Case Report

Weak D type 42 cases found in individuals of European descent

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Patient samples were referred to our immunohematology reference laboratory to investigate the presence of a weak D antigen. In the last 3 years, 26 samples were received. Serology and molecular analyses were performed to identify the weak D variant. *RHD* mRNA from all patients was reverse transcribed, and cDNA was sequenced. The results were compared with a normal *RHD* sequence to identify the polymorphisms causing the weak D phenotype. Five different already known *RHD* variants were observed: weak D type 1 (5 individuals), weak D type 2 (1 individual), weak D type 42 (17 individuals), weak D type 45 (1 individual), and partial D DNB (2 individuals). Surprisingly, weak D type 42 was prevalent in our population, whereas weak D type 1, 2, and 3 are the most prevalent variants elsewhere. Anti-D was found in six cases of weak D type 42. The higher prevalence of weak D type 42 could be the result of a founder effect. Additional studies are needed to estimate the frequency of this variant in the general population. ***Immunohematology* 2011;27:20–24.**

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The D antigen can induce Rh-incompatible blood transfusion reactions and hemolytic disease of the fetus and the newborn (HDFN). It is the next most clinically significant blood group antigen after ABO.[1–5] The lack of *RHD* in 15 percent of Caucasians, 3 to 5 percent of Africans, and less than 1 percent of Asians and the extreme immunogenicity of its encoded D antigen explain the high incompatibility risk, which is of great concern for blood transfusion specialists.[2,3,6]

To prevent alloimmunization of D– recipients and to avoid the administration of D+ blood to patients who already have developed an alloanti-D, blood donor typing should identify all donors expressing the D antigen.[3,4] Unfortunately, the serologic distinction between D+ and D– RBCs is not always straightforward.[7] This can be related to different reagents and to aberrant *RHD* alleles. Although hundreds of *RHD* variants have been described in the literature, they remain rare events with an estimated prevalence of 1 to 2 percent in Caucasians and slightly higher rates in African Americans and Hispanics.[8] As of January 2011, 76 weak D alleles have been described (types 1–76, plus 8 subtypes[9]).

These variants are classified according to the serology profile obtained with different anti-D reagents: weak D, partial D, and DEL, which is only demonstrated by adsorption-elution.[5,10–14] Weak D phenotypes express a reduced amount of D antigen of otherwise unchanged quality as a result of point mutations in the membrane-spanning domains or cytoplasmic loops of the protein. Anti-D alloimmunization is unusual in these individuals.[3,6,8,11,13,15–17]

On the other hand, partial D phenotypes lack one or more epitopes of the D protein and present antigenic modifications caused by *RHD/RHCE* hybrid genes or *RHD* point mutations mostly affecting external loops of the protein.[4–6] These individuals are prone to anti-D alloimmunization.

The weakest D phenotype, DEL, can only be detected by adsorption-elution techniques, from which it gets its name. Individuals with DEL phenotype are routinely typed as D– and are frequent in Asia.[4] Molecular analyses have demonstrated that DEL samples retain an intact *RHD* or have a large *RHD* portion in their genome.[5,13] Transfusion specialists are still debating the potential alloimmunization risk as it relates to the classification of aberrant *RHD* alleles, especially of DEL donors and patients.[5,18,19]

Several studies have concluded that weak D type 1, type 2, and type 3 are the most frequent, at least in Germany, Austria, the Netherlands, and France.[20] In our cohort of patients, weak D type 42 seems to be the most prevalent, with 17 samples identified in the last 3 years. This weak D was first described in two Canadian blood donors by Denomme et al.[7] In this study, we describe the serology and the molecular analyses done for these cases.

Materials and Methods

Serology

From October 2006 to January 2010, 26 Caucasian samples, all multi-generation French Canadians (4 men and 22 women) presenting a weaker D reaction (< 3+ before the indirect antiglobulin test phase (IAT)) or a normal strength

of 4+ with anti-D and negative direct antiglobulin test (DAT) were referred to our immunohematology reference laboratory (IRL).

