) exhibiting a weak D type 4.2.2. Notably, it was found in combination with anti-Hr (RH18) in 8 of the 14 patients (C-12 to C-19). Serologic data related to anti-D investigation are reported in Table 4. Transfusion history and pregnancies related to these individuals are listed in Table 4. To sum up, anti-D was demonstrated to be an alloantibody in two cases (C-6 and C-7). After autologous adsorptions, the anti-D reactivity was not significantly reduced. Autologous controls, DAT, and eluate were negative. In the other 12 cases, no conclusion about the nature (allo- or autoantibody) of anti-D could be made from the incomplete serologic data.

Weak D Type 11

All 18 individuals expressing a weak D type 11 exhibited a D+C+E−c+e+ phenotype. Our results were concordant with previous studies reporting that the weak D type 11 was a weak form of D named DEL (D_{el}) when encoded by a *DCe* haplotype in the Caucasian population.[21]

Table 3. Serologic data of anti-D in weak D type 4.0 individuals

Cases			Anti-D		Autologous controls				Titer/Score*		
Case number/ gender	Transfusion history	Pregnancy	Native RBCs	Papain-treated RBCs	Native RBCs	Papain-treated RBCs	DAT	Direct elution	Before autologous adsorptions	After autologous adsorptions	Number of autologous adsorptions
C-1 Female	Yes	Unk	Negative	Positive	Negative	Negative	Negative	Negative	8/31	4/23	5
C-2 Female	Unk	Yes	Positive	Positive	Negative	Negative	Negative	Negative	128/55	NT	NA
C-3 Female	Yes	Unk	Negative	Positive	Negative	Negative	Negative	Negative	NT	NT	NA
C-4 Female	Yes	Yes	Positive	Positive	Positive	Positive	Positive (IgG)	Anti-D	16/44	1/5	5
C-5 Female	Yes	Unk	Positive	Positive	Negative	Negative	Negative	NT	8/35	1/2	4

*Anti-D tested and titrated against papain-treated RBCs (gel-test).
RBCs = red blood cells; DAT = direct antiglobulin test; Unk = unknown; NT = not tested; NA = not applicable; IgG = immunoglobulin G.

Table 4. Serologic data of anti-D in weak D type 4.2.2 individuals

Cases			Anti-D		Autologous controls				Titer/Score*		
Case number/ gender	Transfusion history	Pregnancy	Native RBCs	Papain-treated RBCs	Native RBCs	Papain-treated RBCs	DAT	Direct elution	Before autologous adsorptions	After autologous adsorptions	Number of autologous adsorptions
C-6 Female	Unk	Yes	Negative	Positive	Negative	Negative	Negative	Negative	8/35	4/23	4
C-7 Female	No	Yes	Positive	Positive	Negative	Negative	Negative	NT	16/40	8/25	3
C-8 Female	Unk	Yes	Positive	Positive	Negative	Negative	Negative	Negative	2/13	NT	NA
C-9 Female	Unk	Yes	Positive	Positive	Negative	Negative	Negative	Negative	NT	NT	NA
C-10 Male	Yes	NA	Positive	Positive	Negative	Negative	Negative	NT	NT	NT	NA
C-11 Male	Yes	NA	Negative	Positive	Positive	Positive	Negative	NT	NT	NT	NA
C-12 Female	Unk	Yes	Negative	Positive	Negative	Negative	Negative	NT	NT	NT	NA
C-13 Female	Unk	Yes	Negative	Positive	Negative	Negative	Negative	NT	NT	NT	NA
C-14 Female	Yes	Yes	Negative	Positive	Negative	Negative	Negative	NT	NT	NT	NA
C-15 Female	No	Yes	Negative	Positive	Negative	Negative	Negative	NT	NT	NT	NA
C-16 Female	No	Yes	Negative	Positive	Negative	Negative	Negative	NT	NT	NT	NA
C-17 Female	No	Yes	Positive	Positive	Negative	Negative	Negative	NT	NT	NT	NA
C-18 Female	No	Yes	Positive	Positive	Negative	Negative	Negative	NT	NT	NT	NA
C-19 Female	Yes	Yes	Negative	Positive	Negative	Negative	Negative	NT	NT	NT	NA

*Anti-D tested and titrated against papain-treated RBCs (gel-test).
RBCs = red blood cells; DAT = direct antiglobulin test; Unk = unknown; NT = not tested; NA = not applicable.

