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Efficacy of murine monoclonal antibodies in RBC phenotyping of DAT-positive samples

E. Lee, K. Hart, G. Burgess, G.R. Halverson, and M.E. Reid

Determining the phenotype of patient RBCs that are positive by the DAT may prove problematic. Antigen typing of RBCs coated with IgG requires direct agglutinating reagents or chemical treatment (such as chloroquine diphosphate [CDP] or citric acid) to remove sufficient IgG to permit testing with IAT-reactive reagents. The citric acid elution method is commonly used in the United States; however, antigens in the Kell system are altered to the extent that they may appear to be absent by this method. There are a limited number of direct agglutinating monoclonal antibodies available. Murine monoclonal antibodies provide an additional tool for typing RBCs with a positive DAT. Five murine monoclonal IgG antibodies (anti-K: MIMA-22, MIMA-23; anti-Kp<sup>+</sup>: MIMA-21, MIMA-27; anti-Fya: MIMA-19) were used in this study. Donor RBCs with known phenotypes were sensitized in vitro with alloanti-D, alloanti-c, and alloanti-K and with 20 autoantibodies (autoanti-D [n=5], autoanti-e [n=5], autoanti-Ce/e [n=5], autoanti-e+D+E [n=1], autoanti-I [n=1], and nonspecific [n=5]) to simulate a positive in vivo DAT. The sensitized RBCs were treated with CDP to remove IgG. To determine the efficacy of the murine monoclonal antibodies when testing DAT-positive samples, both sensitized and CDP-treated RBCs were tested with these monoclonal antibodies by the IAT using anti-mouse IgG. No discrepancies were noted with the unsensitized, sensitized, or CDP-treated RBCs. An exception was noted with a potent autoanti-I, where direct agglutination of the sensitized RBCs was obtained. This study demonstrates the value of using murine monoclonal antibodies to determine the phenotype of RBCs with a positive DAT caused by autoantibodies (e.g., in autoimmune hemolytic anemia) and supports previous studies showing that RBCs sensitized in vivo can be typed without chemical manipulation. Immunohematology 2006;22:161–165.

**Keys Words:** blood groups, murine monoclonal antibodies, anti-mouse IgG, autoantibodies, DAT, chloroquine diphosphate

Determining an accurate RBC phenotype for patients whose RBCs are positive by the DAT can be problematic because these RBCs are already coated in vivo with immunoglobulin, complement, or both; all tests performed will be positive by IAT with antihuman IgG reagents. There are very few IgM directly agglutinating reagents available for the clinically significant antibodies (i.e., anti-K, Jk<sup>a</sup>, Jk<sup>b</sup>, -S, and -Fy<sup>+</sup>). One option is to chemically treat the DAT-positive RBCs to remove IgG autoantibodies. These treatments may use chloroquine diphosphate (CDP), a combination enzyme/reducing agent (ZZAP, National Blood Service Reagents, Birmingham, UK) which is composed of DTT and papain, or EDTA/citric acid (Elu-Kit II, Gamma Biologicals, Inc., Houston, TX). Several other methods are available to remove the antibody for serologic testing; however the RBCs are not suitable for testing after these treatments. CDP and enzyme/reducing agent (ZZAP, National Blood Service Reagents) treatments can cause damage to the RBCs, resulting in the loss of some RBC antigens and possible invalid typing results. Additionally, CDP may not totally remove the coating autoantibody from the RBCs and it does not remove complement component 3 (C3). The proteolytic enzyme, papain, in the enzyme/reducing agent product denatures some MNS and Duffy antigens. The reducing agent, DTT, denatures the antigens in Kell and Yt systems among others. RBCs treated with the reagent combining both these chemicals (ZZAP, National Blood Service Reagents), therefore, have limited applications for use in phenotyping studies. The citric acid elution method is commonly used in the United States, a major drawback being that antigens of the Kell system are significantly weakened by this method. The development of murine monoclonal antibodies has provided an additional tool to allow DAT-positive RBCs to be antigen typed.

**Materials and Methods**

Selected patient samples containing alloantibodies and autoantibodies were obtained from the samples routinely referred to the Red Cell Immunohaematology Laboratory, National Blood Service, Colindale Centre, UK. Samples from three antenatal women with high titers of anti-D, -c, and -K, respectively, were selected for testing. A total of 20 autoantibodies from patients...
with autoimmune hemolytic anemia (AIHA) were also selected: anti-D (n=3), anti-e (n=5), anti-Ce/e (n=5), anti-e+D+E (n=1), anti-I (n=1), and nonspecific (n=5). (See Table 1 as an example of phenotyping results using murine monoclonal antibodies with unsensitized, sensitized, and CDP-treated RBCs for sample 2). All samples were collected in EDTA and were tested within 5 days of collection. The murine monoclonal antibodies used included MIMA-19: (anti-Fya), MIMA-21: (anti-Kpa), MIMA-22: (anti-K), MIMA-23: (anti-K), and MIMA-27: (anti-Kpa). Ten different commercial human monoclonal antibodies were also tested; anti-C, -c, -E, and -e (MS-24, MS-33, MS-258/906, MS-16/MS-21/MS-63 respectively, Biotest AG, Dreieich, Germany); anti-C\textsuperscript{-c}\textsuperscript{+e} and -S (MS-110, MS-15, MS-8, MS-94 respectively, Serologicals Ltd., Livingston, Scotland), anti-K (MS-56, Lorne Laboratories, Reading, UK), and anti-Fya (5T72, Bio-Rad, Marines La Coquette, France).

**Sensitization of donor RBCs**

Aliquots of 500 µL of washed, packed RBCs from nine donors (R1R1 [n=3], R2R2 [n=3], and rr [n=3]) were each incubated with 500 µL of the alloantibody or the autoantibody at 37°C for 60 minutes. Sensitized RBCs were confirmed by obtaining a positive DAT using polyspecific antihuman reagent (AHG, Lorne Laboratories).

**CDP treatment**

CDP (200 mg/mL, pH 5.0 ± 0.1, National Blood Service Reagents) was used to treat RBCs with a positive DAT. Four volumes of CDP were added to 1 volume of washed RBCs, mixed, and incubated at 20°C for a maximum of 2 hours. The strength of the DAT was tested after a 30-minute incubation at 37°C using a monospecific antihuman reagent (anti-IgG, Biotest AG).

**Hemagglutination testing**

All of the treated RBC samples were washed 4 times with 0.9% PBS and resuspended in a low ionic strength solution (LISS, Inverclyde Biologicals, Bellshill, Scotland) at a 2% suspension. Both treated and untreated RBCs were tested with murine monoclonal antibodies by IAT (40 µL:40 µL) and incubated at 20°C for 30 minutes using a sheep-derived IgG reagent directed at mouse IgG (sheep-anti-mouse IgG, The Binding Site, Birmingham, UK, code PC271.X). The sheep-derived reagent was diluted at a ratio of 1 in 50 in a bovine serum albumin solution (1% BSA, Serologicals, Kankakee, IL) in PBS.