The D phenotype was determined by the classic tube technique using up to three sources of anti-D: Gamma (IgM GAMA401 + IgG F8D8, ImmucorGamma, Norcross, GA), Novaclone (DBL; IgM D175–2 + IgG D4151E4, ImmucorGamma), and BioClone (IgM MAD2 + IgG [polyclonal], Ortho-Clinical Diagnostics, Markham, Ontario, Canada). The D antibodies were identified using the IAT gel card technique (ID-Micro Typing System, Ortho-Clinical Diagnostics) with Héma-Québec's red blood cell (RBC) panels. To distinguish autoantibodies from alloantibodies, autoadsorptions were performed if the patient had not been transfused in the last 3 months. An acid elution was performed following the manufacturer's instructions (EluKit Plus, Immucor Gamma) for the samples with positive DAT. The eluate was tested with D+ and D– RBCs.

Molecular Biology

All samples were analyzed at the molecular level in our Research and Development Laboratory. Messenger RNA was studied in all cases. Genomic DNA was analyzed only for the weak D type 42.

RNA

The reticulocyte-enriched buffy coat from a 7-mL EDTA blood tube was transferred to a fresh 2-mL tube containing 1 mL of Trizol (Invitrogen, Burlington, Ontario, Canada) and mixed well, or 500 µL of whole blood was added to RNA*later* (Applied Biosystems Inc. [ABI], Carlsbad, CA). The RNA isolation was performed following the manufacturers' specifications. RNA was quantified, reverse transcribed, and amplified by using the One-Step RT-PCR (Qiagen, Mississauga, Ontario, Canada). Forward primer 397 located in *RHD/RHCE* 5′ end, 5′-cac-agg-atg-agc-tct-aag-tac-3′, and reverse primer 625 located in *RHD* 3′UTR, 5′-taa-atg-gtg-aga-ttc-tcc-tc-3′, were used.[10,21] The full 1446-bp transcript was gel purified and sequenced (ABI 3130*xl* Genetic Analyzer). The sequence was compared with Genbank X63097.

DNA

Two hundred microliters of EDTA-collected blood was kept at –80°C before DNA extraction using QIAamp mini blood kit (Qiagen) following manufacturer's instructions. For weak D type 42 samples, *RHD* exon 9 was amplified from genomic DNA by classic touchdown PCR procedure. The PCR master mix contained 5% glycerol, 1× AmpliTaq Gold Buffer II, 2.5 mM $MgCl_2$, 200 µM dNTPs, 2.5 U AmpliTaq Gold, and 0.35 µM of forward primer 560-RHD-i8-67F, 5′-tga-gat-act-gtc-gtt-ttg-aca-cac-aat-act-tc-3′ located in *RHD* intron 8, and reverse primer 561-RGD-i9+62R, 5′-gtt-tta-ctc-ata-aac-agc-aag-tca-aca-tat-atc-ct-3′, located in *RHD* intron 9.[13] The hybrid Rhesus box was also examined following the process published by Wagner and colleagues.[22]

Weak D Type 42 PCR-SSP

A PCR–sequence-specific primer (SSP) assay was designed to facilitate the analysis of the weak D type 42. Genomic DNA was amplified with forward primer 560 described previously and reverse primer 1034-wRHD-42-as 5′-cta-tca-cgt-taa-tag-gtg-aac-aat-ctt-acC-A-3′ located part in *RHD* exon 9 and part in intron 9. The 'c' was mutated in the primer to avoid a long 'a' stretch. A 202-bp product was amplified only when the weak D type 42 allele was present.[23]

Results

Serology

The samples described in the course of this study were referred to our IRL because of a weak D phenotype or the presence of anti-D in D+ patients with negative DAT. Most consisted of prenatal follow-up and surgery patients for whom an alloimmunization was possible through pregnancy or transfusion. Table 1 summarizes the serology and molecular biology results.

The reactivity with the anti-D reagents ranged from 0 to 4+. Most weak D type 42 samples reacted 1+ to 2+ with Gamma-clone and Novaclone. The BioClone gave no reactions, except with patient 11 (3+). Surprisingly, patient 13 showed no reactivity with the three anti-D sources assayed. No adsorption-elution was attempted to clarify between a DEL and a D– phenotype.