When using the panel of anti-D MoAbs, a very weak positive reaction was obtained with the HIRO3, D7, and P3x249 MoAbs only (data not shown).

Anti-D was detected in 1 of the 18 individuals (5.5%) exhibiting a weak D type 11. This patient produced an autoanti-D (Table 5, C-20). The anti-D reactivity (titer of 32, and score of 53 before autologous adsorptions) was not detected after autologous adsorptions. Autologous controls, DAT, and eluate were negative. No alloantibody against antigens of other blood group systems was detected.

Weak D Type 15

Among the 17 individuals expressing a weak D type 15, 9 exhibited the D+C–E+c+e+ phenotype described by Wagner et al.[9] However, 8 individuals exhibited a D+C+E–c+e+ phenotype. When using monoclonal anti-D or polyclonal anti-D, a negative reaction was observed for all samples.

When using the panel of anti-D MoAbs, a weak positive reaction was obtained with HIRO3, HIRO7, D7, and 415-1E4 IgG MoAbs (data not shown).

Alloanti-D was detected in 1 of the 17 individuals (5.9%) exhibiting a weak D type 15 (Table 5, C-21). After autologous adsorptions, anti-D was present, without significant reduction of either the titer or the score (1 dilution for the titer, and 10 for the score). Autologous controls, DAT, and eluate were negative. No alloantibody against antigens of other blood group systems was detected.

Table 5. Serologic data of anti-D in weak D type 11, 15, or 33 individuals

Cases				Anti-D		Autologous controls				Titer/Score*		
Case number/ gender	Transfusion history	Pregnancy	Weak D type	Native RBCs	Papain-treated RBCs	Native RBCs	Papain-treated RBCs	DAT	Direct elution	Before autologous adsorptions	After autologous adsorptions	Number of autologous adsorptions
C-20 Female	Yes	Unk	Weak D type 11	Negative	Positive	Negative	Negative	Negative	NT	32/53	Neg†	4
C-21 Female	No	Yes	Weak D type 15	Negative	Positive	Negative	Negative	Negative	Negative	64/69	32/62	4
C-22 Female	Yes	Unk	Weak D type 33	Negative	Positive	Positive	Positive	Negative	Anti-D	4/20	Neg	5

*Anti-D tested and titrated against papain-treated RBCs (gel-test).

†Neg indicates titer = 0/score = 0.

RBCs = red blood cells; DAT = direct antiglobulin test; Unk = unknown; NT = not tested.

Weak D Type 33

Only one D+C+E−c+e+ individual was found to express a weak D type 33. When using monoclonal anti-D or polyclonal anti-D, a negative reaction was observed when testing the blood sample from the weak D type 33 individual.

When using the panel of anti-D MoAbs, a positive reaction was obtained when using the P3BROU7 IgM MoAb and all IgG MoAbs except RD7C2.

This weak D type 33 patient produced an autoanti-D (Table 5, C-22). The anti-D reactivity was not detected after autologous adsorptions. Autologous controls were positive. Anti-D was present in the eluate despite a negative DAT. No alloantibody against antigens of other blood group systems was detected.

