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Rh discrepancies caused by variable reactivity of partial and weak D types with different serologic techniques

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BACKGROUND: RhD discrepancies between current and historical results are problematic to resolve. The investigation of 10 discrepancies is reported here.
STUDY DESIGN: Samples identified were those that reacted by automated gel technology and were negative with an FDA-approved reagent. Reactivity with a commercially available panel of monoclonal anti-D was performed. Genomic DNA was evaluated for *RHD* alleles with multiplex *RHD* exon polymerase chain reaction (PCR), weak D PCR-restriction fragment length polymorphism, and *RHD* exon 5 and 7 sequence analyses.
RESULTS: The monoclonal anti-D panel identified two samples as DVa, yet possessed the *DAR* allele. Two weak D Type 1 samples had a similar monoclonal anti-D profile, but only one reacted directly with one of two FDA-approved anti-D. Only two of four weak D Type 2 samples reacted directly with one FDA-approved anti-D, and their D epitope profile differed.
CONCLUSIONS: The monoclonal anti-D reagents did not distinguish between partial and weak D Types 1 and 2. Weak D Types 1 and 2 do not show consistent reactivity with FDA-approved reagents and technology. To limit anti-D alloimmunization, it is recommended that samples yielding an immediate-spin tube test cutoff score of not more than 5 (i.e., ≤1+ agglutination) or a score of not more than 8 (i.e., ≤2+ hemagglutination) by gel technology be considered D– for transfusion and Rh immune globulin prophylaxis. That tube test anti-D reagents react poorly with some Weak D Types 1 and 2 red cells is problematic, inasmuch as they should be considered D+ for transfusion and prenatal care. Molecular tests that distinguish common partial and Weak D types provide the solution to resolving D antigen discrepancies.

RhD variants are classified for clinical purposes into one of three groups: partial D variants (including category), weak D types, or nonfunctional and D-elution alleles.[1] Partial D variants lack D antigen epitopes,[2] and weak D types generally present all D epitopes albeit some epitopes show variability depending on the monoclonal anti-D.[3,4] Individuals who harbor partial D variant alleles have the potential to make alloanti-D,[5] whereas common weak D types do not pose such a risk.[6] The distinction is important because the appropriate assignment of D antigen status determines the selection of blood products and perinatal management to prevent anti-D hemolytic disease of the fetus and newborn.

The intent of FDA-approved anti-D serologic typing reagents is to ensure that the appropriate D antigen status is assigned, such that the most common partial D variants (e.g., DVI) are nonreactive by the immediate-spin (IS) tube test and therefore are classified as D–. Exceptions do exist, however, and in light of changing technologies and reagents along with the multitude of variants with weakened or altered expression, discrepancies are noted.[7,8] When D antigen discrepancies arise clinicians are faced

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with assigning the appropriate D antigen status, so that the appropriate (D– or D+) blood products can be administered. Moreover, in an era of informed consent, the impact of a D antigen discrepancy can create confusion over the use of Rh immune globulin.[9]

The D antigen is a 417 amino acid moiety with a tertiary conformation that can be described in a 30 epitope model.[10,11] More than 175 alleles have been characterized at molecular level.[12] Some D variants have similar epitope profiles when evaluated with monoclonal anti-D reagents. For example, DBS, DFR, and some category DVa variants share some exon 5 amino acid replacements and some D epitopes.[13] For the most part, similar serologic profiles do not create a problem for the interpretation of D antigen status when a partial D variant is suspected. The *DAR* allele is one such allele that is at risk for anti-D alloimmunization due to the lack of D antigen epitopes. With the 37-epitope model, *DAR* is thought to lack epitopes 2, 4, 7, 10, 11, 17, 18, 21, 22, 23, and 31 to 35 and to have an altered expression for many of the remaining epitopes.[14] Thus, specific monoclonal anti-D reagents have become popular reagents to identify partial D variants quickly, which help clinicians make an informed decision on the appropriate D antigen status. Alternatively, molecular analyses including direct nucleotide sequencing have been used to resolve *RHD* discrepancies, although these specialized techniques have a less than ideal turnaround time.[15-18]

We evaluated a group of 10 patients who were identified on the basis of a D antigen discrepancy with historical records or tube test results. We evaluated the expression of the D antigen with three FDA-approved anti-D reagents and a panel of non–FDA-approved monoclonal anti-D reagents and performed molecular analyses to resolve the discrepancies. We show here that the *DAR* allele and category DVa variants have similar monoclonal anti-D profiles. More importantly, we show that an FDA-approved reagent can have variable reactivity with various examples of weak D types, and in some instances, these weak D types can mimic partial D variants in their monoclonal anti-D epitope profile.