In the case of weak D type 1 and weak D type 2, the anti-D reactivity was on average 2+ with the three reagents, except for the sample from patient 18 (4+), which presented autoanti-D and autoanti-C. The situation was different for the weak D type 45 sample and for the two samples with the DNB phenotype: in all three the reactivity was 4+ and an alloanti-D was found.

Molecular Biology

The mRNA sequence analysis of all 26 samples showed a polymorphism that could explain the weak D phenotype or the presence of alloanti-D. Seventeen samples were found to be weak D type 42 (65.4%, 1 man and 16 women), five were weak D type 1 (19.2%, 2 men and 3 women), one sample was found to be weak D type 2 (3.8%, 1 woman), one was weak D type 45 (3.8%, 1 woman), and two were

Table 1. Serology and molecular biology results

Case	Age/Sex	Diagnosis	Pregnancy	Transfusion	Anti-D reaction*			*RHD* variant	Antibodies
					Gamma	Novaclone	BioClone		
1	28/F	Pregnancy	Yes	No	1+	1+	NT	Weak D type 42	No
2	62/F	Renal failure	Yes	Yes	1+	1.5+	NT	Weak D type 42	No
3	74/F	Surgery	No	Yes	NT	2+	NT	Weak D type 42	autoanti-D alloanti-C (no anti-G)
4	30/F	Pregnancy	Yes	No	2+	2+	NT	Weak D type 42	No
5	75/F	Anemia	Yes	Yes	2+	2+	NT	Weak D type 42	autoanti-D alloanti-C (anti-G unlikely)†
6	80/F	Anemia	Yes	Yes	NT	2+	NT	Weak D type 42	No
7	80/M	Angioplasty	No	No	NT	2+	NT	Weak D type 42	No
8	22/F	Postpartum	Yes	No	2+	NT	0	Weak D type 42	No
9	46/F	Surgery	Yes	Yes	NT	2+	NT	Weak D type 42	alloanti-D alloanti-C (anti-G?)
10	20/F	Unknown	Unknown	Unknown	2+	NT	0	Weak D type 42	No
11	26/F	Pregnancy	Yes	No	2+	NT	3+	Weak D type 42	No
12	24/F	Pregnancy	Yes	No	1+	2+	NT	Weak D type 42	No
13	66/F	Surgery	No	Yes	0	0	0	Weak D type 42	alloanti-D alloanti-C (anti-G?)
14	65/F	Surgery	Yes	Yes	1+	2+	NT	Weak D type 42	No
15	80/F	Cancer	Unknown	No	NT	1+	NT	Weak D type 42	autoanti-D alloanti-C (anti-G unlikely)
16	24/F	Pregnancy	Yes	No	1+	1.5+	NT	Weak D type 42	No
17	36/F	Pregnancy	Yes	No	2.5+	2+	0	Weak D type 42	anti-D? alloanti-C (anti-G?)
18	65/F	Hysterectomy	Yes	Yes	NT	4+	NT	Weak D type 1	autoanti-D autoanti-C
19	33/F	Pregnancy	Yes	No	2+	2+	NT	Weak D type 1	No
20	65/M	Unknown	No	Yes	2+	2+	2+	Weak D type 1	No
21	33/F	Pregnancy	Yes	Unknown	2+	2+	2+	Weak D type 1	No
22	18/M	Splenectomy	No	No	NT	1+	NT	Weak D type 1	No
23	32/F	Pregnancy	Unknown	Yes	NT	2+	NT	Weak D type 2	No
24	81/F	Cancer	Unknown	Yes	NT	4+	NT	Weak D type 45	alloanti-D
25	51/F	Surgery	Yes	Yes	NT	4+	NT	DNB	alloanti-D alloanti-K
26	53/M	Aortic rupture	No	Yes	NT	4+	NT	DNB	alloanti-D

*Gamma (ImmucorGamma; IgM GAMA401+IgG F8D8); Novaclone (DBL; IgM D175-2+IgG D4151E4); BioClone (Ortho; IgM MAD2+IgG [polyclonal]).
†Recently transfused.
NT = not tested.