**Table 1.** An example of phenotype results of murine monoclonal antibodies with unsensitized, sensitized, and CDP-treated RBCs (Sample 2)*

<table>
<thead>
<tr>
<th>Sample 2</th>
<th>Aalloanti-K</th>
<th>Donor’s Phenotype</th>
<th>Treated or Untreated RBCs</th>
<th>Anti-K (MS56) Lorne</th>
<th>Anti-Fya (5T72) Biorad</th>
<th>Anti-Kpa MIMA-21</th>
<th>Anti-Kpa MIMA-22</th>
<th>Anti-Kpa MIMA-23</th>
<th>Anti-Kpa MIMA-27</th>
<th>DAT</th>
</tr>
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<tbody>
<tr>
<td>Donor 1</td>
<td>R1R1</td>
<td>+0</td>
<td>Unsensitized</td>
<td>4 4 4 0</td>
<td>NT 0</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Sensitized</td>
<td>5 4 3 0</td>
<td>NT 0</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CDP treated</td>
<td>5 4 3 0</td>
<td>NT 0</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Donor 2</td>
<td>R1R1</td>
<td>+0</td>
<td>Unsensitized</td>
<td>4 4 3 0</td>
<td>NT 0</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Sensitized</td>
<td>5 4 3 0</td>
<td>NT 0</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CDP treated</td>
<td>5 2 3 0</td>
<td>NT 0</td>
<td>0</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Donor 3</td>
<td>R1R1</td>
<td>+0</td>
<td>Unsensitized</td>
<td>4 0 0 0</td>
<td>NT 0</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Sensitized</td>
<td>5 4 0 0</td>
<td>NT 0</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>CDP treated</td>
<td>5 0 0 0</td>
<td>NT 0</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Donor 4</td>
<td>rr</td>
<td>+0</td>
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<td>4 3 3 0</td>
<td>NT 0</td>
<td>0</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Sensitized</td>
<td>5 4 2 0</td>
<td>NT 0</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CDP treated</td>
<td>5 4 3 0</td>
<td>NT 0</td>
<td>0</td>
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<td></td>
<td></td>
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<tr>
<td>Donor 5</td>
<td>rr</td>
<td>+0</td>
<td>Unsensitized</td>
<td>4 3 3 0</td>
<td>NT 0</td>
<td>0</td>
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<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Sensitized</td>
<td>5 4 2 0</td>
<td>NT 0</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>CDP treated</td>
<td>5 4 3 0</td>
<td>NT 0</td>
<td>0</td>
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<tr>
<td>Donor 6</td>
<td>rr</td>
<td>+0</td>
<td>Unsensitized</td>
<td>4 3 3 0</td>
<td>NT 0</td>
<td>0</td>
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<td></td>
<td></td>
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<tr>
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<td></td>
<td></td>
<td>Sensitized</td>
<td>5 4 2 0</td>
<td>NT 0</td>
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<tr>
<td></td>
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<td>CDP treated</td>
<td>5 4 3 0</td>
<td>NT 0</td>
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<tr>
<td>Donor 7</td>
<td>R2R2</td>
<td>+0</td>
<td>Unsensitized</td>
<td>4 4 4 0</td>
<td>NT 0</td>
<td>0</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Sensitized</td>
<td>5 4 3 0</td>
<td>NT 0</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>CDP treated</td>
<td>5 3 3 0</td>
<td>NT 0</td>
<td>0</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Donor 8</td>
<td>R2R2</td>
<td>+0</td>
<td>Unsensitized</td>
<td>4 4 3 0</td>
<td>NT 0</td>
<td>0</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Sensitized</td>
<td>5 4 3 0</td>
<td>NT 0</td>
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<td></td>
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<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>CDP treated</td>
<td>5 3 3 0</td>
<td>NT 0</td>
<td>0</td>
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<td></td>
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<tr>
<td>Donor 9</td>
<td>R2R2</td>
<td>+0</td>
<td>Unsensitized</td>
<td>4 3 3 0</td>
<td>NT 0</td>
<td>0</td>
<td></td>
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<td>NT 0</td>
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<td>CDP treated</td>
<td>5 3 3 0</td>
<td>NT 0</td>
<td>0</td>
<td></td>
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</tr>
</tbody>
</table>

*Grading per system for hemagglutination used by Red Cell Immunohaematology Laboratory, National Blood Service, Colindale Centre, UK

5 Cell button remains in one clump or dislodges into a few large clumps, macroscopically visible
4 Cell button dislodges into numerous large clumps, macroscopically visible
3 Cell button dislodges into many clumps, macroscopically visible
2 Cell button dislodges into finely granular but definite small clumps, macroscopically visible
1 Cell button dislodges into very small agglutinates; these are more distinct if the tube is left on its side to allow the cells to settle, macroscopically visible
< 1 Grainy appearance, agglutinates best seen microscopically
0 Negative result
Adsorption and elution
Two mL of donor RBCs were washed 3 times in PBS, added to 2 mL of sensitizing antibodies, and incubated at 37°C for 60 minutes. To ensure sensitization, a DAT was performed on the sensitized RBCs with polyspecific antihuman reagent (AHG, Lorne Laboratories). An eluate was prepared using an EDTA/citric acid kit (Elu-Kit II, Gamma Biologicals, Inc.). The eluate and last wash were each tested by IAT.

Validation by flow cytometry
The five murine monoclonal antibodies (MIMA-19, MIMA-21, MIMA-22, MIMA-23, and MIMA-27) were validated by flow cytometry. To validate the tests, 4 µL of packed unsensitized, sensitized (DAT-positive), or CDP-treated RBCs were added to 40 µL of MIMA-19, MIMA-23, MIMA-21, MIMA-22, and MIMA-27 in plastic tubes. After 30-minute incubation in a 37°C water bath, the tubes were washed 3 times with 0.9% PBS. Fifty µL of FITC-conjugated F(ab’)2 goat-derived reagent directed at mouse immunoglobulin (goat anti-mouse immunoglobulin DAKO A/S, Denmark, code F0479) was added and the tubes were incubated for 30 minutes at 37°C in the dark. The mixture was washed twice and resuspended in 1% BSA in PBS. The tubes were analyzed by a flow cytometer (FACSCAN, Becton Dickinson, Oxford, UK).