MATERIALS AND METHODS

Sample selection

Samples were initially identified on the basis of a discrepancy between a newly implemented automated gel technology test (ProVue, Ortho Clinical Diagnostics, Raritan, NJ) and historical records. In all instances, the gel Rh typing test indicated that the patient was D+ when the patient's historical record was D–. Other discrepancies were noted when an IS tube test was performed to confirm the Rh type before the release of electronic crossmatch-compatible blood.

Serologic analysis

Once a discrepancy was noted with the automated gel test, the samples were further analyzed by IS tube test with two anti-D reagents (Gamma-clone, Immucor, Inc., Norcross, GA; BioClone, Ortho Clinical Diagnostics, Raritan, NJ) in accordance with the manufacturers' recommendations for the IS tube test. Hemagglutination was graded and scored as described by Marsh.[19] In addition, the red cells (RBCs) were tested with a panel of monoclonal antibodies (MoAbs) indicated as A through L (Alba Bioscience, Edinburgh, UK). Included in the serologic analysis of the discrepancies was a complete Rh C, c, E, e phenotype, with the most probable genotype method used to assign the Rh genotype.[20]

RHD molecular analyses

Samples with a discrepant IS tube test result were analyzed for partial D variants with a multiplex polymerase chain reaction (PCR) protocol for seven RHD-specific exons 3 through 7 and 9 and exon 10.[21,22] Those samples with RHD-specific exons were further analyzed for the presence of weak D Type 1 through Type 3 by PCR-restriction fragment length polymorphism (RFLP).[3,23,24] Samples that could not be assigned an RHD allele were subjected to direct automated sequencing of RHD exon 5.[23] A separate PCR amplification product with the exon 7 primers alone was subjected to direct sequence analysis to evaluate the 1025-nucleotide position for presence of the DAR allele. Comparative analysis with the reference RHD sequence NM_016124 was performed with the pair-wise basic local alignment search tool (BLAST).[25]

RESULTS

Monoclonal anti-D pattern

The MoAbs were chosen on the basis of the potential to provide information for the presence of partial D phenotypes. Table 1 summarizes the reactivity pattern of these monoclonal anti-D reagents evaluated with archived frozen RBCs of various known partial D types including partial DIIIa, DIIIb, and DIIIc; DIVa; and DIVb. Consistent with the manufacturer's monograph, the monoclonal anti-D reagents E and I are nonreactive with partial DVa RBCs. The precise allelic classification of our archived DVa RBCs cannot be determined, however. Monoclonal B was reactive with a single example of partial DIVa but not DIVb. In addition, some of our archived cells reacted with certain monoclonal anti-D in difference to the manufacturer's reaction profile. The following monoclonal anti-D were reactive with our cells: monoclonal A with DBT; C with DVa and DFR; D with DBT; H with DII; and L with RoHAR. The serologic reactivity with the 12 monoclonal anti-D reagents resulted in four patterns among the 10

TABLE 1. Monoclonal anti-D epitope profiles for partial D phenotypes (summarized from in-house testing) and the reactivity with FDA-approved antiserum samples*

	Monoclonal anti-D epitope profile												IS anti-D result		
	A	B	C	D	E	F	G	H	I	J	K	L	P	G	B
DII	+	+	+	+	+	NT	+	+	+	+	+	+	+	+	+
DIIIa	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
DIIIb	0	+	+	+	NT	+	+	+	+	+	+	+	+	+	+
DIIIc	+	+	+	+	NT	+	+	+	+	+	+	+	NT	NT	NT
DIVa	+	+	0	+	0	0	0	0	0	+	+	+	+	+	+
DIVb	+	0	0	+	0	0	0	0	0	+	+	+	NT	NT	NT
DVa	+	+	+	+	0	+	+	+	0	+	+	+	+	+	0
DVI	0	+	0	0	0	+	+	+	0	0	0	0	0	0	0
DVII	+	+	+	+	+	+	+	+	+	0	+	+	NT	NT	NT
DFR	(+)	+	+	+	+	NT	+	+	0	0	+	0	0	0	0
DBT	+	0	0	+	0	NT	0	0	0	+	0	+	+	+	0
RoHAR	0	0	0	0	0	0	0	0	0	0	0	+	+	±	0

* A to L = monoclonal anti-D from Alba BioSciences. P = ProVue gel test; G = Gamma-clone tube test; B = BioClone tube test. + = positive; 0 = negative; (+) = weak; ± = very weak; NT = not tested.