Discussion

From our experience, molecular *RHD* analysis appears to be the only reliable method to identify D variants. The D antigen expression of a given D variant appears variable when tested serologically. Our previous study clearly showed the variable reactivity with anti-D of RBCs expressing weak D type 1 and that of RBCs expressing weak D type 2.[15] In the same way, the present study clearly showed that the reactivity of RBCs expressing weak D type 4.0 or weak D type 4.2.2 ranged from 4+ to 0, despite using a defined validated method with a CE-marked anti-D monoclonal or polyclonal reagent. These data reinforce our feeling that the identification of a D variant should never be based on serologic criteria owing to its variable expression. Consequently, our recommendation would be to perform molecular *RHD* analysis when D expression is weakened when compared with normal D expression, whatever the level of decreased reactivity, or if anti-D is produced by a D+ individual.

Molecular testing has allowed identification of more than 150 different D variants. Based on *RHD* sequence variation, the widely used classification found on RhesusBase has assigned names to D variants, weak D or partial D, referenced in the ISBT allele terminology.[10,19] In our study, the most frequent weak D types were weak D type 1, type 2, type 4.0, type 4.2.2, type 11, and type 15. When available, it is useful to give *RH* haplotype information when describing a novel *RHD* allele. In accordance with data listed on RhesusBase, weak D type 1, type 2, type 4.0, and type 4.2.2 were found to be encoded by *DCe, DcE, Dce,* and *Dce* haplotypes respectively. Weak D type 11 was found to be encoded by a *DCe* haplotype, as expected when expression of this D variant corresponds to a "DEL phenotype."[21] Interestingly, our study first reports that weak D type 15 may be encoded either by the expected *DcE* haplotype or by a *DCe* haplotype. Whether the presence of C in *cis* affects the D antigen density requires further study. In our laboratory, the frequencies of weak D type 1, type 2, type 4.0, type 4.2.2, type 11, and type 15 were 30.1, 23.7, 10.2, 20.3, 3.9, and 3.7 percent, respectively, for the 459 weak D types identified using molecular methods. These particular frequencies are likely biased because of our recruitment strategy toward a population of African ancestry. Actually, weak D type 1, weak D type 2, and weak D type 3 have been reported to be the most prevalent D variants found in the white population.[22–26] In contrast, weak D type 4.0 and weak D type 4.2.2 have been reported to be mostly found in the African population.[10] Thus, our results are in accordance with the D variant frequencies expected when a mixed population is tested.[27–29]

Whether patients carrying certain molecular weak D types are prone to anti-D immunization has been questioned for many years and followed through a Rhesus immunization registry.[10] After our recent study about anti-D immunization in weak D type 1 or type 2 individuals,[15] we perform a complete serologic investigation whenever possible in individuals

expressing a weak D type other than type 1 or type 2. The serologic investigation includes autologous controls, DAT, elution, and titration of anti-D before and after adsorption of serum onto autologous RBCs. In our experience, titration of anti-D before and after adsorption of serum onto autologous RBCs is the most informative test, indicating the nature (allo- or auto-) of the antibody. In this study, anti-D was identified in 19.1 percent of weak D type 4.0 individuals, 15.1 percent of weak D type 4.2.2 individuals, 5.5 percent of weak D type 11 individuals, 5.9 percent of weak D type 15 individuals, and 1 weak D type 33 individual.

In weak D type 4.0 individuals, a complete serologic analysis was performed in three cases. Interestingly, the serologic data demonstrated that anti-D was an alloantibody in one case (C-1), but an autoantibody in the other two cases (C-4 and C-5). To our knowledge, only two cases of weak D type 4.0 individuals developing anti-D have been reported in the Rhesus immunization registry to date. Yet, the allo- or autoantibody nature of anti-D has not been determined. Based on a complete serologic investigation in C-1, we conclude that weak D type 4.0 individuals may produce alloanti-D. The presence of autoanti-D in weak D type 4.0 individuals may not weaken the case for our hypothesis because it may be attributable to an autoimmune reaction unrelated to the expression of this weak D type. However, other complete documented cases are required to definitively conclude this.