Results
Adsorption and elution validation demonstrated autoantibodies that were successfully bound to the donor RBCs and could be eluted off. Flow cytometry results confirmed the selected murine monoclonal antibodies bound specifically to target antigens with unsensitized, sensitized, and CDP-treated RBCs. (See Figures 1a-c.) No nonspecific binding was observed with antigen-negative donor RBCs. (Results not shown).

Table 1 shows the phenotyping results of sample 2 (allo anti-K) using murine monoclonal antibodies with unsensitized, sensitized, and CDP-treated RBCs from nine donors (3 × rr, 3 × R1R1, and 3 × R2R2). Post sensitization, all of the donor’s RBCs were positive by the DAT which confirmed that the donors’ RBCs were coated with the sensitizing antibody in this study. No discrepant results were observed with the murine monoclonal antibodies tested. However, there was insufficient anti-K (MIMA-23) for testing samples number 1 to 3. The false positive reactions with the RBCs from donors 3 and 4 using IgG monoclonal IAT-reactive anti-Fy<sup>α</sup> were not surprising. As the postsensitized donor RBCs were DAT positive, a false positive result would be expected by IAT.

A summary of the phenotyping results for all antibodies tested is shown in Table 2 (actual results not shown). There were no discrepant results obtained with the murine monoclonal antibodies used; however, false positive reactions were obtained with commercial anti-sera and are shown in the Results column.

The test results with unsensitized, sensitized, and CDP-treated RBCs were concordant with one exception: a potent autoanti-I caused direct agglutination of all RBC samples.
Conclusion

In this study, no discrepancies were noted when the murine monoclonal antibodies were tested with unsensitized, sensitized, and CDP-treated RBCs (alloantibodies \( n=3 \), autoantibodies \( n=20 \)). The only exception was a potent autoanti-I that caused direct agglutination of all RBCs, as would be expected. Commercial human monoclonal IgM reagents (anti-K, -Jk\(^a\), -Jk\(^b\), and -S) can produce false positive reactions so it is important to test a low-protein diluent control in parallel with the test as suggested by manufacturers to validate the test results. Polyclonal phenotyping antisera derived from a human source are IAT reactive. The RBCs coated with IgG will give a positive result, using the IAT, which accounted for the false positive reactions.

Although previous studies have shown that phenotypes of RBCs sensitized in vivo by alloantibodies can be determined using murine monoclonal antibodies, the nature and characteristics of autoantibodies present in a patient’s serum may be quite different.\(^{10}\) In this paper, we demonstrated that murine monoclonal antibodies may be used without pretreating the RBCs with enzymes or chemicals. Furthermore, they provide accurate, reproducible results. In summary, murine monoclonal antibodies used in this study and in previous studies show that these antibodies can be used to determine the phenotype of patient RBC samples that are DAT positive, thus saving money and time and improving patient care.

Acknowledgments

This project partially fulfills the requirements of the University of Bristol for the degree of master of science in transfusion and transplantation sciences for Kevin Hart. This work is supported in part by NIH-SCOR Grant HL54459 and by a grant from the MetLife Foundation (GRH and MER).

References


Edmond Lee, MSc, (corresponding author), Senior Biomedical Scientist, Kevin Hart, Biomedical Scientist, Gordon Burgess, Reference Service Manager, Red Cell Immunohaematology, National Blood Service Colindale, Colindale Avenue, London, NW9 5BG, UK; Gregory R. Halverson, Chief Immunohaematologist, and Marion E. Reid, Director, Immunochemistry Laboratory, New York Blood Center, 310 East 67th Street, New York City, New York 10021.

Phone, Fax, and Internet Information: If you have any questions concerning Immunohaematology, Journal of Blood Group Serology and Education, or the Immunohaematology Methods and Procedures manual, contact us by e-mail at immuno@usa.redcross.org. For information concerning the National Reference Laboratory for Blood Group Serology, including the American Rare Donor Program, please contact Sandra Nance, by phone at (215) 451-4362, by fax at (215) 451-2538, or by e-mail at snance@usa.redcross.org
Case report: moderate hemolytic disease of the newborn due to anti-G


Views expressed in this article are those of the author and do not reflect the official policy or position of the Department of the Navy, Department of Defense, or U.S. Government.

The only previously published case of anti-G in a pregnant woman indicated that anti-G alone caused little, if any, fetal or neonatal hemolysis. This report describes an affected fetus with amniotic fluid OD 450 absorbance values in the moderate zone of the Liley prediction graph who required prolonged phototherapy after birth until day of life 20. Anti-G was identified and anti-C and -D excluded in the mother's serum. In contrast to the previous report, this report shows anti-G alone can cause moderate HDN and that fetal monitoring and treatment may be necessary.


Key Words: hemolytic disease, newborn, anti-G, anti-C, anti-D

Antibodies to the antigens in the Rh system are well-known causes of HDN. The D antigen is a very potent immunogen and anti-D can cause severe HDN. Anti-C has also been shown to cause HDN, although generally less severe. First elucidated by Allen and Tippett in 1958, the G antigen is present on almost all D+ or C+ RBCs and absent from virtually all RBCs that are D− and C−.1 The apparent codistribution of the G antigen with either the C or D antigen causes anti-G to appear serologically as anti-C plus anti-D activity.1 Issitt and Tessel were the first to use a serial double-elution procedure using D+C− RBCs followed by D−C+ RBCs to identify the presence of anti-G in approximately 30 percent of sera with anti-C+D activity.1 Case series have shown that anti-D is absent in 10 of 22 (45%)3 and 2 of 7 (30%)4 of alloimmunized pregnant women with apparent anti-D plus anti-C. Although it appears that a significant proportion of apparent anti-D plus anti-C is because of the presence of anti-G, the significance of an apparent titer of anti-D actually due to anti-G in predicting the severity of HDN is not known. The only previously published case of a pregnant woman with anti-G having a titer of 16 against R2R2 RBCs suggested that anti-G alone had caused practically no fetal or neonatal hemolysis.5 We report a case of moderate HDN because of anti-G alone with a titer of 8 against R,R,R RBCs where increased amniotic fluid OD 450 nm absorbance values in the moderate zone of the Liley prediction graph and normal middle cerebral artery Doppler studies were followed by postpartum hyperbilirubinemia, anemia, and the requirement for prolonged phototherapy until day of life 20.