DNBs (7.7%, 1 man and 1 woman). A hybrid Rhesus box was amplified in all cases, indicating a hemizygote *RHD* (Dd).

The cDNA profile of weak D type 42 on agarose gel is characterized by three distinct bands of lower molecular weight compared with normal cDNA (Fig. 1). To facilitate the analysis of the weak D type 42 variant, a PCR-SSP assay was designed. Figure 2 illustrates the clear results obtained for two normal control samples (lanes 3 and 4) and two weak D type 42 samples (lanes 5 and 6).

Discussion

Weak D phenotypes are thought to express quantitative D antigen variant. According to Wagner and Flegel,[22]

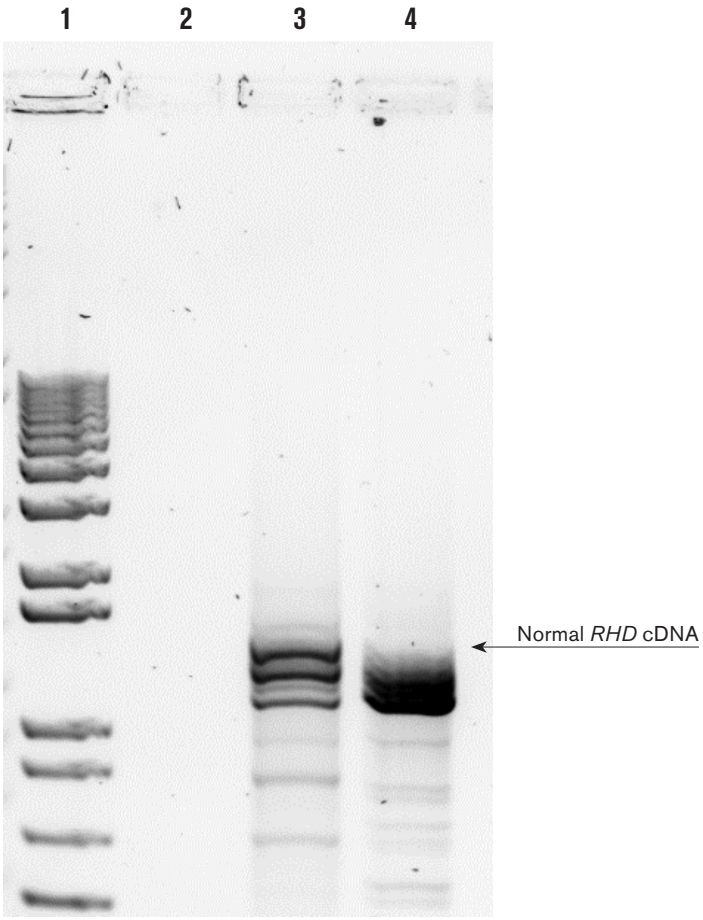

Fig. 1 *RHD* RT-PCR profile. After mRNA isolation, a reverse transcription followed by a PCR amplification was performed. The amplified products were visualized on an agarose gel. In a normal sample (lane 3), three major bands were observed. In a weak D type 42 sample (lane 4), three major bands of lower molecular weight were consistently seen. (Lane 1, molecular weight marker; lane 2, no DNA control; lane 3, normal *RHD* sample; lane 4, weak D type 42 sample [case 4 in Table 1].)