TABLE 2. Results of the monoclonal anti-D epitope profile, FDA-approved anti-D serotyping, and the molecular analyses for samples with a D phenotype discrepancy*

	Allele	MoAb profile	Monoclonal anti-D epitope reactivity												Anti-D score		
			A	B	C	D	E	F	G	H	I	J	K	L	P	G	B
Patients																	
R_or	DAR	DVa	10	10	10	10	0	10	10	10	0	10	10	10	8	8	5
R_or	DAR	DVa	10	10	9	10	0	10	10	10	0	10	10	10	8	8	0
R_1r	Weak D Type 1		10	10	10	10	10	10	10	10	5	10	10	10	8	10	5
R_1r	Weak D Type 1		10	10	10	10	10	10	10	10	5	10	10	10	8	7	0
R_1r	Unknown		10	10	10	10	9	10	10	10	0	6	10	10	8	10	5
R_1r	Unknown		10	10	10	10	10	10	10	10	0	10	10	10	8	10	6
R_2r	Weak D Type 2		10	10	10	10	10	10	10	10	0	9	10	10	8	10	5
R_2r	Weak D Type 2		10	10	10	10	10	10	10	10	5	10	10	10	8	8	5
R_2r	Weak D Type 2		10	10	10	10	10	10	10	10	0	10	10	10	8	7	0
R_2r	Weak D Type 2		10	10	10	10	10	10	10	10	6	10	10	10	8	7	0
Controls																	
R_1r	*RHD+*	Normal	12	12	12	12	11	12	11	11	11	12	12	12	11	11	12
R_2r	*RHD+*	Normal	11	11	11	11	11	11	11	11	11	11	11	11	12	12	12
rr	*RHD–*	Negative	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

* DVa = shares epitope similarity with partial DVa phenotype. P = ProVue gel test; G = Gamma-clone tube test; B = BioClone tube test.

discrepancy samples analyzed (Table 2). RBCs from 2 samples did not react with monoclonal anti-D reagents E and I, suggesting a partial DVa phenotype. RBCs from 4 samples weakly reacted with monoclonal anti-D reagent I alone. The RBCs of 4 samples did not react with monoclonal reagent I; 1 reacted weakly with reagent J.

Reactivity of DAR and weak D types

Multiplex *RHD*-specific PCR indicated that the two serologically DVa samples had nucleotide changes in exon 5 due to a lack of amplification of this exon. Direct nucleotide sequence analyses of exons 5 and 7 revealed a *DAR* (weak D Type 4.2) allele. Two samples were weak D Type 1 and reacted with monoclonal anti-D reagent I; a genotype could not be assigned for these samples. Three other samples did not react with monoclonal anti-D reagent I alone and consisted of one R_1r sample for which exon 5 could not be amplified and two R_2r samples that were weak D Type 2. An additional two R_2r samples demonstrating weak reactivity with monoclonal anti-D reagent I alone were weak D Type 2. Therefore, serologic patterns were inconsistent among the four weak D Type 2 samples analyzed.

Discrepancies by commercial anti-D for partial D and weak D types

The IS tube tests with one FDA-approved reagent were not in agreement for the two DAR samples; one of two was positive with the BioClone reagent. Similarly, one of two weak D Type 1 samples was positive with the BioClone reagent (score, 5). Divergent reactivity was noted also for the four weak D Type 2 samples and the lack of reactivity did not correlate with monoclonal anti-D reagent I. A comparison between the monoclonal anti-D and FDA-

approved reagents are summarized in Table 2. Most importantly, a direct comparison of the results of the three FDA-approved anti-D reagents alone for the samples with the pattern for known partial Ds (Table 1, IS anti-D result) suggests that one DAR, one weak D Type 1, and two weak D Type 2 would be classified as partial DV or DAR types. Therefore, consistent with the manufacturer's reaction profile, it was not possible to categorize the discrepancies as partial or weak D Types 1 or 2 on the basis of serology alone.

DISCUSSION

FDA-approved reagents and technologies are used in the clinical laboratory to ensure the accurate assignment of the D antigen for potential transfusion recipients and pregnant women. The choice of blood products and perinatal care rely heavily on the appropriate serologic D antigen status; patients who express partial D variants should be considered D–. In light of changing reagents and technologies, D antigen discrepancies between existing tests and historical records occur. The implementation of a useful clinical paradigm to resolve these discrepancies is important for appropriate transfusion and perinatal management.

