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An artist of humble origins and a champion of Naturalism in 19th Century France, Jules Bastien-Lepage often portrayed peasants and the poor in a sympathetic though unromantic light. His *Joan of Arc*, painted in 1879, more than 40 years before her canonization, depicts a flesh and blood girl with a real farmhouse and tipped over spinning wheel behind her, in contrast to the three phantasmal saints floating in the trees who guide her. While here she wears no armor, and there is more wonder than fire in her eyes, she would lead French defenders in battle after battle against invading English forces. Schreiber’s letter in this issue concerns CD177 expression in neutrophils, critical in the body’s protection against infection.
First example of an FY*01 allele associated with weakened expression of Fy\textsuperscript{a} on red blood cells

P.A. Arndt, T. Horn, J.A. Keller, R. Young, S.M. Heri, and M.A. Keller

Duffy antigens are important in immunohematology. The reference allele for the Duffy gene (FY) is FY*02, which encodes Fy\textsuperscript{a}. An A>G single nucleotide polymorphism (SNP) at coding nucleotide (c.) 125 in exon 2 defines the FY*01 allele, which encodes the antithetical Fy\textsuperscript{b}. A C>T SNP at c.265 in the FY*02 allele is associated with weakening of Fy\textsuperscript{a} expression on red blood cells (RBCs) (called Fy\textsuperscript{g}). Until recently, this latter change had not been described on a FY*01 background allele. Phenotypematched units were desired for a multi-transfused Vietnamese fetus with α-thalassemia. Genotyping of the fetus using a microarray assay that interrogates three SNPs (c.1-67, c.125, and c.265) in FY yielded indeterminate results for the predicted Duffy phenotype. Genomic sequencing of FY exon 2 showed that the fetal sample had one wild-type FY*01 allele and one new FY*01 allele with the c.265C>T SNP, which until recently had only been found on the FY*02 allele. Genotyping performed on samples from the proband’s parents indicated that the father had the same FY genotype as the fetus. Flow cytometry, which has been previously demonstrated as a useful method to study antigen strength on cells, was used to determine if this new FY*01 allele was associated with reduced Fy\textsuperscript{a} expression on the father’s RBCs. Median fluorescence intensity of the father’s RBCs (called FY*01W.01), associated with weakened expression of FY\textsuperscript{a} on RBCs. Immunohematology 2015;31:103–107.

Key Words: Duffy, Fy\textsuperscript{a}, flow cytometry, genotyping, blood group antigen

The Duffy blood group antigens, expressed on the Duffy antigen receptor for chemokines (DARC), are important, not only in the field of immunohematology where Duffy antibodies can cause transfusion reactions and hemolytic disease of the fetus and newborn, but also in the fields of anthropology, genetics, and disease, where the DARC protein is the receptor for the malarial parasite Plasmodium vivax.\textsuperscript{1} The Duffy gene (FY) consists of two exons on chromosome 1.\textsuperscript{2–4} The reference allele FY*02 (FY*B) encodes for Fy\textsuperscript{a}, Fy3, Fy5, and Fy6. An A>G single nucleotide polymorphism (SNP) at coding nucleotide (c.) 125 in exon 2 defines the FY*01 (FY*A) allele and results in an asparagine to glycine amino acid substitution in the protein (p.42 Asp>Gly) that results in expression of Fy\textsuperscript{a} on red blood cells (RBCs) (Table 1). There are several different mutations leading to the FY*01 allele; FY*02N.01 allele; a t>c SNP at c.–67 located in a transcription factor binding site called a GATA box hinders binding of the transcription factor GATA-1, which results in loss of FY*02 expression on RBCs but not in tissues. Another prevalent Duffy variant is the C>T mutation at c.265 on the FY*02 allele, encoding a p.89 Arg to Cys amino acid substitution that causes weakening of Fy\textsuperscript{b}, Fy3, and Fy6 expression (FY\textsuperscript{g}).\textsuperscript{6–10} The microarray assay, HEA BeadChip\textsuperscript{TM} (BioArray Solutions, Immucor, Norcross, GA), tests for these three notable SNPs to predict the FY phenotype: FY*01W.01, GATA (c.–67t/c), and FY*02W.02. A GATA box mutation has been described on the FY*01 allele that results in the FY(a–b–) phenotype;\textsuperscript{11} but the c.265C>T (FYX) change had not been described on the FY*01 allele until recently.\textsuperscript{6,7}

Flow cytometry has been shown to be a useful method for detecting differences in antigen strength (e.g., attributable to zygosity\textsuperscript{12}) and has been used to study the expression of

<table>
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<tr>
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<tr>
<td>FY<em>02 (FY</em>B)</td>
<td>t  A  C  G</td>
<td>Reference allele; Fy\textsuperscript{a}</td>
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<tr>
<td>FY*02N.01</td>
<td>c  A  C  G</td>
<td>FY(a–b–) erythroid cells only</td>
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<tr>
<td>FY*02W.01</td>
<td>t  A  T  A</td>
<td>Fy(b+\textsuperscript{a}), Fy\textsuperscript{g}</td>
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<tr>
<td>FY*02W.02</td>
<td>t  A  T  A</td>
<td>c.145G&gt;T; Fy(b+\textsuperscript{a}), Fy\textsuperscript{g}</td>
</tr>
<tr>
<td>FY*02W.03</td>
<td>t  A  C  G</td>
<td>c.266G&gt;A; Fy(b+\textsuperscript{a})</td>
</tr>
<tr>
<td>FY*02W.04</td>
<td>t  A  C  G</td>
<td>c.901C&gt;T; Fy(b+\textsuperscript{a})</td>
</tr>
<tr>
<td>FY<em>01 (FY</em>A)</td>
<td>t  G  C  G</td>
<td>Fy\textsuperscript{a}</td>
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<tr>
<td>FY*01W.01</td>
<td>t  G  T  G</td>
<td>Fy(a+\textsuperscript{g})</td>
</tr>
<tr>
<td>FY*01W.02</td>
<td>t  G  T  A</td>
<td>Fy(a+\textsuperscript{g})</td>
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The allele in bold (FY*01W.01) is described in the text.
Duffy antigens on RBCs of different phenotypes or of different ages. We describe the first reported example of an FY*01 allele with the c.265C>T (FyX) change; this allele was shown to be associated with weak expression of Fya as determined by flow cytometry.

**Case Report**

The patient was a Vietnamese male fetus with α-thalassemia. A decision was made to provide the fetus with phenotype-matched RBC units but, because of multiple recent intrauterine transfusions, a genotype was needed to determine the fetus’ predicted phenotype. Molecular typing results (HEA BeadChip, Immucor) indicated the following phenotype: C+ c– E– e+; K– k+ Kp(a–b+) Js(a–b+) Jk(a+b+); M– N+ S– s++; Lu(a–b+); Di(a–b+); Co(a+b–); Do(a+b+).Jo(a+) Hy+; LW(a+b–); SC:1,–2, but the Fya and Fyb results were “indeterminate.” Further molecular studies were performed on the fetal DNA to determine the Fy phenotype. Blood samples were obtained from both the mother and father for molecular and serologic testing.

**Materials and Methods**

**Serologic Testing**

RBCs from the proband’s parents were typed with three different sources of anti-Fya and anti-Fyb (Immucor; Ortho Clinical Diagnostics, Raritan, NJ; American Red Cross, Washington, DC) by the indirect antiglobulin test (IAT). The grading scale included ½+ increments.

**Molecular Testing**

Genomic DNA was isolated from mononuclear cells using a kit (QIAGEN DNA Blood Mini Kit, QiaGen, Valencia, CA). Microarray testing was performed (HEA BeadChip, Immucor) with software (BASIS 3.3, BioArray Solutions Ltd., Warren, NJ). Amplification and Sanger sequencing of FY exon 2 was performed (BigDye Terminator Kit, Life Technologies, Grand Island, NY), and the resulting sequence was aligned to the consensus sequence (Sequencher 5.0, GeneCodes Corp., Ann Arbor, MI). Polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) using BanI was used to interrogate FY*01/FY*02 (c.125G>A) as described by Tournamille et al.

**Flow Cytometry**

A previously described flow cytometric method was used to study the strength of Fya on the proband’s parents’ RBCs; known genotyped and/or phenotyped donor or reagent RBCs were tested in parallel as controls. Briefly, 0.1 mL 5% washed RBCs were incubated with 0.2 mL undiluted polyclonal anti-Fya (Immucor) for 30 minutes at 37°C, washed four times with 0.85% (w/v) phosphate-buffered saline (PBS), then incubated with a dilution of a tagged antihuman globulin (FITC Fab anti-IgG, MP Biomedicals, Aurora, OH) for 30 minutes at room temperature, and washed once with 0.2% bovine serum albumin in PBS. Ten thousand events per sample were acquired by a flow cytometer (FACSort, BD Biosciences, San Jose, CA) using logarithmic amplification of forward scatter, side scatter, and green fluorescence. A gate was set on the forward versus side scatter dot plot to exclude debris, and the median green fluorescence of the gated events was obtained using software (CellQuest Pro, BD Biosciences). In addition to testing by flow cytometry, the RBCs incubated with anti-Fya were also tested by IAT (Anti-IgG, Ortho Clinical Diagnostics).

**Results**

**Serologic Testing**

The mother’s RBCs and the father’s RBCs both typed as Fya(a+b–); both reacted 4+ with all three sources of anti-Fya.

**Molecular Testing**

The molecular testing yielded an indeterminate predicted phenotype for the Duffy antigens on the proband’s sample. The microarray assay (HEA BeadChip, Immucor) results showed the fetal sample to be homozygous for c.125G, associated with the FY*01 (FY*A) phenotype, and heterozygous for c.265C>T change associated with the FY*02W.01 and FY*02W.02 alleles, while being homozygous for c.1-67t. Genomic sequencing of FY exon 2 confirmed the genotype results and determined that the c.298G>A SNP associated with the known FY*02W.01 and FY*02W.02 alleles was not present. The mother’s predicted phenotype by molecular typing was Fya(a+b–), and the sample was negative for the c.265C>T variant. The father’s sample, like that of the fetus, yielded indeterminate calls for Duffy antigens on the microarray assay. PCR-RFLP analysis showed the father’s sample to be homozygous for c.125G and heterozygous for c.265C/T, concordant with the molecular findings.

**Flow Cytometry**

Flow cytometry (median fluorescence) results of the controls [5 Fya(a+b–) donors who genotyped as homozygous for FY*01, 5 Fya(a+b+) donors who were obligate heterozygotes for FY*01, 3 Fya(a+b–) donors who genotyped as FY*01/FY*02N.01 and thus were heterozygous for FY*01, and 2
The Duffy blood group system consists of five antigens (the antithetical Fy\(^a\) and Fy\(^b\); Fy3, Fy5, and Fy6) located on a multipass membrane glycoprotein known as DARC. The four common phenotypes, Fy(a+b–), Fy(a–b+), Fy(a+b+), and Fy(a–b–), are encoded by the FY*01, FY*02, and FY*02N.01 alleles (Table 1). According to Howes et al.,\(^1\) the FY*01 allele is most common in Asia, the FY*02 allele is most common in Europe, and the FY*02N.01 (null) allele is most common in Africa; heterogeneity of all three alleles is greatest in the Americas. A small percentage (2–3%) of Caucasians have the FY*02W.01 allele that codes for weakened expression of Fy\(^a\), Fy3, and Fy6. This Fy\(^a\) phenotype is caused by the c.265C>T mutation that results in a p.89 Arg to Cys amino acid substitution; this change appears to cause reduced levels of DARC in the RBC membrane.\(^6\) The two alleles currently described with this change are FY*02W.01 and FY*02W.02. Both are associated with a second mutation at c.298G>A, which results in a p.100 Ala to Thr change; the FY*02W.02 allele has an additional change at c.145G>T, which results in a p.49 Ala to Ser change. Two more recently described alleles in Caucasians, FY*02W.03 with a c.266G>A (p. 89 Arg to His) change and FY*02W.04 with a c.901C>T (p.303 Pro to Ala) change, also result in Fy(b+) phenotypes.\(^5\)

Although rare mutations in the FY*01 alleles have been described to cause null phenotypes,\(^3,4,7,19\) and there are reports of Southeast Asians with weak Fy\(^a\) on RBCs,\(^2,5,6\) until recently, no FY*01W alleles had been reported. In 2015, Lopez et al.\(^7\) reported an Fy(a+b+) Australian Caucasian blood donor who was noted to have weak expression of Fy\(^a\). This phenotype was shown to be associated with a new allele, FY*01W.02, which had both the c.265C>T and c.298G>A mutations (as seen with FY*02W.01 and FY*02W.02). In 2013, we reported, at an AABB meeting, a recently transfused Vietnamese patient who was found to have an unusual allele, FY*01W.01, with only the critical c.265C>T change.\(^6\) Because of recent transfusions, the proband could not be phenotyped to determine if this allele was associated with weak Fy\(^a\) expression, but his parents were willing to be tested. The Fy(a+b–) mother had a normal FY*01/FY*01W genotype, but the Fy(a+b–) father had the new allele with a normal allele (FY*01/FY*01W.01).