In weak D type 4.2.2 individuals, this report showed these anti-D to be alloantibodies. This conclusion is in accordance with the incomplete serologic data reported in previous studies.[12,20] In the only weak D type 11 individual producing anti-D, the serologic investigation allowed us to conclude that it was an autoantibody. The discordance between our result and the one listed in the Rhesus immunization registry reporting an alloanti-D without serologic data reinforces our hypothesis that discussions about anti-D in weak D patients should systematically be based on the four different tests discussed previously to determine whether anti-D is an allo- or an autoantibody. In the only weak D type 15 individual producing anti-D, data obtained with a complete serologic investigation demonstrated that the anti-D was an alloantibody. This result was in accordance with the data reported by Wagner et al.[12] Finally, in the only weak D type 33 individual producing anti-D, the latter was demonstrated to be an autoantibody.

The documentation of anti-D (auto- or alloantibody) is a major issue for weak D individuals.[30] Consequences are recommendations provided for transfusion strategy and to pregnant women regarding the frequency of the weak D types. The first concern is that anti-D production may only be studied in individuals expressing a D variant whose frequency is high enough for it to be spotted. The second concern is that only complete serologic investigation should be taken into account. Therefore, the demonstration of only autoanti-D in individuals expressing a given weak D type should lead to transfusing patients expressing the same weak D type with D+ RBC units and not giving anti-D immunoprophylaxis to pregnant women. So our recommendation is to transfuse weak D type 1 and type 2 patients with D+ RBC units and not give anti-D immunoprophylaxis to pregnant women of these types.[15] On the other hand, the demonstration of an alloanti-D immunization in an individual expressing a given weak D may lead to transfusing patients expressing the same weak D type with D– RBC units, and to proposing anti-D immunoprophylaxis to pregnant women of these types. Therefore, transfusing weak D type 4.2.2 and type 15 patients with D– RBC units and proposing anti-D immunoprophylaxis to pregnant women should be considered. Considering weak D type 4.0, we recommend the D– transfusion policy and anti-D immunoprophylaxis in our laboratory. However, other cases should be documented at the international level.

Finally, the lack of anti-D immunization is not a prerequisite for labeling a weak D type.[14] However, the notion of anti-D immunization should be taken into account. The present study clearly confirms that the RBCs of individuals with some weak D types carry altered D antigens, as alloanti-D was shown to be produced by patients expressing a D variant associated with *RHD* genetic variants encoding amino acid substitutions in the membrane-spanning or the cytoplasmic domain of the D protein, contrary to the concept that alloanti-D may be produced by patients expressing a D variant associated with *RHD* polymorphisms encoding amino acid substitutions in the extracellular loops of the D protein. These data point out that the discrimination between weak D types and partial D may be a "delicate affair" as the serologic definition, the predicted location of amino acid polymorphisms deduced from molecular sequences, or the notion of anti-D immunization may be flawed. Consequently, to make accurate clinical decisions in terms of transfusion policy and anti-D immunoprophylaxis, alloanti-D production in given weak D types should be the only criterion to consider.

Acknowledgments

The authors would like to thank C. Menanteau and the staff of CNRGS for their technical assistance, C. Salmaslian and M. Delaguette for secretarial assistance, and M. Jaber and C. Castanet for documentation.

