Prevalence of RhD Variants, Confirmed by Molecular Genotyping, in a Multiethnic Prenatal Population

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Key Words: RhD variant; Blood typing; Prenatal; Pregnancy; Genotyping

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Abstract

RhD determination in pregnant women is critical to facilitate Rh immune globulin prophylaxis for RhD-negative women. A single amino acid change in the RhD antigen can cause epitope loss, giving rise to “partial D” variants. Women with some partial D variants may develop anti-D against the missing epitope after pregnancy. RBCs with partial D may type as D-positive or D-negative depending on the reagent used. We screened routine blood bank samples from 501 prenatal patients for RhD variants by 3 commercially available serologic methods. Discordant serologic results were found in 11 cases. Weak D (n = 5) and partial D (n = 5) variants were confirmed by molecular genotyping in all but 1 case. RhD variants, confirmed molecularly, occur in 2.2% of our multiethnic population. Consideration of patients’ ethnic background and close cooperation between pathologists and obstetric providers facilitate optimal prenatal care in these cases.
were serologic discrepancies in D typing, suggestive of a D variant. Knowing that the obstetric population at our institution is very ethnically diverse, we undertook this study to prospectively investigate serologic discrepancies in RhD typing. The goals were to identify the prevalence of D variants in a multiethnic obstetric population and to determine the best Rh test method to appropriately classify D status so that women with D variants capable of making anti-D can be offered RhIG prophylaxis.

Materials and Methods

Samples

We included 501 consecutive EDTA blood specimens submitted to the blood bank for routine type and screen testing from the obstetric clinics and labor and delivery unit during a 3-month period in this study. All samples were tested for RhD by using 3 commercially available FDA-approved methods according to manufacturers’ directions (see the next section). Patients underwent this "triplicate" testing only once during their prenatal course. Patients’ ethnic backgrounds and obstetric and transfusion histories were reviewed. The study was approved by the Boston University Medical Center Institutional Review Board (Boston, MA).

Serologic RhD Typing

Each sample was tested with 4 commercially available and FDA-approved anti-D reagents. Automated solid phase technology for Rh determination was performed on the Galileo (Series 4 and 5, Immucor, Norcross, GA). The 2 anti-D reagents for immediate-spin tube testing included BioClone (Ortho Clinical Diagnostics, Raritan, NJ) and Gamma-clone (Immucor). The specific monoclonal antibodies used in the formulation of these reagents are shown in Table 1. All reagents were used in accordance with the manufacturers’ directions. Manual hemagglutination was graded as follows: 0, no agglutination, even RBC suspension, no hemolysis; 1+, visible macroscopically, button dislodges into many small aggregates, cloudy background; 2+, visible macroscopically, button dislodges into many small to medium aggregates, cloudy background; 3+, visible macroscopically, button breaks up into large aggregates, clear background; and 4+, solid button of cells, no free cells, clear background.

RHD Genotyping

Discrepant serologic results were referred to a reference laboratory (Progenika, Cambridge, MA) for molecular genotyping. Genetic analysis of 10 samples was performed on Progenika’s BloodChip version 2.0 DNA microarray. This test uses oligonucleotide probes to interrogate 75 polymorphisms in the RHD gene that determine the following type and number of variants: 36 D-negative, 41 partial D, and 18 weak D. Briefly, polymerase chain reaction–amplified RHD segments are fragmented, labeled with a fluorescence marker, and hybridized to probes attached to the surface of a functionalyzed glass slide. Fluorescence intensity is detected by a confocal scanner, quantified, and analyzed on proprietary software to determine the genotype and predict the phenotype.

Results

Demographic and Obstetric Data

The median age was 27 years (range, 16-46 years). Ethnic background was as follows: African American, 41.6%; Caucasian, 19.2%; Hispanic-Latino (Dominican Republic, Mexico, and El Salvador), 14.8%; African (Haiti, Algeria, Morocco, Liberia, Ethiopia, Uganda, and Jamaica), 12.0%; Asian (India, Lebanon, Bangladesh, Nepal, and Egypt), 5.8%; Portuguese (Cape Verde and Brazil), 5.8%; and unknown, 0.8%. Just more than half (56.1%) of the patients were undergoing pregnancy termination, 39.7% were receiving routine prenatal care, and 4.2% were being treated for pregnancy abnormality (ectopic pregnancy, fetal demise, and miscarriage). Of the 501 patients, 73.4% were in their first trimester, 18.6% were in their second trimester, and 8.0% were in their third trimester. Only 1 patient had any transfusion history before the visit.