As the proband’s father typed 4+ with anti-Fy\(^a\) due to his FY*01 allele, it would have been difficult to determine the strength of Fy\(^a\) by manual titrations, which have been shown to be poor predictors of antigen strength (e.g., zygosity).\(^13\) Flow cytometry, however, has been used successfully to study Duffy antigens—for example, to demonstrate weak expression of Fy\(^a\), Fy3, and Fy6 on Fy\(^b\) phenotype RBCs;\(^8,10,15,17\) to distinguish zygosity (e.g., FY*01/FY*01 vs. FY*01/FY*02N.01)\(^12,13,15\), and to study Duffy antigen expression on cultured erythroid cells or reticulocytes,\(^14,16,18,20\) after RBC storage and leukocyte reduction,\(^19\) and on ovalocytes.\(^20\) Our flow cytometry studies with known examples of FY*01 homozygotes and heterozygotes showed clear differentiation between the two groups. The proband’s father’s RBCs gave results that clearly fell in the heterozygous group, thus indicating that the FY*01W.01 allele

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**Discussion**

The Duffy blood group system consists of five antigens (the antithetical Fy\(^a\) and Fy\(^b\); Fy3, Fy5, and Fy6) located on a multipass membrane glycoprotein known as DARC. The four common phenotypes, Fy(a+b–), Fy(a–b+), Fy(a+b+), and Fy(a–b–), are encoded by the FY*01, FY*02, and FY*02N.01 alleles (Table 1). According to Howes et al.,\(^1\) the FY*01 allele is most common in Asia, the FY*02 allele is most common in Europe, and the FY*02N.01 (null) allele is most common in Africa; heterogeneity of all three alleles is greatest in the Americas. A small percentage (2–3%) of Caucasians have the FY*02W.01 allele that codes for weakened expression of Fy\(^a\), Fy3, and Fy6. This Fy\(^a\) phenotype is caused by the c.265C>T mutation that results in a p.89 Arg to Cys amino acid substitution; this change appears to cause reduced levels of DARC in the RBC membrane.\(^6\) The two alleles currently described with this change are FY*02W.01 and FY*02W.02. Both are associated with a second mutation at c.298G>A, which results in a p.100 Ala to Thr change; the FY*02W.02 allele has an additional change at c.145G>T, which results in a p.49 Ala to Ser change. Two more recently described alleles in Caucasians, FY*02W.03 with a c.266G>A (p. 89 Arg to His) change and FY*02W.04 with a c.901C>T (p.303 Pro to Ala) change, also result in Fy(b+) phenotypes.\(^5\)

Although rare mutations in the FY*01 alleles have been described to cause null phenotypes,\(^3,4,7,19\) and there are reports of Southeast Asians with weak Fy\(^a\) on RBCs,\(^2,5,6\) until recently, no FY*01W alleles had been reported. In 2015, Lopez et al.\(^7\) reported an Fy(a+b+) Australian Caucasian blood donor who was noted to have weak expression of Fy\(^a\). This phenotype was shown to be associated with a new allele, FY*01W.02, which had both the c.265C>T and c.298G>A mutations (as seen with FY*02W.01 and FY*02W.02). In 2013, we reported, at an AABB meeting, a recently transfused Vietnamese patient who was found to have an unusual allele, FY*01W.01, with only the critical c.265C>T change.\(^6\) Because of recent transfusions, the proband could not be phenotyped to determine if this allele was associated with weak Fy\(^a\) expression, but his parents were willing to be tested. The Fy(a+b–) mother had a normal FY*01/FY*01W genotype, but the Fy(a+b–) father had the new allele with a normal allele (FY*01/FY*01W.01).

As the proband’s father typed 4+ with anti-Fy\(^a\) due to his FY*01 allele, it would have been difficult to determine the strength of Fy\(^a\) by manual titrations, which have been shown to be poor predictors of antigen strength (e.g., zygosity).\(^13\) Flow cytometry, however, has been used successfully to study Duffy antigens—for example, to demonstrate weak expression of Fy\(^a\), Fy3, and Fy6 on Fy\(^b\) phenotype RBCs;\(^8,10,15,17\) to distinguish zygosity (e.g., FY*01/FY*01 vs. FY*01/FY*02N.01)\(^12,13,15\), and to study Duffy antigen expression on cultured erythroid cells or reticulocytes,\(^14,16,18,20\) after RBC storage and leukocyte reduction,\(^19\) and on ovalocytes.\(^20\) Our flow cytometry studies with known examples of FY*01 homozygotes and heterozygotes showed clear differentiation between the two groups. The proband’s father’s RBCs gave results that clearly fell in the heterozygous group, thus indicating that the FY*01W.01 allele
codes for weak expression of Fy\textsuperscript{a}. Interestingly, the proband’s mother’s RBCs gave results much weaker than any of the five FY\textsuperscript{e}01/FY\textsuperscript{e}01 homozygous controls (but still stronger than the eight FY\textsuperscript{e}01 heterozygous controls). She may have an unknown variant FY\textsuperscript{e}01 allele that codes for slightly weaker Fy\textsuperscript{a} expression.

In conclusion, we describe the first example of Fy(a+\textsuperscript{e}). The presence of this phenotype was predicted by Tournamille et al.\textsuperscript{3} based on the description by Shimizu et al.\textsuperscript{25} of some Thai individuals with weak Fy\textsuperscript{a}. Unlike the alleles most commonly associated with Fy(b+\textsuperscript{e}) and the other allele recently described with Fy(a+\textsuperscript{e}), which have at least two mutations (c.265C>T and c.298G>A), the allele we are describing was only associated with one mutation (c.265C>T). This allele was discovered while performing molecular testing on samples from a recently transfused patient. Since the discovery of this allele, two more samples have been identified with the Fy\textsuperscript{e} change on an FY\textsuperscript{e}01 background at the Red Cross National Molecular Laboratory. Both samples were identified by indeterminate calls for the Duffy phenotype on microarray testing, much like the initial case report described here. The first, a female donor, race unknown, demonstrated both c.265C>T and c.298G>A changes on an FY\textsuperscript{e}01 background (like the FY\textsuperscript{e}01W02 allele described by Lopez et al.\textsuperscript{7}). The second case is a 49-year-old male patient, race unknown, with only the c.265C>T change located on the FY\textsuperscript{e}01 background, specifically the FY\textsuperscript{e}01W01 allele. As the use of molecular methods increases in the future, it is possible that more examples of FY\textsuperscript{e}01 alleles associated with Fy(a+\textsuperscript{e}) may be discovered.

Acknowledgments

The authors would like to acknowledge Debbie Bailey (former Assistant Director of the Immunohematology Reference Laboratory, American Red Cross, Southern California Region), for recognizing that flow cytometry would provide useful information in this case, and the late George Garratty (former Scientific Director at the American Red Cross, Southern California Region) for being on the forefront of applying flow cytometry to immunohematology.

References


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Clinical and reference lab characteristics of patients with suspected direct antiglobulin test (DAT)-negative immune hemolytic anemia

M.S. Karafin, G.A. Denomme, M. Schanen, and J.L. Gottschall

Clinical evidence of warm autoimmune hemolytic anemia is present in 1 percent to 10 percent of patients whose direct antiglobulin test (DAT) is negative. The clinical underpinnings associated with DAT-negative immune hemolysis are poorly understood, and the current study aimed to further define the clinical characteristics associated with this form of anemia. A 19-question survey, requesting clinical information about each patient, was retrospectively mailed to all referring labs that had sent patient samples for an enhanced DAT evaluation from January 2011 through June 2013. An enhanced DAT evaluation involved a standard DAT and DATs performed using gel, polyethylene glycol, and 4°C low-ionic strength saline wash. We obtained detailed clinical information from 57 patients with an enhanced DAT investigation. Eighteen of these 57 patients (31.6%) were found to have a positive DAT, 11 (19.3%) of which were found to have a positive enhanced DAT (2 were positive by enhanced methods and negative by standard methods). The reported mean nadir hemoglobin for all 57 patients was 7.8 g/dL (range 3.2–12.7), and lactate dehydrogenase was 827.8 U/L (range 136–6917). Thirty-seven (88.1%) presented with a haptoglobin <10 mg/dL, and 21 (48.8%) reported spherocytes on peripheral smear. About half of the respondents reported using steroids as treatment for the anemia, and 4 of the 18 DAT-positive respondents (23.5%) changed their treatment plan because of the reference laboratory results. One patient died as a result of the reported hemolytic anemia (2.0%). We conclude that immune hemolysis detected by enhanced DAT methods is relatively common, and enhanced DAT methods are valuable tools in the diagnosis and management of patients with DAT-negative hemolytic anemia. Immunohematology 2015;31:108–115.

Key Words: DAT, autoantibodies, Coombs negative hemolytic anemia

The detection of red blood cell (RBC)-bound IgG and/or complement by a direct antiglobulin test (DAT) remains the main assay in the diagnosis of warm autoimmune hemolytic anemia (WAIHA). A positive DAT almost always exists in association with WAIHA and forms the basis for the serologic diagnosis. Several methodologies have been used in the detection of these globulins, but the most common and gold standard method is the conventional tube method. A negative DAT, however, does not exclude the diagnosis of WAIHA, and 1 percent to 10 percent of patients with clinical WAIHA have been reported to have a negative DAT with no detectible serum antibodies.

There have been three hypotheses explaining WAIHA associated with a negative DAT. First, as the standard DAT can only detect about 150 to 200 molecules of IgG per RBC, these patients may carry a lower number of IgG molecules per RBC than the detection threshold for the test, yielding a false-negative tube DAT. Second, these patients may have a low-affinity IgG that dissociates from RBCs during the saline washes performed in the standard DAT procedure. Lastly, these patients may have clinically significant non-IgG immunoglobulins, such as IgA, that are not detected by the standard DAT.

Whether or not the clinical entity of DAT-negative WAIHA differs significantly from DAT-positive WAIHA in terms of underlying pathogenic mechanisms, the severity of presentation, treatments, and clinical outcomes has been rarely studied. To increase our clinical knowledge of DAT-negative hemolytic anemias, we report the serologic results of patient samples that were submitted to our diagnostic reference laboratory for a DAT-negative WAIHA evaluation. We then correlated these reference laboratory results with the results of a 19-question survey that defined additional clinical and laboratory characteristics for these patients.

Materials and Methods

Serologic Detection

We retrospectively evaluated the results of all samples that were received from January 2011 through June 2013 for an enhanced DAT evaluation. The enhanced DAT evaluation at the BloodCenter of Wisconsin immunohematology reference laboratory involves a standard tube DAT, a gel, 4°C low-ionic-strength saline (LISS) wash, and a polyethylene glycol (PEG) DAT for all samples. All of these methods, including the standard DAT, are run in parallel. Standard DAT methods are well documented, and our lab used a polyspecific (rabbit and mouse) antihuman globulin (AHG) (Ortho, Raritan,
NJ), monospecific (monoclonal, mouse) anti-IgG (Immucor, Norcross, GA), anti-C3b, -C3d (Immucor), anti-C3d (Ortho Clinical Diagnostics, Raritan, NJ), and a 10% albumin control (Millipore, Kankakee, IL). The technique will not be further described here. For the enhanced methods, the gel test DAT was performed as described by the manufacturer (ID-MicroTyping System, Ortho Clinical Diagnostics) using the MTS Anti-IgG card™ (rabbit). The 4°C LISS Wash (ELU-KIT II, Immucor) DAT used cold polyspecific AHG (rabbit and mouse antihuman globulin, Ortho Clinical Diagnostics), cold monoclonal anti-IgG (Immucor), and a cold 10% albumin control (Millipore). The 4°C LISS wash technique was performed by washing the sample RBCs four times with ice-cold (4°C) LISS using a refrigerated centrifuge. The RBCs were resuspended to a 2–5% suspension in LISS; one drop was added to two drops of cold anti-IgG or cold 10% albumin, centrifuged, and read immediately. The 10% albumin served as a control for the presence of a cold autoagglutinin; a positive result with the 10% albumin control invalidated the 4°C LISS wash test. The 20% PEG (Fisher Scientific, Fairlawn, NJ) technique (prepared in-house) was performed similarly to techniques described elsewhere.14

Patient Information

An institutional review board–approved 19-question survey was mailed to all referring labs that sent patient samples for an enhanced DAT evaluation from January 2011 through June 2013. Surveys were mailed 1 to 12 months after an enhanced DAT sample was received. The survey requested additional clinical information about each patient, including nadir hemoglobin, bilirubin, lactate dehydrogenase (LDH), haptoglobin, current diagnosis, treatment modality, and patient outcome. The questions included a combination of multiple choice and free response answers. The survey questions and answer choices are available in Figure 1.