Case Report

A 31-year-old group O, rr (dce/dce), gravida 5 pregnant woman was referred to the maternal-fetal medicine department at our facility at 17 weeks, 6 days gestational age because of a history of recurrent spontaneous abortions. Prenatal antibody screening test results from the referring hospital had revealed anti-C and anti-D with titers of 64 and 32, respectively. The patient had received Rh immunoglobulin (RhIG) prophylaxis after all procedures and abortions except for a spontaneous abortion at 6 weeks' gestation before the current pregnancy. She denied ever receiving a blood transfusion. Because of her history of multiple pregnancy losses and a positive antinuclear antibody screen, the patient was placed on prophylactic subcutaneous heparin. Fetal middle cerebral artery peak systolic velocity (MCA PSV), measured by ultrasound Doppler at 21 weeks, 6 days' gestation, was below the median value for gestational age, suggesting no significant fetal anemia. Given these findings, amniocentesis was not performed and the patient was instructed to return in three weeks. The patient returned at 25 weeks, 1 day gestational age, at which time it was noted that fetal growth lagged established dating by two weeks. The patient returned at 25 weeks, 1 day gestational age, at which time it was noted that fetal growth lagged established dating by two weeks. However, MCA PSV was still without evidence of fetal anemia and there was no
evidence of hydrops by ultrasound. The patient was offered amniocentesis to rule out chromosomal anomaly as the etiology for fetal growth restriction. Amniotic fluid was also analyzed for \( \Delta OD_{450} \) which was 0.120. This value is in the lower portion of the affected zone of OD450 curve by Queenan,6 which is valid for pregnancies earlier than 27 weeks’ gestation. These results suggest that the fetus was affected with HDN but was not severely anemic. Serial amniocentesis at 28, 30, and 32 weeks all returned with \( \Delta OD_{450} \) values in the low-affected zone of the Queenan chart and low zone 2 of the Liley chart while MCA PSV values also remained within the normal range, suggesting no worsening fetal hemolysis or anemia. The fetus was also followed for asymmetric intrauterine growth restriction by serial ultrasound. The decision was made to deliver the fetus by Caesarean section at 33 weeks’ gestational age secondary to oligohydramnios with an amniotic fluid index of 3.4 cm (normal 5–25 cm) and absent end-diastolic flow in the umbilical artery on Doppler ultrasound. A viable male infant weighing 1300 g with APGAR scores of 8 and 9, whose RBCs typed as group O, D+C+E–c+E, was delivered. The physical exam showed no ascites or peripheral edema. A peripheral serum ultrasound. A viable male infant weighing 1300 g with APGAR scores of 8 and 9, whose RBCs typed as group O, D+C+E–c+E+, was delivered. The physical exam showed no ascites or peripheral edema. A peripheral serum sample collected on day 1 of life showed a bilirubin of 3.6 mg/dL (1.0–10.5 mg/dL), Hb of 15.7 g/dL (13.5–19.5 g/dL), and Hct of 47.3% (42–60%). Phototherapy was initiated with a preliminary diagnosis of moderate HDN. While on phototherapy, the bilirubin would decrease, maintaining levels below 10 mg/dL. However, when phototherapy was discontinued, the bilirubin would increase, reaching a maximum of 18.1 g/dL on day of life 13. The direct bilirubin level was consistently between 0.0 and 0.1 mg/dL, supporting an absence of obstructive liver disease. On day of life 20, phototherapy was stopped and the bilirubin remained stable at 16.5 to 16.7 mg/dL over the course of six days until it started to decrease. The Hb and Hct continually declined to values of 8.8 gm/dL (10.0–18.0 gm/dL) and 25.7% (31–55%), respectively, on day of life 30. The neonate received no exchange transfusions or RBC transfusions. At a follow-up visit at age 3 months, the infant’s Hb and Hct were 11.5 g/dL and 34.6%, respectively and the infant was not jaundiced. The mother received RhiG prophylaxis before discharge.

Materials and Methods
A peripartum maternal serum sample was used to determine antibody specificity and titers. The specificity testing was performed at the American Red Cross Blood Services, Greater Chesapeake and Potomac Region reference laboratory. The serum was first tested to determine initial reactivity to R0R0 (Dce/dce) commercial reagent RBCs and to exclude other clinically significant antibodies by both LISS- and PEG-AHG, according to the manufacturer’s protocol (Panocell and Gamma PEG, ImmucorGamma, Houston, TX; ORTHO Antibody Enhancement Solution, Ortho-Clinical Diagnostics, Raritan, NJ). The sample was tested against rare r’r RBCs. The patient’s serum was then adsorbed onto ficin (Sigma-Aldrich, St. Louis, MO)-treated r’r’ (dCe/dCe) donor RBCs five times to adsorb anti-C, anti-G, or both to exhaustion. The absorbed serum was tested against two R0r (Dce/dce) reagent RBCs. The eluate prepared from the first adsorbing aliquot of r’r’ (dCe/dCe) RBCs was adsorbed onto ficin-treated R0R0 (DcE/DcE) donor RBCs to adsorb out anti-G, if present (Gamma ELU–KIT II, Gamma Biologicals, Inc., Houston, TX). The absorbed eluate was tested against two r’r’ (dCe/dCe) reagent RBCs. Titrations were performed on the anti-C and anti-D using r’r’ (dCe/dCe) and R0R0 (Dce/dce) reagent RBCs (Immucor) and a saline-AHG method with 1-hour incubation at 37°C.

The DAT (Anti-IgG, Murine Monoclonal Gamma-Clone, ImmucorGamma, Norcross, GA) was performed on the infant’s cord blood sample that was manually washed 6 times. The eluate was tested against an antibody identification panel (Panocell–16, Immucor) to identify anti-D and anti-C activity and to exclude other clinically significant antibodies.

ABO and Rh typing of RBC samples from the mother and infant were performed with monoclonal reagents according to manufacturers’ instructions (Immucor and Gamma Biologicals, Inc.).

Results
Testing of the patient’s serum and of the eluate from the infant’s cord blood sample with selected panel RBCs revealed reactivity with R0R0 (Dce/Dce) RBCs and excluded other clinically significant antibodies. Testing with r’r’, D–G+ RBCs was strongly reactive. After antibody adsorption from the patient’s serum with ficin-treated r’r’ (dCe/dCe) donor RBCs, reactivity against R0r (Dce/dce) RBCs was not detected, excluding the presence of anti-D. An eluate prepared from the r’r’ (dCe/dCe) adsorbing RBCs showed no reactivity with r’r’ (dCe/dCe) RBCs after adsorption of anti-G with ficin-treated R0R0 (Dce/dce) RBCs, thereby excluding the presence of anti-C. Using this double-
elution procedure and eliminating contributions from anti-C or anti-D through serial adsorptions, it was shown that anti-G alone was responsible for the reactivity with \( \text{R}_1 \text{R}_1 (\text{DcE}/\text{DcE}) \) RBCs and \( \text{r}^\prime \text{r} \) RBCs (Table 1). The anti-G titer with \( \text{r}^\prime \text{r} \) (dCe/dCe) RBCs was 16; the titer with \( \text{R}_2 \text{R}_2 (\text{DcE}/\text{DcE}) \) RBCs was 8. The DAT of the infant's RBCs was 3+ and the eluate demonstrated 3+ reactivity with \( \text{R}_1 \text{R}_1 (\text{DcE}/\text{DcE}) \), \( \text{r}^\prime \text{r} \) (dCe/dce), and \( \text{r}^\prime \text{r} \) (dCe/dCe) RBCs, 2+ reactivity with \( \text{R}_0 \text{r} (\text{Dce}/\text{dce}) \) and \( \text{R}_2 \text{R}_2 (\text{DcE}/\text{DcE}) \) reagent RBCs and no reactivity with seven different \( \text{r}^\prime \text{r} \) (dCe/dce) and \( \text{rr} \) (dce/dce) reagent RBCs.