We found D antigen discrepancies between a recently implemented gel technology in our institution and historical records or the result of tube test anti-D performed as part of the electronic crossmatch process. We used a large series of monoclonal anti-D reagents to evaluate the epitope profiles of these samples as a way to resolve discrepancies. In addition, we performed a multiplex sequence-specific priming PCR, PCR-RFLP, and sequenced exon 5 to identify common partial and weak D types.

Our studies found that the DAR phenotype and the partial D category DVa have similar monoclonal anti-D epitope profiles. This observation is not surprising given the common nucleotide changes in exon 5 shared by these two partial D variants. The nucleotide change at position 1025 of exon 7 confirmed the presence of the *DAR* allele, and the assignment did not have an impact on the choice of blood products or perinatal care. Serologic reactivity with BioClone anti-D, however, showed a disparity for the two DAR samples. One sample reacted (score 5) and the other did not react with the IS test method. The most probable genotype of both samples was R_or and because the assignment is based on probability, the disparity possibly could be explained on the basis of one sample representing the R_oR_o phenotype, that is, homozygous for *RHD*. For the monoclonal anti-D profile and molecular analyses to be consistent with the presence of two *RHD* alleles, however, both would have to represent the *DAR* allele as suggested in the product monograph. We have had one other R_or sample expressing *DAR* that did not react with the BioClone reagent, which was not tested with the monoclonal anti-D reagents (data not shown). Regardless, weak or negative reactivity with one FDA-approved anti-D caused us to perform the monoclonal anti-D profile, which indicated a partial D phenotype and helped to assign an D– status.

Two examples of Weak D type 1 and several examples of Weak D type 2 showed disparity with an FDA-approved anti-D. Both weak D Types 1 and 2 show heterogeneity in their reactivity with the BioClone reagent. The two weak D Type 1 samples differed in their IS tube test reactivity with the BioClone reagent, and a similar observation was seen with the weak D Type 2; two of four did not react with the BioClone reagent. Moreover, the lack of reactivity with the BioClone reagent was inconsistent with a monoclonal anti-D that was nonreactive with two of four weak D Type 2 (Table 2).

An exhaustive evaluation of D antigen density and epitope distribution among partial D has been performed in the past.[2,26-29] The variability of expression for similar examples of weak D Types 1 and 2 is lacking, however. Moreover, reduced reactivity with monoclonal anti-D reagents can be viewed as indicative of a partial D allele. Therefore, if results of the monoclonal anti-D profile were applied to some examples of weak D Types 1 and 2, then they would be deemed D– and the patients would receive D– blood or be given Rh immune globulin.

The most likely reason for the variable expression among similar weak D types is unknown. Aside from the variable expression known to exist for partial D, previous studies showed that the expression of the D antigen varies.[30,31] Other than the cis-trans effect exerted in the presence of *RHCe*,[32] no molecular mechanism for this variation has been identified. Possibly, there are noncoding differences among similar variants that may affect the half life of the mRNA, or alternatively, there may be subtle differences in some other moiety of the Rh complex, which affects the expression of the D antigen as has been previously hypothesized.[30] In our opinion, tube tests have the highest degree of subjectivity and result in the most variability. The definition of "immediate spin" is difficult to define when one considers that samples can be tested alone or as part of a batch. Moreover, small differences in the testing temperature, protein concentration, and other technical factors such as minor centrifugation speed differences or how the RBC pellet is dislodged can affect the degree of agglutination and likely contribute to the variable results for manual tube tests even for repeat tests performed on the same sample.

The variation in expression and ultimately the D antigen status of patients who harbor alleles of weak D alleles still pose a challenge for the transfusion service. In our hands, a change in method (introduction of gel technology) resulted in Rh discrepancies. The data presented

here of our investigations show that a serologic solution to the positive identification of weak and partial D variants is unlikely. Therefore, we recommend that samples yielding an IS tube test cutoff score of not more than 5 (i.e., ≤1+ hemagglutination) or a score of not more than 8 (i.e., ≤2+ hemagglutination) by gel technology, be considered D– for the purpose of transfusion and Rh immune globulin prophylaxis.

Unfortunately, serology alone does not resolve the issue of those weak D types not at risk of making anti-D. Molecular tests that can distinguish common partial and weak D types provide the best solution to the resolution of an accurate D antigen status. In our small study, the D antigen status of 8 of 10 patients was resolved with three molecular tests. Future work should focus on improvements to the turnaround time for resolving Rh discrepancies.

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