Statistical Analysis

Patient and enhanced DAT data were summarized as means, standard deviations, and percents, as applicable. A comparison between those with and without a DAT-positive test result was performed. Frequency data were analyzed using a Fisher’s exact test, and continuous data were analyzed using an independent-sample t test. A p ≤ 0.05 was considered statistically significant.

Results

Serologic Findings

We received and performed an enhanced DAT on 447 samples from January 2011 until June 2013. The samples came from 213 female patients (47.7%) and 234 (52.3%) male patients. The average reported patient age for these reference lab samples was 51.1 years (±22.2 years) and ranged from 1 year to 99 years of age.

The summary of serological data is presented in Table 1. Of the 447 samples submitted for an enhanced DAT evaluation, 103 (23.0%) were positive for at least one standard DAT method, and of these, 28 (27.2%) were found to be positive for only complement. We further identified that 107 (23.9%) of the 447 samples were positive for at least one of the enhanced methods. Only 37 (34.6%, 8.3% overall) of the 107 samples were positive with an enhanced method but negative with the standard polyspecific DAT method, and only 8 (7.5%) samples were positive with an enhanced method when positive for only complement using standard methods. Five (13.5%) of the 37 were positive for a combination of enhanced methods, 18 (48.6%) were positive only with gel, 14 (37.8%) were positive only with 4°C LISS, and none were positive with PEG alone (two samples were positive by PEG and gel).

Clinical Findings

Of the 447 surveys mailed, we received additional clinical information regarding 57 DAT-negative patients (response rate: 12.6%). There were more female than male patients (37 female, 64.9%) in this cohort, and the average reported age

<table>
<thead>
<tr>
<th>Results (N=447)</th>
<th>Number positive (%)</th>
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<tbody>
<tr>
<td>Any positive standard polyclonal AHG</td>
<td>103 (23.0)</td>
</tr>
<tr>
<td>Any positive standard C3 or IgG</td>
<td>85 (19.0)</td>
</tr>
<tr>
<td>Any positive enhanced test</td>
<td>107 (23.9)</td>
</tr>
<tr>
<td>• Standard DAT negative</td>
<td>37 (8.3)</td>
</tr>
<tr>
<td>Any positive for multiple enhanced methods</td>
<td>50 (11.2)</td>
</tr>
<tr>
<td>• Standard DAT negative</td>
<td>5 (1.1)</td>
</tr>
<tr>
<td>Any gel test positive</td>
<td>39 (8.7)</td>
</tr>
<tr>
<td>• Standard DAT negative</td>
<td>18 (4.0)</td>
</tr>
<tr>
<td>Any 4°C LISS test positive</td>
<td>17 (3.8)</td>
</tr>
<tr>
<td>• Standard DAT negative</td>
<td>14 (3.1)</td>
</tr>
<tr>
<td>Any PEG test positive</td>
<td>1 (0.2)</td>
</tr>
<tr>
<td>• Standard DAT negative</td>
<td>0</td>
</tr>
</tbody>
</table>

DAT = direct antiglobulin test; AHG = antihuman globulin; LISS = low-ionic-strength saline; PEG = polyethylene glycol.
was 48 (±25.2) years with a range of 1 to 90 years. Of the 57 patients, 39 (68.4%) were negative by all testing methods. Of those who tested positive, 16 (28.1%) had a positive standard polyspecific DAT, with 4 (7.0%) being positive for the standard polyspecific DAT alone, 6 (10.5%) being positive for IgG alone, 3 (5.3%) being positive for C3 alone, and 3 (5.3%) having both IgG and C3 coating their RBCs. Eleven of the 18 patients with a positive DAT (61.1%) had at least one positive enhanced DAT (4 [22.2%] 4°C LISS positive, 6 [33.3%] PEG positive, and 11 [61.1%] gel positive). Only 2 (18.2%) of the 11 respondents had a positive enhanced DAT (both gel only) with a negative standard polyspecific DAT in our laboratory (Table 2).
Antibody specificities were further classified by the reference lab when possible (Table 2). Of the 18 total respondents who had a positive DAT (enhanced and/or standard), serum or eluate evidence of warm autoantibodies was noted in 9 (50%) of these patients, followed by 5 (27.8%) cold autoantibodies, 3 (16.7%) “low affinity” warm autoantibodies (suggested by a positive enhanced LISS DAT, and negative standard monoclonal IgG DAT), and 1 (5.6%) alloantibody (anti-E). Two of the 18 patients were also found to have evidence of a drug-induced hemolytic anemia (cefotetan and carboplatin) as the cause of their DAT-negative evaluations. The two patients who had a negative standard polyspecific DAT, but a positive enhanced DAT, were both suggested to have warm autoantibodies (one was also found to have an anti-I in the serum).

A summary of selected survey questions for the 57 patients is presented in Table 3. Overall, patients presented with anemia (hemoglobin mean 7.8 g/dL ± 2.1, range 3.2–12.7 g/dL), hyperbilirubinemia (mean 4.1 mg/dL ± 4.32), and evidence of hemolysis, as demonstrated by an elevation in LDH, low haptoglobin, and the presence of spherocytes. A majority of respondents reported that the patient did not have a known disease process associated with a hemolytic anemia (32 respondents, 56.1%), and of those who did, hematologic malignancy (lymphoma, chronic lymphocytic leukemia, chronic myelocytic leukemia, B-cell or T-cell acute lymphocytic leukemia, multiple myeloma, hemophagocytic syndrome) was the most commonly reported diagnosis (15 respondents, 26.3%). Other reported associated diagnoses included colon/lung/ovarian cancer, sickle cell disease, artificial heart valves, and hypothyroidism. About half of the patients were treated with steroids for their anemia, and most started this therapy prior to the enhanced DAT evaluation (64.3%). After the DAT evaluation was reported, 85.7 percent (42 respondents) reported not changing their selected therapies, and 77.6 percent (38 respondents) did not alter their reported diagnosis. The most common reported diagnosis after DAT was hematologic malignancy (12 patients, 25.5%), followed by WAIHA (8 patients, 17.5%), immune hemolysis (5 patients, 10.5%), or sepsis (5 patients, 10.5%). Less frequently reported diagnoses included solid organ malignancy, microangiopathic hemolysis, mechanical hemolysis, congenital red cell defects, prematurity,
coronary artery disease, occult bleeding, and vasculitis. Most patients recovered or became stable with treatment (64.7%), and while 14 patients were reported as deceased, only 1 death was reported as caused by the hemolytic process (1.8%). The average recovery time was 2.7 months, with a reported range of 1 week to 1 year.

We statistically compared the survey responses of those 18 patients who were found to have a positive reference lab DAT (any method) from those who did not. While the numbers were small, we found no significant difference in patient gender distribution or age (Table 3). While we found that those with a positive DAT had a lower mean nadir hemoglobin, higher mean LDH, and higher mean bilirubin than those who had a negative DAT, none of these differences reached statistical significance ($p > 0.05$). Moreover, the frequency of reported associated diagnoses, treatments used, and patient outcome did not differ based on the DAT result. In contrast, we did find that there was a significant difference in physician responses to the results. Specifically, we found that 7 of 17 (41.2%) respondents changed their patient’s working diagnosis when the DAT was reported to be positive, whereas only 4 of 32 (12.5%) reported a change in diagnosis after a negative DAT result ($p = 0.03$).

We also found a trend where more respondents (DAT positive: 4 of 17, 23.5%, DAT negative: 3 of 32, 9.4%, $p = 0.2$) changed...
treatment plans when the DAT was reported to be positive. Of those who changed their treatment plan because of a positive DAT result, 50 percent (2/4) specifically added steroids as a treatment, and 1 discontinued steroid therapy (patient with the drug-induced hemolytic anemia). Only one of the three respondents (33%) who changed a treatment plan because of the negative DAT result specifically reported discontinuing steroid use.

Discussion

The current study aimed to provide a clinical-pathologic correlation for patients with suspected immune DAT-negative hemolytic anemia, regardless of the cause. Of the 447 samples evaluated during the study interval, we found that about 1 in 4 DAT-negative referrals were positive with enhanced methods. This finding was complicated by the fact that most of these positive studies were also positive with standard DAT methods in our reference lab and that only 8.3 percent of samples were positive using DAT-enhanced methods alone. Our survey revealed that the clinical characteristics of patients with a DAT-positive and DAT-negative evaluation did not differ significantly, though knowledge of a positive enhanced DAT result caused significantly greater changes to the patient’s reported diagnosis, and altered treatment plans.

The phenomenon of clinically evident WAIHA with a negative DAT has been known for decades. Worledge and Blajchman (1972) reported that 3 percent of 333 patients with AIHA were DAT negative; Chaplin reported a similar incidence; Petz and Garratty studied 347 patients with suspected AIHA and found an incidence of 7 percent (11% if including all referred samples); and Boccardi et al. reported that 11 percent of their patients with WAIHA were DAT negative. Our data, finding 8.3 percent of cases to be DAT negative using standard methods, are consistent with these previous studies.

As the methods used for detecting warm autoantibodies have improved, many reference labs are now using a combination of enhanced methods, such as those performed by our reference laboratory, to detect low-level antibodies, low-affinity antibodies, and non-IgG antibodies. Our serologic findings are similar to those published previously using standard methods. First, studies, such as that performed by Leger et al. and Garratty et al., found that many cases submitted for a suspected DAT-negative hemolytic anemia (10–50%) had a positive standard DAT result when handled by their reference laboratory. These differences are significant, because they demonstrate and confirm that differences in technique and methodology can dramatically influence the quality of hospital laboratory DAT results. In our laboratory, we also found that 23 percent of samples submitted for a DAT-negative hemolytic anemia had a positive standard DAT, supporting these previous observations. Second, we found a similar proportion of positive DAT results using enhanced methods. In the study performed by Leger et al., 4.9 percent of samples were positive by LISS alone, 2.0 percent by polybrene alone, and 0 percent by gel alone. When excluding those that were positive by standard DAT methods, we also found that 3.1 percent of samples were positive only with 4°C LISS, none were positive with PEG alone, and 4.0 percent were positive only with gel.

To our knowledge, our study is the first in the U.S. to evaluate clinical information on a cohort of 57 patients with a suspected DAT-negative hemolytic anemia and then correlate these findings with reference laboratory serologic results. The findings from our survey are consistent with, and expand upon, the observations from other previous studies. Specifically, DAT-negative WAIHA has been associated with non-Hodgkin’s lymphomas, certain chemotherapeutic drugs (such as fludarabine, cyclophosphamide, and rituximab), certain hematopoietic stem cell transplants, autoimmune diseases such as Sjogren syndrome, kidney transplants, certain solid organ malignancies, and pregnancy. Similarly, our 18 DAT-positive respondents reported that their hemolytic anemia was associated with acute leukemia status post–bone marrow transplant, myelodysplastic syndrome, and sickle cell disease.

We also confirmed that steroids are the most common treatment for our patient cohort and that these treatments generally resulted in disease remission or stability. This observation is consistent with what has been previously reported. Case studies reveal that patients with DAT-negative hemolytic anemia are generally responsive to steroids, but recurrences of the hemolytic anemia were generally responsive to steroids, and treatment failures (one requiring splenectomy) have been reported. To our knowledge, the largest study to investigate the clinical characteristics and outcomes for DAT-negative hemolytic anemia was performed by Kamesaki et al. In their retrospective review, they identified 154 patients over a 7-year period with DAT-negative hemolytic anemia. They found that 91 percent responded to steroids, 70 percent entered remission in 4 weeks, and 84 percent survived at least 1 year. While fewer of our cohort reported that they used steroids (about 50%), we still found that when steroids were used, more than half demonstrated remission or stability of disease. The differences between our study, and that done by Kamesaki et al. are likely
attributable to the differences in the lab methods used to detect DAT-negative hemolytic anemia and the differences in how we categorized DAT-positive from DAT-negative patient cohorts.