Table 1

<table>
<thead>
<tr>
<th>Method</th>
<th>Source</th>
<th>IgM Anti-D</th>
<th>IgG Anti-D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manual-tube hemagglutination</td>
<td>BioClone, Ortho Clinical Diagnostics, Raritan, NJ</td>
<td>MAD2</td>
<td>Polyclonal</td>
</tr>
<tr>
<td>Manual-tube hemagglutination</td>
<td>Gamma-clone, Immucor, Norcross, GA</td>
<td>GAMA401</td>
<td>F8D8</td>
</tr>
<tr>
<td>Automated solid phase</td>
<td>Galileo Series 4, Immucor</td>
<td>MS201</td>
<td>MS26</td>
</tr>
<tr>
<td>Automated solid phase</td>
<td>Galileo Series 5, Immucor</td>
<td>Th28</td>
<td>MS26</td>
</tr>
</tbody>
</table>

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Serologic D Typing

D status was assigned as positive in 441 patients (88.0%) by the 3 methods (consistently strong agglutination ≥2+); 49 patients (9.8%) were assigned as negative. Serologic discrepancies were found in 11 patients [Table 2]. Six discrepancies were noted between the tube typing reagents, and 5 discrepancies were noted between tube and automated solid phase methods. Samples from all 11 patients were initially classified as D-positive based on the initial test of record, which is tube testing with Gamma-clone.

Molecular RHD Genotyping and Prevalence of Partial D and Weak D Variants

Of the 11 discrepant cases, 10 were confirmed by molecular genotyping as partial D (5 cases) or weak D (5 cases) (Table 2). This gives a D variant prevalence of 2.2%, as detected by initial serologic discrepancies. Weak D type 4.0 was the most frequent, followed by DAR (2 homozygous and 1 heterozygous), DV type 1 (also called D’vaka), and weak D type 3.0. Details of the 10 confirmed D variant cases are given in [Table 3]. Allele linkage analysis showed that 6 of the D variants are in linkage with RHCE variants, the most frequent RHCE variant allele being ceAR (with DAR) followed by VS+ (with weak D type 4.0), and r’S (with weak D type 4.0).

Correlation Between Serologic and Molecular Results

Correlation between serologic and molecular results is shown in Table 3. The 2 samples homozygous for DV type 1 reacted strongly (≥3+) with Gamma-clone and by automated solid phase but weakly (<2+) with BioClone. The 2 samples homozygous for DAR reacted only with Gamma-clone (2+). The samples with weak D demonstrated more variable reactivity with the manual tube reagents, eg, 1 sample with weak D type 4.0 reacted 3+ in Gamma-clone and 1+ in BioClone, whereas 2 other samples with weak D type 4.0 reacted 2+ in Gamma-clone and BioClone. The weak D samples reacted weakly (1+) or were negative in automated solid phase.

Discussion

Appropriate assignment of RhD antigen status is critical so that RhIG can be offered to D-negative women. However, the serologic distinction between D-positive and D-negative RBCs is not always straightforward in the case of D variants. Commercially available anti-D reagents can react differently with D variant antigens.7,12-14

| Table 3 |

Results of Serologic and Molecular D Determination in Samples With Initial Phenotypic Discrepancies*  

<table>
<thead>
<tr>
<th>Ethnic Background</th>
<th>RHD Genotype</th>
<th>RhD Phenotype</th>
<th>Immediate-Spin Tube</th>
<th>Automated Solid Phase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Gamma-clone</td>
<td>BioClone</td>
</tr>
<tr>
<td>African American</td>
<td>Weak D type 4.0</td>
<td>Weak D</td>
<td>1+</td>
<td>0</td>
</tr>
<tr>
<td>African (Haitian)</td>
<td>Weak D type 4.0</td>
<td>Weak D</td>
<td>3+</td>
<td>1+</td>
</tr>
<tr>
<td>Hispanic-Latino</td>
<td>Weak D type 4.0</td>
<td>Weak D</td>
<td>2+</td>
<td>2+</td>
</tr>
<tr>
<td>Hispanic-Latino</td>
<td>Weak D type 4.0</td>
<td>Weak D</td>
<td>2+</td>
<td>2+</td>
</tr>
<tr>
<td>Caucasian</td>
<td>Weak D type 3.0</td>
<td>Weak D</td>
<td>1+</td>
<td>1+</td>
</tr>
<tr>
<td>African American</td>
<td>DAR</td>
<td>Partial D</td>
<td>2+</td>
<td>0</td>
</tr>
<tr>
<td>African American</td>
<td>DAR</td>
<td>Partial D</td>
<td>2+</td>
<td>0</td>
</tr>
<tr>
<td>African American</td>
<td>DAR</td>
<td>Partial D</td>
<td>1+</td>
<td>1+</td>
</tr>
<tr>
<td>Portuguese</td>
<td>DV type 1</td>
<td>Partial D</td>
<td>3+</td>
<td>1+</td>
</tr>
<tr>
<td>African American</td>
<td>Unknown</td>
<td>Unknown</td>
<td>2+</td>
<td>0</td>
</tr>
</tbody>
</table>