References

1. Klein HG, Anstee DJ. Mollison's blood transfusion in clinical medicine. 11th ed. Malden, MA: Blackwell Publishing Ltd, 2005.
2. 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;94:3986–96.
3. Scott ML, Voak D, Liu W, Jones JW, Avent ND. Epitopes on Rh proteins. Vox Sang 2000;78(Suppl 2):117–20.
4. Issitt PD, Anstee DJ. Applied blood group serology. 4th ed. Miami, FL: Montgomery Scientific Publications, 1998.
5. Frohn C, Dümbgen L, Brand JM, Görg S, Luhm J, Kirchner H. Probability of anti-D development in D– patients receiving D+ RBCs. Transfusion 2003;43:893–8.
6. Yazer MH, Triulzi DJ. Detection of anti-D in D– recipients transfused with D+ red blood cells. Transfusion 2007;47:2197–201.
7. 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;48:1318–24.
8. Daniels G. Human blood groups. 2nd ed. Oxford, UK: Blackwell Science; 2002.
9. Wagner FF, Gassner C, Müller TH, Schönitzer D, Schunter F, Flegel WA. Molecular basis of weak D phenotypes. Blood 1999;93:385–93.
10. Wagner FF. RhesusBase, version 2.0. Available at http://www.uni-ulm.de/%7Efwagner/RH/RB/. Accessed February 14, 2013.
11. Stratton F. New Rh alleomorph. Nature 1946;158:25–8.
12. Wagner FF, Frohmajer A, Ladewig B, et al. Weak D alleles express distinct phenotypes. Blood 2000;95:2699–708.
13. McGann H, Wenk RE. Alloimmunization to the D antigen by a patient with weak D type 21. Immunohematology 2010;26:27–9.
14. Flegel WA. How I manage donors and patients with a weak D phenotype. Curr Opin Hematol 2006;13:476–83.
15. Pham BN, 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;51:2679–85.
16. Ansart-Pirenne H, Asso-Bonnet M, Le Pennec PY, Roussel M, Patereau C, Noizat-Pirenne F. RhD variants in Caucasians: consequences for checking clinically relevant alleles. Transfusion 2004;44:1282–6.
17. Legler TJ, Maas JH, Köhler M, et al. *RHD* sequencing: a new tool for decision making on transfusion therapy and provision of Rh prophylaxis. Transfus Med 2001;11:383–8.
18. 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;100:4223–31.
19. International Society of Blood Transfusion (ISBT). Blood group allele terminology. Available at http://www.isbtweb.org/working-parties/red-cell-immunogenetics-and-blood-group-terminology/blood-group-terminology/blood-group-allele-terminology/. Accessed February 14, 2013.
20. Hemker MB, Ligthart PC, Berger L, van Rhenen DJ, van der Schoot CE, Wijk PA. 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;94:4337–42.
21. Wagner FF, Frohmajer A, Flegel WA. RHD positive haplotypes in D negative Europeans. BMC Genet 2001;2:10–24.
22. Flegel WA, Wagner FF. Molecular biology of partial D and weak D: implications for blood bank practice. Clin Lab 2002;48:53–9.
23. 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.
24. Le Maréchal C, Guerry C, Benech C, et al. Identification of 12 novel RHD alleles in western France by denaturing high-performance liquid chromatography analysis. Transfusion 2007;47:858–63.
25. Christiansen M, Samuelsen B, Christiansen L, Morbjerg T, Bredahl C, Grunnet N. Correlation between serology and genetics of weak D types in Denmark. Transfusion 2008;48:187–93.
26. Flegel WA. Blood group genotyping in Germany. Transfusion 2007;47(1 Suppl):47S–53S.
27. Chen Q, Flegel WA. Random survey for RHD alleles among D+ European persons. Transfusion 2005;45:1183–91.
28. 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;45:1554–60.
29. Dogic V, Bingulac-Popovic J, Babic I, et al. Distribution of weak D types in the Croatian population. Transfus Med 2011;21:278–9.
30. Garratty G. Do we need to be more concerned about weak D antigens? Transfusion 2005;45:1547–51.

Bach-Nga Pham (corresponding author), MD, PhD, Head of Unit, Michèle Roussel, Dominique Gien, Maryline Ripaux, and Carine Auxerre, Technicians, and Pierre-Yves Le Pennec, MD, Institut National de la Transfusion Sanguine (INTS), CNRGS, 20 rue Bouvier; BP 79, 75522 Paris cedex 11, France; and Christine Andre-Botte, MD, Etablissement Français du Sang, Lorraine Champagne, France.