Our study has some notable limitations. First, despite our best efforts, our survey response rate was quite low. Identified reasons for this low response rate included unexpected changes to physician address that could not be further clarified, and large numbers of corporate referring labs that did not have access to patient data. The limited response rate added a possible selection bias that cannot be fully accounted for. However, we feel that our data are generally representative, since our findings are similar to other published literature on this topic. Second, most of our respondents had DATs that were positive by both the enhanced and the standard methods. Consequently, the subsequent patient data reviewed does not represent the clinical characteristics of truly DAT-negative hemolytic anemia. What we can say, however, is that the data reviewed here likely represent the characteristics of those patients who had a negative DAT at some point in their diagnostic workup and were suspected to have a DAT-negative hemolytic anemia. Lastly, while we evaluated hundreds of DAT-negative samples, we could only provide clinical correlations for a small number of cases. Because of sample size and the varying test methods used, we were not able to determine the cause for the negative DAT in these patients (i.e., low number of IgG molecules or significant non-IgG immunoglobulins). IgA-induced hemolytic anemia, specifically, was not represented in our cohort, and only two cases of low-affinity antibodies (LISS positive, monoclonal IgG negative) were evaluated in our survey sample.

In conclusion, this study suggests that using a battery of enhanced methods to evaluate cases of possible DAT-negative autoimmune hemolytic anemia is valuable, and changes to diagnosis and treatment plans were identified when a DAT-positive result was obtained from a formerly DAT-negative patient. Moreover, the use of enhanced techniques in the 103 cases with positive standard DATs increased the confidence that immunoglobulin was truly binding to red cells. Clearly, no single test can be used to predict the presence of significant autoantibodies, and our study supports an increasing body of literature that DAT-negative and DAT-positive hemolytic anemias appear similar in etiology, treatment, and general outcome. Additional larger prospective studies are needed to further elucidate the qualities that make patients with DAT negative hemolytic anemia unique.

References


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Blocked D phenomenon and relevance of maternal serologic testing

A. Jain, V. Kumawat, and N. Marwaha

A blood requisition for double-volume exchange transfusion was received for a 2-day-old male child born to a 29-year-old multiparous female (P_{2002}) referred to our institute having neonatal jaundice with encephalopathy; no maternal sample was received. The neonatal blood sample was typed as group A, D−, and the direct antiglobulin test (DAT) was strongly positive (4+) using the gel method. Mono-specific DAT showed the presence of IgG antibodies on neonatal red blood cells (RBCs). Acid elution and gentle heat elution (at 56°C) confirmed the presence of anti-D on neonatal RBCs. The baby received two exchange transfusions with group O, D−, packed RBCs compatible with his own serum. Later, on day 3, the neonate’s mother was typed as group AB, D−, and her serum revealed the presence of alloanti-D, -C, and -S reactive in the anti-human globulin phase. The anti-D titer was 1024. This report highlights the “blocking” phenomenon caused by maternal anti-D in a case of hemolytic disease of fetus and newborn with a positive DAT. Immunohematology 2015;31:116–118.

**Key Words:** blocked D phenomenon, maternal serologic testing, antibody screening

Of all red blood cell (RBC) antigens, D is second only to ABO in importance in blood transfusion. The Rh antigens are fully expressed at the time of birth, unlike the weak expression of ABO in neonates.1 A cumulative dose of not more than about 0.03 mL RBCs is capable of inducing primary D immunization.2 False-negative typing results caused by potent antibody-blocking antigen sites are uncommon when using modern monoclonal blood-grouping reagents. Nevertheless, it is well known that blocking of fetal D with potent maternal anti-D (also known as “blocked D phenomenon”)3 can occur and attempted typing of these fetal RBCs using IgM anti-D can yield false-negative results. We report here a case of blocked D in a neonatal blood sample in a suspected case of Rh hemolytic disease of fetus and newborn (HDFN) that was detected in our hospital.

**Case Report**

A 2-day-old male child was admitted through the pediatric emergency department of our institute after referral from a nearby district hospital with a diagnosis of neonatal jaundice with encephalopathy. The neonate was born to a 29-year-old multiparous female (P_{2002}) with a previous live male child who was currently 2 years old. First delivery was unsupervised full-term vaginal delivery with no history of neonatal jaundice. No anti-D immunoprophylaxis was given at the first delivery. Index neonate was also born after a full-term normal vaginal delivery with a birth weight of 3 kg. Total serum bilirubin was 25 mg/dL when the neonate was admitted through the pediatric emergency department. The neonate was lethargic with excessive crying and poor feeding. Blood requisition for double-volume exchange transfusion was received at our blood bank without the mother’s sample since she was not available.

**Results**

In the pre-transfusion testing laboratory, the neonatal RBCs were typed for ABO and D by the conventional tube method using commercially available reagents; this showed the blood type as group A, D−. The direct antiglobulin test (DAT) was performed using the gel method (Anti-IgG + C3d, LISS-Coombs AHG Card, Bio-Rad, Cressier, Switzerland), which gave a strong positive (4+) result. Monospecific DAT (DC screen II, Bio-Rad) showed the presence of IgG on the neonatal RBCs (Fig. 1). Subsequently, the D typing was repeated with anti-D sera from various manufacturers and was found to be negative with all of them (Fig. 2). Antibody screening (Diacell, Bio-Rad) and identification (Diapanel, Bio-Rad) of the neonate’s serum revealed presence of anti-D. Acid elution (Diacidel, Bio-Rad) and gentle heat elution (at 56°C) of the neonatal’s RBCs confirmed the presence of anti-D on the RBCs.

Because this was an emergency situation and also because of the limited sample volume, extended Rh-matched (C, c, E, e) and K-matched, compatible group O, D−, RBCs along with group AB, D+, plasma were issued for exchange transfusion. After two exchange transfusions, the bilirubin lowered to 17.7 mg/dL from 24.6 mg/dL and the hematocrit improved from 30 percent to 50 percent. The neonate’s mother was subsequently available on day 3, and she was typed as group AB, D−, with a probable Rh phenotype of rr (dce/dce) (Table 1). Antibody
screening (Diacell) and identification (Diapanel) of maternal serum revealed presence of multiple alloantibodies. Testing with enzyme-treated cells and using the “select cell process” confirmed the presence of alloanti-D, -C, and -S in maternal serum. These were found to be reactive in the antihuman globulin (AHG) phase. Maternal titer of anti-D was found to be 1024 by the conventional tube method. The father was typed as group B, D+, with an extended typing of C+, c−, E−, e+.

On day 4 of life, in view of rising bilirubin (21.5 mg/dL), a third exchange transfusion was done with an AHG phase–compatible, group O, D−, packed RBC component that was Rh-matched (C, c, E, e), K-matched, and also S−. The baby subsequently recovered well and was finally discharged from the hospital on day 8 of life.

Discussion

ABO and D testing can be regarded as the most important serologic tests performed in transfusion services on pretransfusion samples. Of the alloantibodies that have been implicated in HDFN, anti-D is usually associated with the most severe disease. In our case, despite the mother being sensitized to three clinically significant alloantibodies, it was primarily the anti-D that was responsible for HDFN, because it was the only antibody to be identified in the neonatal red cell eluate.

The blocking phenomenon caused by maternal anti-D should be suspected in a case of Rh-HDFN if fetal or neonatal RBCs give a strongly positive result in a DAT but a negative result on D typing with saline reactive anti-D. This phenomenon may lead to a delay in making an antenatal or postnatal diagnosis of Rh-HDFN by routine serologic investigation. The first description of D blocking phenomenon was given by Wiener in 1944. Recently, Verma et al. described the blocked D phenomenon on fetal RBCs from a group AB, D−, female who had a high anti-D titer (256) and was planned for intrauterine transfusion because of fetal anemia. In their report, the fetal blood sample gave a strong positive DAT (4+) using the gel method and was typed as D−. The fetal parameters improved after the transfusion, but

Table 1. Extended phenotyping

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<th>K</th>
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nd = not done.
the mother developed an additional alloantibody (anti-Jk\(^b\)). Sulochana et al.\(^5\) described a case in which a D-negative result was found repeatedly in a newborn baby with severe HDFN born to a second gravida group B, D–, mother. The authors reported that the mother had an anti-D titer of 1024 along with alloanti-C, which was determined only after the baby was born and underwent exchange transfusions. There was no antenatal follow-up of the patient. In our case as well, the antibody specificities could be completely determined only after testing the maternal sample. We found three alloantibodies (anti-D, anti-C, and anti-S) that were reactive in the AHG phase and thus were clinically significant. The paternal RBC typing revealed that he was D+, C+, and S+, which might have been inherited by the fetus causing alloimmunization in the mother.

The baby recovered well after three exchange transfusions and was discharged. The serologic testing for “weak D” on the neonate’s RBCs during the period of positive DAT was not of much significance. However, the later follow-up of the baby’s RBCs for D typing and extended phenotyping once the transfused cells had cleared from the baby’s circulation could not be performed. Thus, a regular antibody screen and follow-up in the antenatal period is of utmost importance to ensure appropriate fetal management.

The blocking phenomenon is not limited to anti-D. Blocking phenomenon caused by Kell blood group antibodies have been reported (e.g., two cases of false-negative K1 typing of fetal cells caused by blocking maternal IgG anti-K\(^6–8\)). With the immunogenicity of D being only second to ABO, accuracy in D typing is critical in transfusion medicine. British Committee for Standards in Haematology\(^9\) antenatal grouping and screening guidelines provide guidance to identify potentially harmful cases of HDFN.

In conclusion, the blocking phenomenon caused by maternal anti-D should be suspected in a possible case of Rh-HDFN if fetal or neonatal RBCs give a strongly positive result in a DAT but typing for D is negative.

References


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Anti-Jk3 in a Filipino man

S. McCaskill, S. Wise, and S. Tinsley

A 62-year-old Filipino man with a history of chronic obstructive pulmonary disease, hypertension, and hyperlipidemia was admitted to the emergency department at Hospital A with recurrent fevers, weakness, and jaundice. The patient was evaluated and eventually discharged with a diagnosis of possible drug-induced hepatitis. One month later, the patient was admitted to Hospital B for recurrent fevers and weakness. The patient’s hemoglobin was 3.8 g/dL. Six units of packed red blood cells (RBCs) were ordered for transfusion. The patient’s sample typed as group B, D+, and the antibody screen was negative. All six units of packed RBCs appeared compatible (at immediate spin) and were transfused to the patient. His hemoglobin level 4 days post-transfusion was 9.3 g/dL, and the patient was discharged. The patient returned after a week for follow-up and his hemoglobin was found to have dropped to 8.5 g/dL, which continued to fall until it reached 7.0 g/dL. Additional packed RBCs were ordered for transfusion. During subsequent pre-transfusion compatibility testing, the antibody screen was found to be positive (all screening cells reactive at the anti-human globulin phase). An antibody identification panel was performed. The patient’s serum was found to react with all panel cells tested, including the autocontrol tube. A direct antiglobulin test revealed the presence of both anti-IgG and anti-C3 coating the patient’s RBCs. The specimen was then sent to a reference laboratory for further testing. Results from the reference lab testing revealed the presence of anti-Jk3 in the patient’s serum. The patient was placed on steroids, and his reticulocyte count increased with no further signs of extravascular hemolysis. No additional transfusions were necessary. He was eventually discharged with a hemoglobin of 13.6 g/dL. The purpose of this case study is to report the findings of an extremely rare but clinically significant antibody, anti-Jk3.