**Table 1. Adsorption and elution procedure**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Panel RBCs</th>
<th>Results</th>
<th>Conclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peripartum maternal serum sample</td>
<td>( \text{R}_1 \text{R}_1 (\text{DcE}/\text{DcE}) )</td>
<td>Strongly reactive</td>
<td>Anti-C, D, or G present</td>
</tr>
<tr>
<td>Peripartum maternal serum sample</td>
<td>( \text{r}^\prime \text{r} ) (dCe/dCe)</td>
<td>Strongly reactive</td>
<td>Anti-G present (+/- Anti-C or D)</td>
</tr>
<tr>
<td>Absorbed serum, after adsorption onto ficin-treated ( \text{r}^\prime \text{r} ) (dCe/dCe) RBCs to adsorb anti-C, anti-G, or both</td>
<td>( \text{R}_0 \text{r} (\text{Dce}/\text{dce}) )</td>
<td>Nonreactive</td>
<td>Anti-D excluded</td>
</tr>
<tr>
<td>Eluate from ( \text{r}^\prime \text{r} ) (dCe/dCe) adsorbing RBCs, after adsorption onto ficin-treated ( \text{R}_1 \text{R}_1 (\text{DcE}/\text{DcE}) ) RBCs to adsorb anti-G</td>
<td>( \text{r}^\prime \text{r} ) (dCe/dCe)</td>
<td>Nonreactive</td>
<td>Anti-C excluded</td>
</tr>
</tbody>
</table>

**Discussion**

Most cases of HDN associated with anti-G have been in association with anti-D, anti-C or both.\(^{4,5,7-9}\) Cash and colleagues\(^5\) described the first case where anti-G alone was identified as the cause of a positive DAT in an infant which resulted in the absence of clinical evidence of HDN. In their case report, the newborn did not require transfusion and the bilirubin reached a peak of 11.9 mg/dL at day of life 4. Although the presence of anti-C was not definitively excluded in the mother, the newborn’s RBCs were C–. The titer of the antibody was 64 against \( \text{r}^\prime \text{r} \) RBCs and 16 against \( \text{R}_2 \text{R}_2 \) RBCs. They concluded that anti-G alone without anti-C, anti-D, or both may not be sufficient to cause severe HDN and suggested the question of whether identification of anti-G and exclusion of anti-C and anti-D could indicate a benign clinical course and alter clinical management such that amniocentesis would not be indicated.

The clinical significance of anti-G remains controversial. Palfi et al.\(^6\) identified anti-G+C in 4 of 27 samples in their study of alloimmunized pregnant women, none of which caused severe HDN. They proposed that low concentration of antibody, the occurrence of IgM antibodies, or both, were possible explanations for this finding. In agreement with the case reported by Cash et al.,\(^5\) they concluded that anti-G+C alloimmunization may have a decreased risk of HDN. This has been disputed by others. Hadley et al.\(^7\) reported a case of severe HDN due to anti-G+C in a D– newborn with anemia who required multiple exchange transfusions. The levels and functional activities of both anti-G and anti-C were evaluated with the IAT, the chemiluminescence test (CLT), and flow-cytometric techniques. They found that 6 of 7 anti-G-containing eluates bound higher levels of IgG anti-G to \( \text{r}^\prime \text{r} \) (C+) RBCs than to \( \text{R}_0 \text{r} \) (D+) RBCs, which paralleled their results using flow-cytometric analysis. Using the CLT, they found the response of \( \text{r}^\prime \text{r} \) (C+) RBCs sensitized with an eluate containing anti-G (69%) to be consistent with severe HDN. However, \( \text{r}^\prime \text{r} \) (C+) RBCs sensitized with anti-C after adsorption with \( \text{R}_2 \text{R}_2 \) RBCs showed a weak CL response that was not consistent with HDN. They also tested 28 serum samples from alloimmunized pregnant women with over 5 IU/mL anti-C+D with 2 of 28 containing levels of anti-G that were consistent with moderate to severe HDN by the CLT. They concluded that anti-G may cause moderate to severe HDN in those women with greater than 5 IU/mL anti-C+D (approximately 7%) and that HDN caused by anti-G is probably not rare. Similarly Lenkiewicz et al.\(^8\) reported a case of moderate HDN due to anti-G+C in a D–C+ newborn with hyperbilirubinemia requiring phototherapy. The levels and functional activities of both anti-G and anti-C were evaluated with the IAT and the CLT. Their results showed both the level and the functional activity of anti-G to be greater than those of anti-C. They concluded that anti-G, and not anti-C, was responsible for the moderate HDN and that anti-G should be regarded as clinically significant in the alloimmunized pregnant woman.

We report a second case of HDN caused solely by anti-G but in an infant who expressed both the C and D antigens on his RBCs. By using a serial double-elution procedure, we identified anti-G alone as the sole antibody present. Differential adsorption techniques were performed, excluding the possibility of a concomitant anti-D or anti-C contributing to the hemolysis. In our case, hyperbilirubinemia requiring a
prolonged stay in the hospital for phototherapy argues that anti-G alone is sufficient to cause at least a moderate HDN in an infant that expresses both the D and C antigens on his RBCs. However, it would seem that differentiation of anti-G from anti-D and anti-C would not be relevant for decisions of patient monitoring.

Our case suggests that differentiating anti-D plus anti-C from anti-G may not be relevant for the purpose of suggesting an indication for amniocentesis given the increasing availability of MCA PSV to predict fetal anemia. Recent articles have suggested that MCA PSV may be superior to amniotic fluid ∆OD$_{450}$ for the diagnosis of fetal anemia in cases of RBC alloimmunization in the hands of an experienced ultrasonographer$^{11,12}$ and many centers have now replaced serial amniocentesis using ∆OD$_{450}$ with serial MCA Doppler. In our case, despite the fact that the patient was referred to our institution with an anti-D titer of 32 and anti-C titer of 64, which would be considered critical if performed in our lab, a decision was made to not perform amniocentesis because of the reassuring MCA PSV results. Only later, when the patient had an additional indication for amniocentesis, was the ∆OD$_{450}$ measurement made. The patient was then followed for the remainder of her pregnancy with both MCA PSV and amniocentesis, the results of which indicated a stable level of hemolysis without the development of significant anemia.