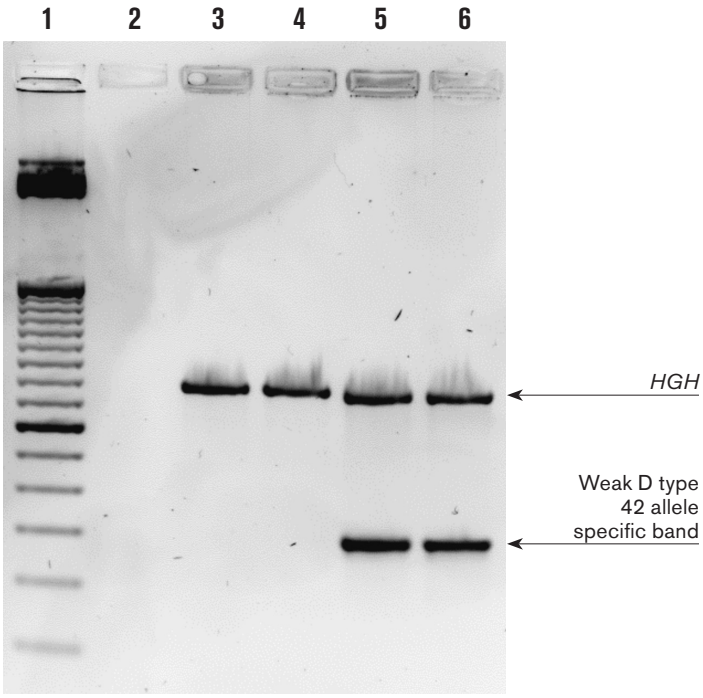

Fig. 2 PCR-SSP assay for weak D type 42. This multiplex PCR assay was done on genomic DNA. The amplified *HGH* internal control was present in all samples (432 bp). The band specific for the weak D type 42 allele at 202 bp was observed only when this variant allele was present. (Lane 1, molecular weight marker; lane 2, no DNA control; lanes 3 and 4, normal *RHD* samples; lanes 5 and 6, weak D type 42 samples [cases 7 and 8 in Table 1].)

position 1226, involved in the weak D type 42 variant (1226A>T, K409M), should be located inside the cell, which meets the weak D definition. In the original publication describing this D variant, no alloantibodies were reported.[7] In our study, anti-D as well as anti-C was present in the serum of six patients. Some were classified as alloanti-D or -C, whereas others were autoanti-D or -C. Anti-G was not ruled out in these cases because adsorption-elution is only performed on prenatal cases according to our procedures.

The weak D type 1 patients had no anti-D, except patient 18, who had autoanti-D. The only patient with a weak D type 45 had alloanti-D as did the two patients with the DNB. Other D variants have been reported to be associated with alloimmunization.[3,6,8,11,13,15–17]

The variation in the D typing observed among weak D type 42 patients could be attributable to the D antigen density at the RBC surface. No flow cytometry analyses were performed to prove this hypothesis. In the case of patient 13, no reactions were seen with the three anti-D sources used. Because the DAT on the patient's sample was positive, the analyses were not tested by the IAT. An adsorption-elution would have been useful to establish a clear D type for this patient (DEL or D), but additional sample was not available.

During the course of this study, we have been aware of only one case of HDFN involving a weak D type 42 mother (patient 17). This patient had a suspected anti-D and an alloanti-C. We could not determine whether the HDFN was caused by the suspected anti-D or the alloanti-C.

During the years, several studies have shown that weak D types 1, 2, and 3 are the most prevalent in Caucasians. In our multi-generation French Canadian population, we see a different result, in which weak D type 42 seems to be the most frequent. This could be caused by a founder effect directly linked to our history. Immigration to Quebec started in the early 17th century. Most immigrants came from France. By the end of the 18th century, land along the St. Lawrence River was occupied. To make room for the continued arrival of new immigrants, territories were opened for colonization and closed a few years later when enough families had settled. This geographic particularity

limited access to a diversified genetic background, causing a founder effect. It is particularly observed in genetic diseases.[24]

Our blood donor population consists of 99.6 percent multi-generation French Canadians (Yvan Charbonneau, Ing., VP Exploitation Héma-Québec, personal communication). It would be interesting to evaluate the prevalence of the weak D type 42 in the general population. A preliminary study found no weak D type 42 samples (data not shown) among 500 D+ blood donors originating from different regions of the province. This study should be pursued in the near future.

Additional note: Since this work was submitted, more samples were analyzed. The total number of weak D type 42 samples has now reached 50. Additional *RHD* variant samples were found: nineteen weak D type 1, four weak D type 2, and six other unique variants.

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