Key Words: magnetic resonance cholangiopancreatography, MRCP, antihuman globulin, AHG, direct antiglobulin test, DAT, intravenous immunoglobulin, IVIG, delayed hemolytic transfusion reaction, DHTR, breakpoint cluster region protein, BCR, Janus kinases 2 gene, JAK2, Abelson murine leukemia viral oncogene homolog 1, ABL1

The Kidd blood group system is defined by two antigens, Jka and Jkb, that yield four different phenotypes: Jk(a+b+), Jk(a+b−), Jk(a−b+), and Jk(a−b−). Jk(a−b−) is also known as Jk−3 or the Jknull phenotype. The antigens Jka and Jkb were discovered by Allen, Diamond, and Niedziela in 1951 and the Jk(a−b−) phenotype was discovered soon after by Pinkerton and Mermod in 1959. Individuals with the Jk(a−b−) phenotype lack both Jka and Jkb. The Jk(a−b−) phenotype can result from the homozygous inheritance of recessive alleles that produce neither Jka nor Jkb or from the inheritance of a dominant inhibitor gene, In(Jk), reported to have no association with the Jk locus but causes a suppression of the expression of Kidd antigens. The Jk(a−b−) phenotype is seen more frequently in people of Polynesian, Filipino, and Chinese ancestry. The Jk(a−b−) phenotype is rare among white and black populations and is more common among Polynesians, Filipinos, and Chinese. The Jk(a−b−) phenotype frequency among the white population is less than 0.1 percent. In Taiwan, the Jk(a−b−) phenotype was found to be 1.0 percent among the Rukai tribe and 1.2 percent among the Paiwan tribe. Among Polynesians, the Jk(a−b−) phenotype frequency is 1.4 percent. The Jk(a−b−) phenotype has also been reported to occur more frequently in Indian, Brazilian, Japanese, and Thai populations.

Alloantibodies to Kidd antigens are clinically significant in transfusion recipients and women who are pregnant. The antibodies are produced in reaction to antigen exposure during a previous transfusion or pregnancy and remain in low titer in plasma until secondary exposure occurs. Kidd antibodies are capable of binding complement and are known to cause both immediate and delayed hemolytic transfusion reactions (DHTRs) as well as hemolytic disease of the fetus and newborn (HDFN). The production of anti-Jk3 occurs only in individuals who have inherited recessive alleles causing the Jk(a−b−) phenotype; those who have inherited the dominant inhibitor gene In(Jk) cannot make anti-Jk3. Anti-Jk3 is best detected by the indirect antiglobulin test (IAT) and can be enhanced with enzyme pretreatment of RBCs used in antibody identification procedures.

Case Report

A 62-year-old Filipino man with a history of chronic obstructive pulmonary disease, hypertension, and hyperlipidemia was admitted to the emergency department at Hospital A with recurrent fevers, weakness, and jaundice. His liver function tests were elevated: aspartate aminotransferase (AST) = 64 IU/L (normal 1–40 IU/L), alanine aminotransferase (ALT) = 64 IU/L (normal 7–55 IU/L), gamma glutamyl transferase (GGT) = 83 IU/L (normal 17–46 IU/L), alkaline phosphatase (ALP) = 119 U/L (normal 28–106 U/L), bilirubin (direct) = 0.3 mg/dL (normal 0–0.3 mg/dL), and bilirubin (total) = 1.4 mg/dL (normal 0.2–1.3 mg/dL). The complete blood count revealed a hemoglobin of 7.3 g/dL. The patient was placed on steroids, and his reticulocyte count increased with no further signs of extravascular hemolysis. No additional transfusions were necessary. He was eventually discharged with a hemoglobin of 13.6 g/dL. The purpose of this case study is to report the findings of an extremely rare but clinically significant antibody, anti-Jk3.

Key Words: magnetic resonance cholangiopancreatography, MRCP, antihuman globulin, AHG, direct antiglobulin test, DAT, intravenous immunoglobulin, IVIG, delayed hemolytic transfusion reaction, DHTR, breakpoint cluster region protein, BCR, Janus kinases 2 gene, JAK2, Abelson murine leukemia viral oncogene homolog 1, ABL1
The patient was subsequently discharged. The patient was admitted to Hospital B 1 month later with recurrent fevers and weakness. His hemoglobin was 3.8 g/dL. A peripheral blood smear showed signs of leukocytosis (29K) (normal range male: 4.5–11.0K) and thrombocytosis (normal range: 150–450K). A bone marrow biopsy did show hypocellularity with atypical megakaryocytes, hyperplasia, and erythroblastopenia. An IgG plasma cell dyscrasia was noted with 13 percent to 15 percent plasma cells (flow and immunofixation positive, fluorescence in situ hybridization [FISH] negative) with serum protein electrophoresis of 1.5 (reference range: 0.7–1.5 g/dL).

Results of genetic testing revealed the patient to be JAK2-negative and FISH for BCR/ABL–negative, but positive for three copies of ABL1 w/trisomy 9/9q, which can be associated with BCR/ABL1-negative chronic myeloproliferative disease, myelodysplastic syndrome, acute myelogenous leukemia, and, rarely, B-cell acute lymphoblastic leukemia. Tests for parvovirus B19 (serum IgM and immunohistochemistry) on the bone marrow biopsy showed no evidence of biliary obstruction/dilation or stones. Other serologic testing for infectious disease markers and autoimmune disorders was negative.

The patient returned to Hospital B 1 week later for follow-up with an oncologist, and his hemoglobin was found to have dropped to 8.5 g/dL, which continued to fall until it reached 7.0 g/dL (approximately 19 days after transfusion). Additional packed RBCs were ordered for transfusion. During subsequent compatibility testing procedures, the antibody screen was found to be positive with all screening cells at the antihuman globulin (AHG) phase of testing. An antibody identification panel was performed. The patient’s serum was found to react with all panel cells tested including the autocontrol tube at the AHG phase of testing. A direct antiglobulin test (DAT) panel was performed using polyspecific (anti-IgG + anti-C3) and monospecific AHG reagents (anti-IgG, anti-C3). The results of the DATs showed both anti-IgG and anti-C3 coating the patient’s RBCs. The specimen was then sent to a reference laboratory for further testing. Results from the reference lab testing using a polyethylene glycol (PEG)-IAT method revealed the presence of anti-Jk3 in the patient’s serum. An elution was not performed because the patient had not been transfused within the previous 2 weeks. Aliquots of the patient’s serum were adsorbed using allogeneic cells. Clinically significant antibodies to other major blood group antigens were excluded by the PEG-IAT using the adsorbed plasma. Phenotyping was performed on autologous neocytes recovered by microhematocrit centrifugation. Results of the phenotyping showed the patient to be Jk(a–b–). Genomic sequencing was also performed on the patient’s RBCs to determine the genotype that would allow for the prediction of the patient’s phenotype. Results of the genetic testing revealed the presence of two variant alleles that were detected by JK-cDNA analysis and genomic sequencing. Jk*02N.01 carried the intron 5c.342-1g>a variant that is associated with exon 6 skipping and a null Jk(b–) phenotype. The Jk*01 allele carried the two known changes of c.130G>A and c.893G>A, not normally reported on the same allele. The JkC.130A single-nucleotide polymorphism (SNP) is associated with weakened antigen expression, and the c.893A SNP is associated with a null Jk(a–) phenotype. It was recommended, if transfusion was necessary, to select ABO/Rh compatible units negative for both Jk and JkC.
Discussion

This case report focuses on a clinically significant but infrequent antibody known as anti-Jk3. The clinical significance of anti-Jk3 is that it displays weak reactivity in vitro but has the capacity to induce severe RBC destruction in vivo in the presence of Jk\(a^+\) and/or Jk\(b^+\). Anti-Jk3 will present as a “panagglutinin” because the antibody will react with all panel cells, since all panel cells are Jk(a+b−), Jk(a−b+), or Jk(a+b+).\(^5,10\) The autocontrol tube may be either positive or negative depending on whether or not the patient has been recently transfused. The DAT will usually be positive for both anti-IgG and anti-C3. If the patient has been recently transfused (within the last 3 months), the use of allogeneic cells for adsorption procedures is necessary to rule out antibodies to other major blood group antigens. The decision to perform an elution rests with a facility’s protocol(s). Some facilities may elect to do an elution on any patient specimen with positive DAT results, whereas other facilities may be more selective in terms of number of days following a recent transfusion.

The patient was questioned concerning transfusion of blood at other facilities and reported that he had never received a transfusion prior to the units that were given at Hospital B. The timing of this DHTR is consistent with the formation of the antibody specificity and phenotyping of the patient’s RBCs. Because most blood banks and transfusion services have limited resources, confirmation of the antibody specificity and phenotyping of the patient’s RBCs may need to be performed by a reference laboratory. Only blood that is Jk(a−b−) should be transfused to patients with anti-Jk3. Because of the rareness of the Jk(a−b−) phenotype, it is difficult to find blood for these patients. Finding compatible units for such patients will require the use of rare donor registries. Siblings of the patient can also be tested to see if they might be of the same phenotype as the patient.

In this case, IVIG was given to the patient because of the suspected parvovirus infection. This is one of the off-label uses of IVIG as described by Hillyer et al.\(^14\) Also, because IVIG is prepared from pools of donor plasma, it may contain antibodies to blood group antigens (e.g., anti-A, -B, -D, and -K; panagglutinins). In this particular case, the IVIG did not interfere in the serologic testing used to identify the antibody present in the patient’s serum.

Conclusion

This case report presents the findings of an extremely rare but clinically significant antibody. Anti-Jk3 is capable of causing severe DHTRs and HDFN. This antibody is difficult to identify in that it will present as a “panagglutinin,” meaning that it will react with all panel cells tested, since they are typically either Jk(a+) or Jk(b+) or both. The autocontrol may be positive if the patient has been recently transfused. If the autocontrol is positive, a DAT should be performed to characterize the protein coating the patient’s RBCs. An elution should also be performed to identify the antibody coating the patient’s RBCs, since it is not safe to assume that the antibody present in the patient’s serum is the same antibody that is coating the patient’s RBCs. Because most blood banks and transfusion services have limited resources, confirmation of the antibody specificity and phenotyping of the patient’s RBCs may need to be performed by a reference laboratory. Only blood that is Jk(a−b−) should be transfused to patients with anti-Jk3. Because of the difficulty in finding compatible units for such patients, rare donor registries will need to be consulted in the event that transfusion is required. The use of IVIG for patient treatment must be taken into consideration when performing serologic workup for suspected transfusion reactions because of contaminating antibodies that may be present in the formulation that could interfere with serologic testing.

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References


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Severe hemolytic disease of the fetus and newborn due to anti-C+G


Case Report

Anti-G is commonly present with anti-D and/or anti-C and can confuse serological investigations. In general, anti-G is not considered a likely cause of severe hemolytic disease of the fetus and newborn (HDFN), but it is important to differentiate it from anti-D in women who should be administered anti-D immunoglobulin prophylaxis. We report one woman with three pregnancies severely affected by anti-C+G requiring intrauterine treatment and a review of the literature. In our case, the identification of the correct antibody was delayed because the differentiation of anti-C+G and anti-D+C was not considered important during pregnancy since the father was D−. In addition, anti-C+G and anti-G titer levels were not found to be as reliable as is generally considered in Rh immunization. Severe HDFN occurred at a maternal anti-C+G antibody titer of 8 and anti-G titer of 1 in comparison with the critical titer level of 16 or more in our laboratory. Close collaboration between the immunohematology laboratory and the obstetric unit is essential. In previously affected families, early assessment for fetal anemia is required even when titers are low. Immunohematology 2015;31:123–127.

Key Words: anti-G, anti-C, HDFN, alloimmunization, intrauterine transfusion

Anti-D is the most common antibody responsible for severe hemolytic disease of the fetus and newborn (HDFN), but antibodies against other antigens belonging to the Rh blood group system can also cause HDFN. G, first described by Allen and Tippett,1 is part of the Rh blood group system and is dependent on expression of both the RHCE*Ce and RHD alleles. There are some exceptions, but most individuals who do not carry an RHD or RHCE*Ce allele would be expected to be negative for G. Anti-G often presents with anti-D and/or anti-C, but seldom alone. Anti-G should be suspected in cases where the anti-C titer is higher than the anti-D titer and confirmed by additional tests.2,3 Anti-G is present in approximately 30 percent of cases with apparent anti-D+C.4

Anti-G has been associated with HDFN. A few cases have been reported where anti-G was the probable cause of severe HDFN, but usually the disease is mild and does not require therapeutic intervention in the offspring.3,4−9 A case initially considered anti-D+C may turn out to be anti-C+G instead, and in these cases, anti-D prophylaxis should be administered to prevent anti-D alloimmunization and manifestation of HDFN in subsequent pregnancies.

In this case report, we describe the outcomes of three pregnancies of a Caucasian woman and her African husband, where anti-C+G caused severe HDFN requiring intrauterine (IU) transfusions.