Despite the noninvasiveness and increased availability of MCA PSV, current recommendations still do not consider use of this modality until a critical titer is reached. Thus, titration of the antibodies is a critical step, although, in cases of anti-D plus anti-C, the critical titer is not known. Complicating this situation is the fact that, although review articles would make one tend to believe that the situation with anti-D is well established where defined titers (32$^{12}$ or 8 to 32$^{13}$) are recommended and standard methodology is recommended (use of R$_2$R$_2$ reagent RBCs$^{15}$), laboratories are still reporting widely discrepant results as our case illustrates (referral laboratory titer result of 64 vs. our result of 8). Further complicating the situation with anti-D plus anti-C is the variation in the phenotype of the RBCs used to determine and compare the anti-D and anti-C titers (R$_2$R$_2$ and r’r$^{5,13}$ R$_3$R$_3$ and R$_2$R$_2$, $^3$ R$_2$ and r’r RBCs$^{2,4,9,10}$). The recommendations of the subcommittee of the Scientific Section Coordinating Committee of the AABB recommended the use of r’r and R$_2$R$_2$ RBCs when comparing the titer of anti-C with anti-D,$^15$ although we used r’r’ RBCs and R$_3$R$_3$ RBCs in the interest of comparing RBCs that would have more comparable (homozygous) expression of antigens. In commenting on the comparability of our anti-D titer result to other labs, we think it is relevant that we have been participating in the College of American Pathologists Survey of anti-D titers and our results have been consistent with the largest peer groups. Therefore, we think our titer result would likely be consistent with most labs that participated in this survey. Hopefully, increased participation in titer proficiency surveys will improve the consistency of titer results among laboratories and facilitate comparison of titer results in case studies.

Finally, although we do not feel that identifying anti-G and excluding anti-D and anti-C has relevance in deciding whether or not to nonserologically monitor a pregnancy for HDN, especially with the availability of MCA PSV, we agree with the recommendations of Shirey et al.$^4$ that identifying or excluding anti-D is relevant. One study has indicated that although anti-G seems to mask the antigenic sites of C and D, it does not prevent the eventual development of anti-D, meaning that RhIG would be indicated in a patient with anti-G but without anti-D.$^9$ Also, there are medicolegal reasons for the exclusion of anti-D, such as excluding questions of paternity for a D– couple, avoiding inadequate prophylaxis with RhIG, and avoiding confusion regarding previous transfusion history as anti-G and anti-C can be found in recipients of D– products.$^9,10$

References


Aaron R. Huber, D.O., National Naval Medical Center, Bethesda, MD; George T. Leonard, Jr., M.D., Ph.D. (corresponding author), National Capital Consortium Pathology Residency, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, MD 20814-4799; Rita W. Driggers, M.D., National Naval Medical Center, Bethesda, MD; Sakhone B. Learn, BB(ASCP), Red Cross Blood Services, Greater Chesapeake and Potomac Region, Baltimore, MD; and Colleen W. Gilstad, M.D., National Naval Medical Center, Bethesda, MD, 20814-4799.

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The MNS blood group antigens are expressed in the RBC membrane on glycophorin A (GPA), glycophorin B (GPB), or combinations of both. GPA expresses the M or N antigen, whereas GPB expresses the S or s antigen and the N antigen (N'). Both glycophorin genes (GYPA and GYPB) are located on the long arm of chromosome 4 and share 95 percent sequence identity. This high degree of sequence identity, together with the rare involvement of a third homologous gene (GYPE), provides an increased chance of recombination, resulting in hybrid molecules that often carry one or more novel antigens. Some of the antigens in the MNS system result from a single nucleotide substitution. The MNS blood group system now consists of more than 40 distinct antigens. This review summarizes the molecular basis associated with some of the antigens in the MNS blood group system.


Key Words: MNS blood group, MNS antigens, glycophorin genes, hybrid glycophorins, genetic mechanism, molecular basis

MNS Blood Group System

The MNS blood group system was the second blood group system to be discovered.1 The MNS antigens are carried on glycophorin A (GPA), glycophorin B (GPB), or hybrids of GPA and GPB, and are fully developed at birth. Currently, this blood group system consists of more than 40 distinct antigens (Table 1), and is second only to the Rh blood group system in its complexity.2 The antigens of the MNS system arise from single nucleotide substitution, unequal crossing over, or gene conversion between the glycophorin genes (Table 2).

Glycophorin A and Glycophorin B

GPA is similar to GPA, but consists of 72 amino acids, has an approximate molecular weight of 25 kDa (by SDS-PAGE) and is also organized into three domains: an extracellular N-terminal domain of 44 amino acids, a hydrophobic membrane-spanning domain of 20 amino acids, and a short C-terminal cytoplasmic domain of 8 amino acids. There are an estimated 2 × 10^5 copies of GPB present on the RBC membrane.3,4 On intact RBCs, GPA is susceptible to cleavage by trypsin at amino acid residue 31 and 39 but is resistant to α-chymotrypsin cleavage whereas GPB is resistant to trypsin cleavage but sensitive to α-chymotrypsin at amino acid residue 32.3,6 Thus, these proteolytic enzymes are useful in laboratory testing to identify antibodies in the MNS blood group system.

Function of Glycophorin A and Glycophorin B

GPA and GPB contribute most of the carbohydrate on the RBC membrane. The amino-terminal domains of GPA and GPB carry O-glycans while only GPA carries an asparagine-linked-glycan (N-glycan).3 The O-glycans are smaller molecules than N-glycans and are attached to serine or threonine. In glycophorins, it is the O-glycans that carry most of the sialic acid and contribute to the net negative charge of the RBC through the high sialic acid content of each glycoprotein. The negative charge keeps RBCs from sticking to each other and to the endothelial cells of the blood vessels.5,7 The negatively charged glycocalyx also protects the RBC from invasion by bacteria and other pathogens.8,9 GPA-deficient RBCs are more resistant to invasion by Plasmodium falciparum merozoites due to the reduction of sialic acid on the RBCs because sialic acid appears to be essential for adhesion of the parasite to the RBC.10–14 The sialic acid attached to GPA and GPB has been reported to be the target of the influenza virus8 and the encephalomyocarditis virus.15 Moreover, GPA plays the role of chaperone for band 3 transport to the RBC membrane.16,17
Gene Family