Psi, RHD pseudogene.  
* The grading scale was as follows: 0, no agglutination, even RBC suspension, no hemolysis; 1+, visible macroscopically, button dislodges into many small aggregates, cloudy background; 2+, visible macroscopically, button dislodges into many small to medium aggregates, cloudy background; 3+, visible macroscopically, button breaks up into large aggregates, clear background; and 4+, solid button of cells, no free cells, clear background.
Denomme et al, using 2 anti-D reagents, found 55 serologic discrepancies (0.2%) among 34,000 routine blood bank samples in a diverse multiethnic population in Toronto, Canada. Molecular analysis confirmed these RhD variants as DAR, DVa, DAR, and DFR and weak D types 1 to type 5. The authors propose that 2 different anti-D reagents should be used routinely to establish the Rh status for obstetric patients and potential transfusion recipients. Our D variant prevalence of 2.2% is 10 times higher; this difference is most likely attributable to ethnic diversity. The prevalence of D variants in our study was 2.6% (7/268) in Africans/African Americans, 1.0% (1/96) in Caucasians, and 2.7% (2/74) in Hispanics-Latinos. Mezokh et al recently identified D variant alleles in homozygous or heterozygous form in 8.8% (35/400) of African American blood donors by a high-throughput RBC genotyping platform. The D variant alleles in their African American population included weak D type 4.0 as the most frequent, followed by DV type 1, DAR, and weak D type 3.0—exactly the same variants found in our study. Because we performed molecular genotyping only in cases with serologic discrepancies, the true prevalence of D variants in our population most likely exceeds 2.2%.

There are around 60 known partial D variants. The DAR variant consists of a D allele with 3 point mutations, in which D-specific nucleotides are replaced by CE-specific nucleotides. It is commonly associated with a variant RHCE gene called ceAR. The frequency of the DAR phenotype is 1.5% in the African population, much higher than the frequency of partial D variants in a Caucasian population (0.1%-0.001%). In our study, all cases of DAR were linked with ceAR and did not react in automated solid phase testing. Because people with DAR may form anti-D when exposed to wild-type D antigen, pregnant women and transfusion recipients expressing the DAR variant should be regarded as D-negative for prenatal and transfusion management.

DV type 1 (DVa), another partial D variant, has been reported in white, black, and Japanese people. The molecular basis for DV type 1 has been reported, in which the entire RHD exon 5 (DVa Huf) or a portion of it (DVa Kou) is replaced by the RHCE equivalent, resulting in a hybrid RHDE-CE-D transcript. There is no reported case of HDFN in a woman with DV type 1.

Our study was precipitated by the finding of anti-D in a D-positive pregnant woman and by the increasing discrepancies we have noted since adding the automated solid phase platform to traditional tube testing. We use the 2 methods interchangeably, depending on the urgency of the compatibility testing result. Samples from the labor and delivery unit were prioritized as stat testing, which was then performed manually. In the case of patients with a D variant, often a discrepancy would be noted when the current result was compared with the historic result earlier in the pregnancy because outpatient prenatal ABO/Rh testing was almost always batched for automated solid phase testing. For internal consistency, our laboratory will most likely eliminate the Gamma-clone reagent in favor of using Series 4 or Series 5 reagents, which are approved for manual and automated solid phase testing. This strategy will classify the DAR variant as D-negative, which is ideal because women with this variant should be offered RhG prophylaxis. Our 5 patients with partial D variants have a median of 4 prior pregnancies and have not been sensitized despite having been treated as RhD-positive to date.

Our proposed new classification strategy may unnecessarily classify some weak D variants as D-negative. Weak D variants express reduced quantities of the D antigen as a result of mutations in the protein’s transmembrane or intracellular domains. There are more than 50 different mutations that cause weak D expression. As the name implies, weak D RBCs react weakly or not at all in the immediate-spin phase of hemagglutination. Weak D variants, predominantly types 1 and 2, occur in an estimated 0.2% to 1% of Caucasians and are not typically associated with anti-D development. However, anti-D has been reported in people with weak D types 4.2 and 15; these variants are better classified as partial D. In fact, the D variants DAR and weak D type 4.2 have the same 3 nucleotide changes. The risk of women with weak D variants developing Rh immunization is so low that most authors hold that women with weak D do not require RhG prophylaxis. Similarly, people with weak D can safely receive D-positive blood. According to the American Association of Blood Banks, weak D testing is not required in pregnant women but is mandatory in blood donor and cord blood testing. Flegel et al recommend that pregnant women with previous weak D-positive test results receive RhIG in their current pregnancy if they continue to have test results less than 2+ in immediate-spin hemagglutination D testing.

The possibility of D variants must be considered by general clinical pathologists practicing transfusion medicine if there is a significant discrepancy in the strength of reactions obtained with different anti-D reagents or a discrepancy between current test and historical test results or if anti-D is detected in a patient who is serologically typed as RhD-positive. If RhD status is discrepant, the patient should be treated as D-negative until the problem is resolved. Some authorities recommend interpreting a 1+ or weaker D testing result as D-negative, although this may not be explicitly stated in the manufacturers’ directions. Molecular Rh genotyping is now available to resolve serologic discrepancies. Women found to have partial D variants should be classified as D-negative for prenatal management. Consideration of patients’ ethnic backgrounds and close cooperation between pathologists and obstetric providers facilitate optimal prenatal and transfusion management.
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References