Case Report

The patient had a history of two miscarriages and one extraterine pregnancy. Subsequently, she had a normal pregnancy, where antenatal antibody screening was negative. In the following pregnancies, alloimmunization was identified, resulting in three cases of severe HDFN. In this case report, we present these affected pregnancies, designated as the first, second, and third (Table 1). According to the national protocol for antenatal antibody screening, maternal sampling began at 8–12 weeks of gestation in all pregnancies. The postnatal blood samples were drawn on the day of delivery.

In her first affected pregnancy (in 2009), the antibody screening was positive for what were at the time assumed to be anti-D and anti-C. Followed monthly, the antibody titers remained at moderate levels (4–8) until 34 weeks of gestation. Because the titer level was below the critical level of 16, ultrasound examinations to detect fetal anemia were not considered necessary. At 37 weeks of gestation, an emergency caesarean section was performed because of reduced fetal movements and a sinusoidal heart rate pattern in cardiotocography. A D− boy was delivered with a hemoglobin (Hb) of 3.1 g/dL and a positive direct antiglobulin test (DAT). Unexpectedly, the anti-D+C titer on a sample taken 5 days prior to the delivery and available postnatally was 128. Immediately postnatally, the anti-D+C titer was 64. The anti-C titer was higher than the anti-D titer, as had been the case already during the pregnancy, thus prompting suspicion of anti-G instead of anti-D. The infant recovered well after successful treatment with red blood cell (RBC) transfusion and treatment with intravenous immunoglobulin (IVIG).

At the beginning of her second affected pregnancy (in 2012), anti-D−like antibody, anti-C, and anti-Jkα were
Table 1. The courses and outcomes of three pregnancies (in the years 2009, 2012, and 2013) of an alloimmunized D–C–c+E–e+Jk(a–b+) mother

<table>
<thead>
<tr>
<th>Year</th>
<th>Antibody</th>
<th>Titer</th>
<th>Titer cells</th>
<th>Antibody</th>
<th>Titer</th>
<th>Titer cells</th>
<th>Antibody</th>
<th>Titer</th>
<th>Titer cells</th>
</tr>
</thead>
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<tr>
<td>2009</td>
<td>Anti-D+C</td>
<td>4–8</td>
<td>R,r</td>
<td>Anti-D+C</td>
<td>16</td>
<td>R,r</td>
<td>Anti-C+G</td>
<td>8–16</td>
<td>R,r</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>8–16</td>
<td>r,r</td>
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<td></td>
<td></td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1–2</td>
<td>R,r</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Anti-Jkα</td>
<td></td>
<td>r</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Follow-up</td>
<td>Monthly antibody levels</td>
<td>Monthly antibody levels</td>
<td>Monthly antibody levels</td>
<td></td>
<td></td>
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<tr>
<td>Intervention</td>
<td>Emergency CS at 37 weeks (reduced fetal movements, sinusoid cardiotocography)</td>
<td>IU × 5, Hb (between 24 and 34 weeks), induction of labor at 34 weeks</td>
<td>IU × 3, Hb (between 31 and 35 weeks), induction of labor at 36 weeks</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Delivery</td>
<td>D– boy, DAT+ 3.1 g/dL</td>
<td>D– girl, DAT– 14.1 g/dL</td>
<td>D– boy, DAT+ 14 g/dL</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Prenatal work-up</td>
<td>Anti-D+C</td>
<td>128</td>
<td>R,r</td>
<td>Anti-D+C</td>
<td>8</td>
<td>R,r</td>
<td>Anti-C+G</td>
<td>4</td>
<td>R,r</td>
</tr>
<tr>
<td></td>
<td>64–128</td>
<td>r,r</td>
<td></td>
<td></td>
<td>1</td>
<td>R,r</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>64</td>
<td>r,r</td>
<td></td>
<td>Anti-G</td>
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<td>r,r</td>
<td>Anti-Jkα</td>
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<td>r,r</td>
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<tr>
<td></td>
<td>128</td>
<td>R,r</td>
<td></td>
<td>Anti-C+G</td>
<td>2</td>
<td>r,r</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Anti-C+G</td>
<td>4</td>
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<tr>
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<td>Anti-Jkα</td>
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<td>r,r</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Anti-Jkα</td>
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<td>r,r</td>
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<tr>
<td>Postnatal work-up</td>
<td>Anti-D+C</td>
<td>64</td>
<td>r,r</td>
<td>Anti-D+C</td>
<td>64</td>
<td>r,r</td>
<td>Anti-C+G</td>
<td>4</td>
<td>r,r</td>
</tr>
<tr>
<td></td>
<td>Anti-D+C</td>
<td>128</td>
<td>R,r</td>
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</tr>
<tr>
<td></td>
<td>Anti-C+G</td>
<td>128</td>
<td>R,r</td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>Anti-Jkα</td>
<td>1</td>
<td>r,r</td>
<td></td>
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<tr>
<td></td>
<td>Anti-Jkα</td>
<td>4</td>
<td>r,r</td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Anti-Jkα</td>
<td>1</td>
<td>r,r</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment</td>
<td>IVIG, red cell transfusion</td>
<td>IVIG, phototherapy, red cell transfusion ×2</td>
<td>IVIG ×3, phototherapy</td>
<td></td>
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</tbody>
</table>

*Sample taken 5 days prior to the delivery; result available 1 day postpartum.
†Sample taken on the day of delivery.
‡Anti-C+G confirmed postnatally.
§Sample taken from umbilical cord.

MCA = middle cerebral artery; PSV = peak systolic velocity, cm/sec; MoM = multiples of median; CS = caesarean section; IU = intrauterine; Hb = hemoglobin; DAT = direct antiglobulin test; IVIG = intravenous immunoglobulin.

identified. The anti-D+C titer was 16 and the anti-Jkα titer was 1. The antibody levels were followed monthly/fortnightly, and they remained unchanged during the pregnancy. Because the father was D–C+, at 16+2 weeks of gestation the fetus was genotyped from amniotic fluid sampling and found positive for a RHD-CE-D hybrid gene that is predicted to express no D and instead expresses an altered C. The fetus was then carefully monitored noninvasively with ultrasound examinations. No evidence of fetal anemia was apparent until 23 weeks of gestation, when the peak systolic velocity (PSV) in the middle cerebral artery (MCA) Doppler examinations exceeded 1.5 multiples of median (MoM). In cordocentesis at 24+1 weeks, the fetal Hb was 7.4 g/dL. A total of five IU transfusions were performed successfully between 24+1 and 32+6 weeks of gestation. Vaginal delivery was induced at 36+4 weeks and a slightly premature but otherwise healthy girl was born with a Hb of 14.1 g/dL and a negative DAT. Neonatal treatment included IVIG, phototherapy, and two top-up transfusions. Anti-C+G was confirmed postnatally.

At the beginning of her third affected pregnancy, in 2013, anti-C, anti-G, and anti-Jkα were identified. During the pregnancy, the titers remained stable: 8–16 for anti-C+G, 2 for anti-G, and 1 for anti-Jkα. The fetus was genotyped from amniotic cells as a RHD-CE-D hybrid at 21+1 weeks of gestation. Weekly ultrasound examinations were begun at 22+6 weeks. Middle cerebral artery PSV stayed under 1.29 MoM until 30+0 weeks, when it reached 1.4 MoM. Given the mother’s history, the last finding gave reason to suspect anemia, and the first IU transfusion was then scheduled. The Hb at the first transfusion was 8.8 g/dL. A total of three IU transfusions were performed between 30+5 and 34+6 weeks of gestation. Labor was induced at 36+0 weeks, and a healthy R. Jernman et al.
boy was born with a Hb of 14 g/dL and a positive DAT. The child received IVIG treatment (× 3) and phototherapy, with the latter continued at home after discharge from the hospital.

**Materials and Methods**

All blood samples and amniotic fluid samples were analyzed in the Finnish Red Cross Blood Service (in Helsinki), which is a national reference laboratory to which the antenatal screening for RBC antibodies is centralized. In the second affected pregnancy, in 2012, a blood sample was also sent to the International Blood Group Reference Laboratory (IBGRL) in Bristol, UK, where absorption studies were performed to differentiate anti-G from anti-D and anti-C.

ABO and D typing of RBC samples from the mother were performed with an analyzer (PK7300, Olympus Corporation, Tokyo, Japan) on microtiter plates.

Antibody screening, identification, and DAT studies were carried out with a gel-based analyzer (DiaMed ID GelStation, DiaMed, Cressier, Switzerland). Antibody screening and identification were performed using a gel method (LISS/Coombs cards, Bio-Rad, Cressier, Switzerland) for untreated RBCs. Antibody identification with enzyme (papain)-treated RBCs in a gel method (NaCl card, DiaMed) was also used. A DAT was performed on the infant's cord blood sample using polyspecific antihuman globulin (AHG) (anti-IgG and anti-C3D, Bio-Rad) in a gel method (LISS/Coombs cards). Serologic phenotyping was carried out with specific reagent gel cards (Bio-Rad).

Titrations were performed using the tube method for indirect antiglobulin test and R, r reagent RBCs for anti-D, anti-D+D, and anti-D; r’r for anti-C; and R, r for anti-G (because reagent cells with only C or G antigen were not available for routine use). During the first affected pregnancy, only R, r cells were used in titration of anti-D+C, but after delivery of the severely anemic D− child, titration was also performed with r’r cells. In the second affected pregnancy, cells used for titrating anti-D+C, anti-C, and anti-G were R, r, r’r, and R, r, respectively, throughout the pregnancy. In the third affected pregnancy, the titer cells used were R, r for anti-C+G and R, r for anti-G throughout the pregnancy. Titration for anti-Jk was performed with rr Jk(a+b+) reagent RBCs.

Genotyping of the fetal RHD and RHCE genes was carried out from amniotic cells using polymerase chain reaction with sequence specific primers (PCR-SSP) (PCR-SSP, Inn-Train, Kronberg, Germany) according to the manufacturer’s instructions.

**Results**

The Caucasian mother was phenotyped as D−C−c+E−e+; Jka(b−) and the African father as D−C+c+E−e+; Jk(a+b−). The father carried the RHD-C-E-D hybrid, which encodes an altered C that reacts more weakly than a conventional C when using serologic methods. The father was the same in all pregnancies.

In the first affected pregnancy, the anti-D+C titer remained 4–8 using D+C+ (R, r) cells followed monthly. The titer was 128 using D−C+ (r’r) cells on a sample taken 5 days prior to the delivery and 64 immediately postnatally.

In the second affected pregnancy, the anti-D+C titer was 16 using D+C+ (R, r) and D−C+ (r’r) cells, and 2 with D+C− (R, r) cells. A maternal blood sample had also been sent to the IBGRL in Bristol, UK, where anti-C+G and anti-Jk were confirmed and anti-D ruled out. The postpartum maternal anti-C+G titer was 8, and anti-G and anti-Jk titers were both 1; the anti-C+G titer from cord blood was 4 and anti-G titer was 1; anti-Jk was not detected.

During the third affected pregnancy, the antibody titers remained stable: 8–16 for anti-C+G using D+C+ (R, r) cells, 2 for anti-G using D+C− (R, r) cells, and 1 for anti-Jk. The postpartum maternal anti-C+G and anti-G titers were 4 and 2, respectively; anti-Jk titer was 1 and not obtained for analysis from the cord blood sample.

**Discussion**

In our patient, anti-C was the dominant antibody in all affected pregnancies, including the first, as could be confirmed retrospectively. The finding of a stronger anti-C compared with anti-D led to the suspicion of anti-G. In the first affected pregnancy, antibody levels remained low until a rapid increase in the final weeks before term, resulting in signs of fetal distress and an emergency caesarean section of a severely anemic child. The last titer level 3 weeks earlier was 8 but had risen to 128 by the time of delivery. In the following pregnancies, despite the fact that the antibody titers remained at moderate levels (2–16), IU transfusions were required.