The genes encoding GPA (GYPA) and GPB (GYPB) are homologous and are located on the long arm of chromosome 4.18 Although it may not encode a RBC product, a third homologous gene, GYPE, which is adjacent to GYPB, can contribute to hybrid genes. The GYPB has seven exons, GYPE has six exons (of which exon 3 is a pseudoexon or non-coding exon), and GYPE has six exons (of which exons 3 and 4 are pseudoexons).19–22 For each of the GYP genes, exon 1 and part of the 5′ end of exon 2 encode the leader sequence for the corresponding glycophorin, exons 2 to 4 encode the extracellular domains, exon 5 encodes the transmembrane domains of each glycophorin, and exon 6 and part of the 5′ end of exon 7 of GYPB encode the cytoplasmic domain of GPA (Fig. 1: Modified from Reid ME, 1994).23

Polymorphic Antigens

M, N, S, and s antigens

GPA carries M and N antigens. M has serine and glycine while N has leucine and glutamic acid at position 1 and 5, respectively.24,25 The first 26 amino acids of GPB are identical to GPA carrying the N

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**Table 1.** Antigens of the MNS blood group system

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Historical name</th>
<th>ISBT number</th>
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<td>M</td>
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</tr>
<tr>
<td>N</td>
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<td></td>
</tr>
<tr>
<td>S</td>
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<td>Or</td>
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<tr>
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<td>Low – T17R</td>
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**Table 2.** Molecular mechanisms and associated antigens

<table>
<thead>
<tr>
<th>Molecular mechanism</th>
<th>Associated antigens</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single nucleotide substitution</td>
<td>GPA: Vr, M′′, R′′, Ny′′, Or, ERIK, Os′′, ENEP/HAG, ENAV/MARS GPB: S/s, M′′, s″, Mit</td>
</tr>
<tr>
<td>Two or more nucleotide substitution</td>
<td>M/N</td>
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<tr>
<td>Unequal crossing over</td>
<td>St′, Dantu, Hil, TSEN, MINY, SAT</td>
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<tr>
<td>Gene conversion</td>
<td>He, M′′′, Vw/Hut/ENEH, Mur, M′′′′, M′′′′′′′′, St′, Hil, Hop, Nop, DANE, MINY, MUT</td>
</tr>
</tbody>
</table>

**Fig. 1.** Genomic organization of GYPA, GYPB, and GYPE genes.
antigen. GPB carries S or s antigens. The Ss polymorphism of GPB depends on a single amino acid substitution at position 29; S has methionine and s has threonine (Table 3).

### Table 3. Amino acid polymorphisms of GPA and GPB

<table>
<thead>
<tr>
<th>Glycophorin</th>
<th>Gene</th>
<th>Common variants</th>
<th>Amino acid polymorphisms</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPA</td>
<td>GYP</td>
<td>GPA¹ 1 Ser, 5 Gly</td>
<td>GPA² 1 Leu, 5 Glu</td>
</tr>
<tr>
<td>GPB</td>
<td>GYPB</td>
<td>GPB¹ 29 Met</td>
<td>GPB² 29 Thr</td>
</tr>
</tbody>
</table>

**High-Prevalence Antigens**

**U antigen**

The U antigen is commonly found in all populations. The U– phenotype is found among Blacks and its prevalence in certain regions of Africa is as high as 35 percent. For expression, the U determinant requires the presence of GPB amino acids 33 to 39 and possibly an interaction with another membrane protein, the Rh associated glycoprotein (RhAG). The U– phenotype is associated with an absence of GPB or with certain altered forms of GPB. U– RBCs (except those that are Dantu+ and some Rhnull/Rhmod RBCs) are S–s–. Of S–s– RBCs, approximately 16 percent have weak expression of U antigen (U+var) encoded by a hybrid GYP gene. Of these U+var samples approximately 23 percent are He+. Anti-En is a global term used for antibodies that detect high prevalence antigens on GPA. The sensitivity of these antigens to enzyme treatment is dependent on their location on GPA. Thus, Enα antigens are trypsin sensitive (TS), ficin sensitive (FS), and ficin resistant (FR). Enα antigens are absent from GPA-deficient RBCs and those carrying certain variants of GPA.

**Enα antigen**

Anti-Enα is a global term for antibodies that detect high prevalence antigens on GPA. The sensitivity of these antigens to enzyme treatment is dependent on their location on GPA. Thus, Enα antigens are trypsin sensitive (TS), ficin sensitive (FS), and ficin resistant (FR). Enα antigens are absent from GPA-deficient RBCs and those carrying certain variants of GPA.

**Genetic Mechanisms Giving Rise to Variant Phenotypes**

Variant phenotypes may occur as a consequence of a single amino acid substitution, crossing over, gene conversion, or gene deletion (Table 2). A novel sequence of amino acids exposed on the outside surface of the RBC can result in the expression of novel antigens.

**GPB gene deletions giving rise to null phenotype RBCs**

A deletion of GYP (exon 2 to 7) and GPB (exon 1) gives rise to the rare En(a–)Fin phenotype and RBCs from these individuals lack GPA and, thus, antigenic determinants associated with GPA. Deletion of GPB (exon 2 to 6) and GYPE (exon 1) precludes production of GPB and RBCs from these individuals have the S–s–U– phenotype. A deletion of GYP (exon 2 to 7), GPB (exon 1 to 6) and GYPE (exon 1) results in the M'Mk genotype and RBCs from these individuals lack both GPA and GPB and, thus lack all MNS blood group antigens (Table 4).

**Crossing over and gene conversion**

The GYP, GPB, and GYPE share more than 95 percent sequence identity, span at least 150 kb of DNA,
and are adjacent at the MNS locus in the order of 5′-GYPA-GYPB-GYPE-3′. GYPE probably does not encode a RBC membrane component but it does participate in gene rearrangements resulting in variant alleles. Sequence homology between the glycophorin genes increases the chance of recombination because of unequal crossing over or gene conversion events (Figure 2: Adapted with permission from Cheng-Han Huang).31,39 Unequal crossing over is a mutual exchange of nucleotides between misaligned homologous genes during meiosis; this mechanism occurs between regions of homology with generation of two recombinants in reciprocal arrangement. A chromosome carrying the hybrid GYP(A-B) does not have GYP(A) and GYP(B) (Lepore type). In contrast, a chromosome carrying a hybrid GYP(B-A) also has both GYP(A) and GYP(B) (Anti-Lepore type).

Gene conversion can occur during the process of DNA repair between homologous genes during meiosis.31,40 Gene conversion is the transfer of nucleotides from one gene to another gene and does not result in a reciprocal product. Gene conversion can cause insertion of nucleotides from GYP(A) into the GYP(B) or GYP(B) into the GYP(A). As a result of this mechanism, the chromosome carrying a GYP(B-A-B) also carries a GYP(A) but not GYP(B), whereas the chromosome carrying a GYP(A-B-A) also carries a GYP(B) but not GYP(A). During gene conversion, a consensus splice sequence may be altered and a number of recombinant products have been described. These alterations sometimes cause transcription of part of the GYP(B) pseudogene 3 and hence allow translation of a novel sequence of amino acids.31 Gene conversion gives rise to hybrid genes that encode novel glycophorin molecules carrying certain low-prevalence antigens in the MNS blood group system (Table 6).