Though there are several case reports in the literature of HDFN caused by anti-G/anti-C+G, to our knowledge, only one of them required IU transfusion: a mother with a history of several affected pregnancies presented with severe HDFN caused by anti-C+G in a twin pregnancy. Maternal IVIG and plasmapheresis were required before IU RBC transfusions could be initiated, and despite intensive monitoring, one twin
was lost and the other needed prolonged treatment including phototherapy, RBC transfusions, and erythropoietin injections. In a case report by Hadley et al., anti-G was found to be the cause of severe HDFN; cordocentesis was planned but found impossible to perform, and after delivery, the infant required several exchange transfusions. The third reported severe case of HDFN with anti-C+G was described by Jakobowicz and Simmons. Thus, anti-G can be clinically significant in pregnancy and may contribute to the development of moderate or severe HDFN, although most reported cases have been mild. For example, Muller et al. reported a typical case where anti-C+G was mistaken for anti-D+C in a primigravida who had received RBC transfusions prior to her pregnancy. After delivery of a D– baby, anti-C+G was identified in maternal blood by adsorption and elution, but no anti-D was detected.\textsuperscript{3} Lenkiewicz and Zupanska reported a pregnancy where anti-C+G antibodies were responsible for moderate hemolytic disease of the newborn, and, based on titration results, anti-G levels were much higher than anti-C.\textsuperscript{10}

Anti-G is only rarely the single antibody responsible for HDFN, but is more often expressed with anti-D, anti-C, or both. Palfi and Gunnarsson analyzed the D/C/G antibody combinations in 27 pregnancies and found that anti-G was present in 24 cases, and in 4 of the 27 cases, anti-C+G was identified without anti-D; anti-G was not found alone in any of these cases.\textsuperscript{8} Interestingly, Huber and coworkers reported a patient who did have anti-G as the sole cause of moderate hemolytic disease of the newborn—anti-D and anti-C were excluded.\textsuperscript{11}

In our case, the affected fetuses had inherited the hybrid allele \textit{RHD-CE-D} from their father. The allele codes a D– and weak C\textsuperscript{+} phenotype. G is intact. Thus, both anti-C and anti-G could find the target antigens on the RBCs of the fetuses.

Furthermore, our case demonstrates that anti-C or anti-G titers are poor predictors of the outcome in an immunized pregnancy. In our facility, monitoring of pregnancies at high risk for HDFN is planned in collaboration with the immunohematology laboratory and the obstetric unit. Antibody screening, identification, and follow-up of antibody levels are carried out monthly or every 2 weeks. Usually, if critical antibody titer levels of 16 are reached, fetal ultrasound examinations begin at 18 weeks of gestation and continue weekly/fortnightly throughout the pregnancy. Fetal well-being is assessed with serial ultrasound for signs of hydrops and Doppler measurement of the PSV of the MCA form the basis for follow-up in immunized pregnancies. In anemic fetuses, lower blood viscosity and increased cardiac output result in a higher PSV; a threshold value of 1.5 MoM is predictive of moderate to severe anemia, whereas levels lower than 1.29 are considered normal.\textsuperscript{12,13} This noninvasive method has replaced the need for assessment of amniotic bilirubin levels. Moreover, fetal \textit{RHD} and \textit{RHCE} genotyping from maternal serum is now feasible, although blood groups other than Rh still need to be analyzed from amniocytes.\textsuperscript{13}

Appropriate titer cells are important in the estimation of the role of anti-G. A significant number of cases where apparent anti-D+C are identified may actually contain only anti-C+G and lack anti-D.\textsuperscript{14} Anti-G should be considered if anti-C titer levels exceed or reach the levels of anti-D titers.\textsuperscript{3} In our case, cells used in titration were R\textsubscript{r} for anti-D+C, R\textsubscript{r} for anti-D and anti-G, and r\textsubscript{r} for anti-C. The titer levels obtained with R\textsubscript{r} cells were consistently higher than with R\textsubscript{r} in all three pregnancies, indicating anti-C as the stronger antibody. In general, in our laboratory, when anti-D+C are identified, only R\textsubscript{r} cells are used in titration. Anti-G should be suspected prior to titration if the antibody identification panel shows stronger reactions for anti-C than anti-D. Then, if titrations performed with R\textsubscript{r}, R\textsubscript{r}, and/or r\textsubscript{r} cells also indicate the possibility of anti-G, additional tests should be carried out. In our laboratory, anti-G is demonstrated and anti-D ruled out with absorption studies performed concurrently with D–C+ and D+C– reagent RBCs. In many laboratories, differential absorption and elution is the method of choice, and is recommended for the differentiation of anti-G from anti-D.\textsuperscript{2–4,6,10,11} Furthermore, rare r\textsuperscript{–r} cells may also be used in titration.\textsuperscript{5,7,11} National and international blood group reference laboratories may be of use in cases where antibody identification by in-house methods is challenging.

Identifying anti-G in serum that initially seems to contain anti-D+C is important because D– women shown to have anti-C+G but not anti-D should receive anti-D immunoglobulin prophylaxis when carrying or having delivered a D+ child. Failure to recognize these cases endangers future pregnancies, since alloimmunization may develop. Furthermore, if the father is D–, the identification of anti-C+G should be discussed, because an incorrect report of anti-D+C may lead to unnecessary distress, paternity testing, and social consequences. The number of reported anti-G cases is still low. Nevertheless, anti-G is more common than has traditionally been thought, and may contribute to the development of severe HDFN.

In conclusion, if a pregnant woman seems to have anti-D and anti-C, with anti-C being the stronger antibody, it is important to test for anti-G. Primarily, if anti-D can be excluded, the woman requires anti-D prophylaxis. In addition, anti-G can, especially in combination with anti-C, cause severe HDFN, even with low titer levels.
References


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Human neutrophils are central to the defense against invading microbes. Neutrophils are not homogenous, but exist in subgroups such as low-density neutrophils or subsets with differential olfactomedin-4 and CD177 expression. CD177 is a neutrophil-specific glycoprotein that is expressed intracellularly and on the neutrophil membrane in 95 percent of all individuals—3 to 5 percent of persons are CD177-deficient. In the former, the expression pattern is bimodal with distinct CD177-positive and CD177-negative subsets. The positive subset ranges from 0 to 100 percent, but remains stable in a given individual. Clinically, CD177 is relevant to anti-neutrophil cytoplasmic antibodies vasculitis, neutropenia in newborns, graft failure in bone marrow recipients, drug-induced neutropenia, and transfusion-related lung injury. A role for CD177 in neutrophil migration was suggested by engaging platelet-endothelial-cell-adhesion molecule-1 on endothelium.

Mechanisms controlling the size of the CD177-positive neutrophil subset are unknown. To test whether or not the percentage of CD177-positive neutrophils changes during inflammation, we performed serial CD177 expression studies in a large patient cohort with acute bacterial sepsis and analyzed the data in relation to disease severity. Peripheral blood was obtained after local ethics board approval (EA1/144/10) from 96 patients in the intensive care unit with acute bacterial sepsis (Sequential Organ Failure score 10.0 ± 4.8). We included 33 disease controls with non-infectious systemic inflammatory response syndrome (solid organ transplantation, acute ischemic stroke, myocardial infarction) and 103 normal controls, respectively. The proportion of CD177-positive neutrophils and the mean fluorescence intensity (MFI) were determined by flow cytometry (PE anti-CD177 [clone:MEM-166], Biolegend, San Diego, CA). Fifty-six sepsis patients died, 36 before the scheduled 28-day follow-up flow cytometry assessment.

We found that sepsis patients, but not disease controls, displayed a significantly higher percentage of CD177-positive neutrophils and higher CD177 MFI at the initial assessment, compared with normal persons (Fig. 1A–C). Furthermore, the CD177-positive percentage and MFI changed concordant with clinical outcome. Both CD177-positive percentage and MFI decreased significantly in the 34 patients with inflammation resolution (Fig. 1D, E). C-reactive protein (CRP) decreased in these patients from 21.8 to 3.5 mg/dL and leukocytes from 11.8 to 8.6 × 10⁹/L. In contrast, both CD177 parameters remained elevated in the 26 patients who lacked clinical improvement (CRP from 23.1 to 20.2 mg/dL and leukocytes from 15.8 to 14.2 × 10⁹/L; Fig. 1C, D). One particularly informative patient was admitted with non-infectious systemic inflammatory response syndrome after myocardial infarction and developed Klebsiella pneumoniae pneumonia at day 6 and subsequent sepsis at day 17. CD177-positive neutrophil percentage and MFI increased from 52 percent/39MFI to 74 percent/80MFI at day 6 and finally to 83 percent/109MFI at day 17. The patient recovered, and, at day 39, the values had decreased to 58 percent/44MFI.

Taken together, our data indicate that a high percentage of CD177-positive neutrophils undergo dynamic changes during severe bacterial sepsis and that these changes affect clinical outcome. Possibly, the cytokine milieu modulates CD177 expression. Consistent with this assumption, extended, but not short-term, granulocyte-colony stimulating factor administration in healthy patients increased the CD177-positive neutrophil percentage. Further studies should be done to identify the exact mechanisms that control CD177 expression.

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To the Editor

Alternative to providing ABO-incompatible donors for patients in end-stage renal disease: renal transplant registries, the need of the hour

Transplantation antigens can be classified into major histocompatibility antigens, ABO and Rh blood group antigens, and minor histocompatibility antigens. ABO compatibility—generally not considered critical in hematopoietic cell (HPC) transplants because pluripotent and early committed HPCs do not express ABO antigens—is, however, an important consideration in solid organ transplants. Removal of patient anti-A and/or -B that would be incompatible with the transplant is usually achieved by therapeutic plasma exchanges (TPEs) or by immunoabsorption, which specifically removes anti-A and/or -B by affinity column. We report a case of a nonproductive major ABO incompatible (ABO-I) renal transplant (included in category I for American Society for Apheresis guidelines for apheresis) in a patient started on TPEs and immunosuppressive regimen to lower the titer of incompatible antibody.

A 36-year-old male patient, with a known history of type II diabetes mellitus for 16 years, was diagnosed with chronic renal failure progressing to end-stage renal disease (ESRD). His blood group was O, D+. He had a family history of diabetes mellitus (in his mother and younger sister) as well as diabetic retinopathy and diabetic neuropathy. He was currently on insulin therapy. The patient had a history of oliguria for a year and was being maintained on hemodialysis. On investigation, he was found to have anemia and azotemia: serum urea = 112 mg% (normal range: 10–50 mg%); creatinine = 5.68 mg% (normal range: 0.8–1.8 mg%).

The patient had planned to receive a renal transplant from his brother (not a known diabetic) who typed as group O, D+. However, his brother refused to donate. Subsequently, his wife, who types as group B, D+, was worked up as a donor. Because the baseline anti-B titers in the patient’s serum against pooled reagent B cells were 8 (IgM) and 512 (IgG), he was prepared for desensitization to the ABO-I renal transplant. TPEs (centrifugal TPE on Cobe Spectra cell separator) were planned for the patient to reduce the anti-B titer. Simultaneously, he was put on an immunosuppressive regimen 2 weeks before the transplant that included rituximab (375 mg/m²) single dose, intravenous immunoglobulin (IVIG 100 mg/kg), tacrolimus (4 g/day), and mycophenolate mofetil (2 g/day). A total of 15 TPEs were performed; 11 were performed every alternate day and 4 were performed at 2-day intervals. The procedures involved exchanging 1 to 1.5 times the patient’s plasma volume with group B, D+, fresh-frozen plasmas (FFPs) that were screened by enzyme-linked immunosorbent assay (ELISA) and nucleic acid testing (NAT) for transfusion transmitted infection (TTI). The units of FFP were purposely group specific (B) to gain the added advantage of antibody neutralization by the soluble B antigenic substance in plasma. The patient did not experience any major complications during the procedures. Anti-B titers were repeated after every TPE procedure. Figure 1 shows the anti-B titers (IgG) at antihuman globulin (AHG) phase with respect to each TPE. Anti-B titers after the 15th TPE procedure were 2 (IgM) and 128 (IgG), respectively. After this, no further procedures were performed, and the renal transplant was not performed.

Renal transplant is the treatment of choice in ESRD. It is difficult to maintain the patient on long-term dialysis. Because ABO antigens are present on the surface of the endothelium of vessels and basement membranes of renal tubular cells, they constitute a strong histocompatibility barrier for renal transplants. Major incompatibility refers to the presence of preexisting natural antibodies in the recipient against the donor’s A and/or B blood group antigens, which can lead to hyperacute/acute humoral rejection of the organ because of endothelial damage. Minor incompatibility occurs when the organ donor has naturally occurring ABO antibodies against the recipient, which can cause passenger lymphocyte syndrome.

Limitations in donor availability for stem cell or organ transplantation require that ABO-I donors be used. Although ABO-I transplantation entails increased expense when compared with maintenance dialysis, the benefits of a successful transplant and the subsequent health-related quality of life make it clearly cost-effective. For ABO-I renal transplantation to be effective, pre-existing patient antibodies directed at antigen(s) on the donor organ must be minimized. The goal is to bring the recipient to a titer below a set target and prevent production of additional ABO antibodies before and after transplantation. The most significant
Various pre-conditioning protocols are used in different combinations to reduce the titer of circulating ABO antibody (IgG) to less than or equal to 16, permitting engraftment of ABO-I kidney transplants. TPE along with immunosuppressive regimens is the most commonly used method worldwide. TPE is a nonselective procedure, since it removes not only ABO antibodies but nonspecific antibodies as well. Selective methods such as double filtration plasmapheresis and antigen-specific immunoadsorption using immunoabsorption columns (Glycosorb-ABO columns, Glycorex Transplantation, Lund, Sweden) are available at few centers in India.

Our patient who typed as group O, D+, planned for renal transplant using a live renal donor who typed B, D+. Because of the major ABO incompatibility, the patient was started on a TPE and immunosuppressive regimen. The baseline anti-B titer (IgG) was high (512). Transplantation was to be performed when the anti-B titer reached 4. However, this could not be achieved.

In a retrospective study that included 46 individuals who had ABO-I renal transplants, it was seen that patients with higher initial antibody levels or rebounding titers between treatments received more TPEs. In a study from Johns Hopkins, a protocol was proposed for the required number of pre- and post-transplant TPE procedures based on pre-TPE ABO IgG antibody titers. The authors observed that patients with higher pre-TPE ABO titers had to undergo more therapeutic procedures to remove the antibody. Lawrence et al. undertook a prospective study on 56 patients awaiting ABO-I renal transplants to determine a
cutoff titer above which patients should not enter the ABO-I program, using the relationship between the starting ABO IgG titer and the number of TPEs required. They introduced a cutoff titer of 256 for baseline ABO antibody for consideration of ABO-I renal transplants. They emphasized that patients with higher titers should not be exposed to costly, prolonged courses of immunosuppression and TPE without the guarantee of successful transplantation. Despite these considerations, we decided to go ahead with the preparation protocol in our patient, since the renal failure was progressive and an ABO-compatible renal donor was not available.

For the first two TPE procedures, the titer was 512; the antibody titers then remained stable at 64, decreased to 32 after the seventh procedure, and returned to 64 through the tenth procedure. The lowest titer recorded was 8 after the 11th procedure, which was followed by a subsequent increase to 128 after the 15th procedure. Thereafter procedures were stopped.

There are few case reports in the literature with successful ABO-I renal transplant in patients with high-titer ABO antibodies. In a case report from India, a renal transplant was successfully performed in a patient who typed as group O, D−, who received an organ from his mother, who was group B, D+. The baseline anti-B titer was 512, and it decreased to 8 after 11 TPEs and immunosuppression. In a case report from Japan, successful ABO-I renal transplantation was performed in three patients with high (>512) ABO antibody titer using a preconditioning protocol consisting of rituximab infusions, splenectomy, plasmapheresis, and pharmacologic immunosuppression. It was the same scenario with our patient, with the only difference being that splenectomy was not performed in our case.

We conclude that in a patient whose ABO titer exceeds the cutoff and the trial of TPE fails to decrease the titer to the desired level, the patient should be counseled and offered alternative routes to transplantation. In patients with blood group O, renal transplants from A2 blood group individuals are preferred as compared with other blood groups because of the low expression of A antigen on the graft. A novel approach of blood group antigen-neutralizing therapy has been introduced in which A and B blood group antigens on red blood cells and in kidney tissues are neutralized by blood group antigen–targeting peptide (BATP). In New Zealand, a kidney transplant chain has been developed to overcome such incompatibilities through recipients “exchanging” incompatible for compatible donors. A paired and pooled kidney scheme allows matching a potential kidney recipient having a willing but incompatible donor to another incompatible pair resulting in both recipients receiving a compatible kidney. List Donation involves exchange of a live donor kidney for a deceased donor kidney. Non-Directed Donors is a strategy to allocate donors who desire to donate a kidney but do not have a designated recipient. Thus, the time is opportune to develop a national Kidney Paired Donation program in India with improved coordination among various transplant centers, since the use of unrelated living donors has great potential to increase the donor pool. Maintaining renal transplant donor registries that have a database of voluntary renal donors can greatly facilitate such a program.

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<td><a href="http://www.fbsblood.org">www.fbsblood.org</a></td>
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</tr>
<tr>
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</tr>
</tbody>
</table>

Revised February 2013
Diagnostic testing for:
- Neonatal alloimmune thrombocytopenia (NAIT)
- Posttransfusion purpura (PTP)
- Refractoriness to platelet transfusion
- Heparin-induced thrombocytopenia (HIT)
- Alloimmune idiopathic thrombocytopenia purpura (AITP)

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  - detection of heparin-induced antibodies (PF4 ELISA)
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- Solid phase red cell adherence (SPRCA) assay
- Molecular analysis for HPA-1a/1b

For further information, contact:
Platelet Serology Laboratory (215) 451-4205
Sandra Nance (215) 451-4362
Sandra.Nance@redcross.org

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- Granulocyte agglutination (GA)
- Granulocyte immunofluorescence by flow cytometry (GIF)
- Monoclonal antibody immobilization of neutrophil antigens (MAINA)

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- HLA (PRA) Class I and Class II antibody detection

For further information, contact:
Neutrophil Serology Laboratory (651) 291-6797
Randy Schuller (651) 291-6758
Randy.Schuller@redcross.org

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e-mail:
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or write to:
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American Red Cross Biomedical Services
Connecticut Region
209 Farmington Ave.
Farmington, CT 06032

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Fax: (215) 451-2538

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ardp@redcross.org

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Fax: (215) 451-2538
immuno@redcross.org

Quality Control of Cryoprecipitated–AHF
Phone, business hours:
(215) 451-4903
Fax: (215) 451-2538
A. For describing an allele which has not been described in a peer-reviewed publication and for which an allele name or provisional allele name has been assigned by the ISBT Working Party on Blood Group Allele Terminology (http://www.isbtweb.org/working-parties/red-cell-immunogenetics-and-blood-group-terminology/blood-group-terminology/blood-group-allele-terminology/)

B. Preparation
1. Title: Allele Name (Allele Detail)
   ex. RHCE*01.01 (RHCE*ce48C)
2. Author Names (initials and last name of each (no degrees, ALL CAPS)

C. Text
1. Case Report
   i. Clinical and immunohematologic data
   ii. Race/ethnicity and country of origin of proband, if known
2. Materials and Methods
   Description of appropriate controls, procedures, methods, equipment, reagents, etc. Equipment and reagents should be identified in parentheses by model or lot and manufacturer’s name, city, and state. Do not use patient names or hospital numbers.
3. Results
   Complete the Table Below:

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Allele Name</th>
<th>Nucleotide(s)</th>
<th>Exon(s)</th>
<th>Amino Acid(s)</th>
<th>Allele Detail</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>e weak</td>
<td>RHCE*01.01</td>
<td>48G&gt;C</td>
<td>1</td>
<td>Trp16Cys</td>
<td>RHCE*ce48C</td>
<td>1</td>
</tr>
</tbody>
</table>

   Column 1: Describe the immunohematologic phenotype (ex. weak or negative for an antigen).
   Column 2: List the allele name or provisional allele name.
   Column 3: List the nucleotide number and the change, using the reference sequence (see ISBT Blood Group Allele Terminology Pages for reference sequence ID).
   Column 4: List the exons where changes in nucleotide sequence were detected.
   Column 5: List the amino acids that are predicted to be changed, using the three-letter amino acid code.
   Column 6: List the non-consensus nucleotides after the gene name and asterisk.
   Column 7: If this allele was described in a meeting abstract, please assign a reference number and list in the Reference section.

4. Additional Information
   i. Indicate whether the variant is listed in the dbSNP database (http://www.ncbi.nlm.nih.gov/snp/); if so, provide rs number and any population frequency information, if available.
   ii. Indicate whether the authors performed any population screening and if so, what the allele and genotype frequencies were.
   iii. Indicate whether the authors developed a genotyping assay to screen for this variant and if so, describe in detail here.
   iv. Indicate whether this variant was found associated with other variants already reported (ex. RHCE*ce48C,1025T is often linked to RHD*DIVa-2)

D. Acknowledgments

E. References

F. Author Information
   List first name, middle initial, last name, highest degree, position held, institution and department, and complete address (including ZIP code) for all authors. List country when applicable.
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Before submitting a manuscript, consult current issues of *Immunohematology* for style. Number the pages consecutively, beginning with the title page.

II. SCIENTIFIC ARTICLE, REVIEW, OR CASE REPORT WITH LITERATURE REVIEW
A. Each component of the manuscript must start on a new page in the following order:
   1. Title page
   2. Abstract
   3. Text
   4. Acknowledgments
   5. References
   6. Author information
   7. Tables
   8. Figures

B. Preparation of manuscript
   1. Title page
      a. Full title of manuscript with only first letter of first word capitalized (bold title)
      b. Initials and last name of each author (no degrees; all CAPS), e.g., M.T. JONES, J.H. BROWN, AND S.R. SMITH
      c. Running title of ≤40 characters, including spaces
      d. Three to ten key words
   2. Abstract
      a. One paragraph, no longer than 300 words
      b. Purpose, methods, findings, and conclusion of study
   3. Key words
      a. List under abstract
   4. Text (serial pages): Most manuscripts can usually, but not necessarily, be divided into sections (as described below). Survey results and review papers may need individualized sections
      a. Introduction — Purpose and rationale for study, including pertinent background references
      b. Case Report (if indicated by study) — Clinical and/or hematologic data and background serology/molecular
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      d. Results — Presentation of concise and sequential results, referring to pertinent tables and/or figures, if applicable
      e. Discussion — Implication and limitations of the study, links to other studies; if appropriate, link conclusions to purpose of study as stated in introduction
   5. Acknowledgments: Acknowledge those who have made substantial contributions to the study, including secretarial assistance; list any grants.
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      b. Number references consecutively in the order they occur in the text.
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      a. Head each with a brief title; capitalize the first letter of first word (e.g., Table 1. Results of…) use no punctuation at the end of the title.
      b. Use short headings for each column needed and capitalize first letter of first word. Omit vertical lines.
      c. Place explanation in footnotes (sequence: *, †, ‡, §, ¶, **, ††).
   8. Figures
      a. Figures can be submitted either by e-mail or as photographs (5 ×7” glossy).
      b. Place caption for a figure on a separate page (e.g. Fig. 1 Results of…), ending with a period. If figure is submitted as a glossy, place first author’s name and figure number on back of each glossy submitted.
      c. When plotting points on a figure, use the following symbols if possible:
         ◯ ● △ ▲ ▼ □
   9. Author information
      a. List first name, middle initial, last name, highest degree, position held, institution and department, and complete address (including ZIP code) for all authors. List country when applicable. Provide e-mail addresses of all authors.

III. EDUCATIONAL FORUM
A. All submitted manuscripts should be approximately 2000 to 2500 words with pertinent references. Submissions may include:
   1. An immunohematologic case that illustrates a sound investigative approach with clinical correlation, reflecting appropriate collaboration to sharpen problem solving skills
   2. Annotated conference proceedings
B. Preparation of manuscript
   1. Title page
      a. Capitalize first word of title.
      b. Initials and last name of each author (no degrees; all CAPs)
   2. Text
      a. Case should be written as progressive disclosure and may include the following headings, as appropriate
         i. Clinical Case Presentation: Clinical information and differential diagnosis
         ii. Immunohematologic Evaluation and Results: Serology and molecular testing
         iii. Interpretation: Include interpretation of laboratory results, correlating with clinical findings
         iv. Recommended Therapy: Include both transfusion and nontransfusion-based therapies
         v. Discussion: Brief review of literature with unique features of this case
         vi. Reference: Limited to those directly pertinent
         vii. Author information (see II.B.9.)
         viii. Tables (see II.B.7.)

IV. LETTER TO THE EDITOR
A. Preparation
   1. Heading (To the Editor)
   2. Title (first word capitalized)
   3. Text (written in letter [paragraph] format)
   4. Author(s) (type flush right; for first author: name, degree, institution, address [including city, state, Zip code and country]; for other authors: name, degree, institution, city and state)
   5. References (limited to ten)
   6. Table or figure (limited to one)

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