Low-Prevalence Antigens

He

The He antigen is found in about 3 percent of African Americans but is very rare in Caucasians.41 Protein sequencing showed that GPHe is identical to GBP except for an alteration at the amino-terminus; the amino acids leucine, threonine, and glutamic acid found in positions 1, 4, and 5 of GBP are replaced by threonine, serine, and glycine, respectively.12 In GPHe, the presence of a glycine residue at position 5 is recognized by some anti-M. DNA analysis has shown that He antigen is due to gene conversion resulting in a GYP(B-A-B) or a GYP(B-A-ΨB-A) hybrid. In addition to the classic He+S+ and He+s+ variants, four other GYPB variants of GPHe have been described as follows.

GPHe(P2) is characterized by two mutations in GYPB: a C>G change at the 3′ end of exon 5, which creates a cryptic acceptor splice site, and a G>T change at position +5 of intron 5, which alters the consensus donor splice site.43 These two mutations cause skipping of exon 5 and a shift in the open reading frame with chain elongation. Consequently, a portion of the 3′ untranslated region is read during protein synthesis, causing GPHe(P2) to have a new transmembrane hydrophobic sequence. This variant does not express the S antigen or the U antigen. GPHe(P2) has not been detected in the RBC membrane; however, these S−s−RBCs are He+w because of the expression of low levels
of GPB.He. GP.He(NY) is characterized by a partial deletion of exon 5 that alters the open reading frame and is predicted to encode a protein of 43 amino acids, which has not been demonstrated in the RBC membrane. The S–s– RBCs are He+" caused by the expression of low levels of GPB.He.35

GP.He(GL) is characterized by two nucleotide changes: a T>G mutation at nucleotide -6 of the acceptor splice in intron 3, which leads to skipping of exon 4, and a C>G mutation in exon 5. The latter point mutation results not only in a predicted Thr>Ser substitution at position 65 but in the creation of a new acceptor splice site; partial inactivation of the normal splice site results in four different cDNAs. The full-length transcript GP.He-1, which is equivalent to GPB in molecular size, contains a Thr>Ser substitution at position 65 and encodes He, S, and U antigens. Transcript GP.He-2 codes for a polypeptide with an intact transmembrane segment but, because of the deficiency of exon 4, the encoded protein lacks the sequence defining the S and U antigens as well as the cleavage sites of α-chymotrypsin.3 Transcripts GP.He-3 and GP.He-4 are low-level transcripts with major deletions which probably prevent insertion of their putative protein products into the membrane.44

GP.Cal is an example of a gene conversion event resulting in a GP(B-A-ΨB-A) hybrid that carries both He and St" antigens. The GYP(A-B) recombination site is in exon 2; the mature protein, after cleavage of the leader peptide, is GP(A-A). GYPB also contributes the pseudoexon, which is out-spliced.45 The 5' portion of GP.Cal is similar to GP.He with a GP(B-A-B) arrangement, whereas its 3' portion has a GP(B-A) configuration identical to the GPSat.44

SAT

The SAT antigen is associated with two different glycophorin isoforms.47 GPTK is a GP(A-B) hybrid composed of exons 1 to 4 of GYPB and exons 5 to 6 of GYPB.48 This transcript encodes 104 amino acids with a hexapeptide sequence “Ser-Glu-Pro-Ala-Pro-Val” at positions 69 to 74 produced by the junction between GPA and GPB, and encodes the SAT antigen. GPSAT is associated with a GYP(A-B-A) hybrid which does not encode a GPA-B-A hybrid (Table 6). This variant is characterized by an insert, between exon 4 of GYPB and exon 5 of GYPB, of nine bases (three amino acids) originating from the 5' end of exon 5 of GYPB. The tripeptide sequence “Ala-Pro-Val” inserted into the GPA molecule creates the SAT-specific sequence.49

<table>
<thead>
<tr>
<th>Molecular basis</th>
<th>Glycophorin</th>
<th>Phenotype symbol</th>
<th>Antigens associated with hybrid</th>
</tr>
</thead>
<tbody>
<tr>
<td>GYP(A-B)</td>
<td>GP(A-B)</td>
<td>GPHe (Mi.V)</td>
<td>Hil, MINY</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GPJL (Mi.XI)</td>
<td>TSEN, MINY</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GPTK</td>
<td>SAT</td>
</tr>
<tr>
<td>GYP(B-A)</td>
<td>GP(B-A)</td>
<td>GPSch (M')</td>
<td>St&quot;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GP.Dantu</td>
<td>(see Table 7)</td>
</tr>
<tr>
<td>GYP(A-B-A)</td>
<td>GP(A-B-A)</td>
<td>GPM</td>
<td>M&quot;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GPKI</td>
<td>Hil</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GP(B-A)</td>
<td>SAT</td>
</tr>
<tr>
<td>GYP(B-A-B)</td>
<td>GP(B-A-B)</td>
<td>GPMur (M.III)</td>
<td>Mi&quot;, Mur, MUT, Hil, MINY</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GPBun (M.VI)</td>
<td>Mi&quot;, Mur, MUT, Hop, Hil, MINY</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GPHe (M.IX)</td>
<td>Mi&quot;, MUR, HIL, MINY</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GPHop (M.IV)</td>
<td>Mi&quot;, Mur, MUT, Hop, TSEN, MINY</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GPZan (M&quot;)</td>
<td>St&quot;</td>
</tr>
<tr>
<td>GYP(B-ΨB-A)</td>
<td>GP(A-A)</td>
<td>GP.Dantu (P2, GL)</td>
<td>He</td>
</tr>
<tr>
<td>GYP(A-ΨB-A)</td>
<td>GP(A-A)</td>
<td>GPVw (M.I)</td>
<td>Mi&quot;, Vw</td>
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<tr>
<td></td>
<td></td>
<td>GP.Hut (M.II)</td>
<td>Mi&quot;, Hut, MUT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GP.Nob (M.VII)</td>
<td>Nob</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GP.Joh (M.VIII)</td>
<td>Nob, Hop</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GP.Dane (M.IX)</td>
<td>Mur, DANE</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GPZan (M&quot;)</td>
<td>St&quot;</td>
</tr>
<tr>
<td>GYP(A-ΨB-A)</td>
<td>GP(A-A)</td>
<td>GPEBH</td>
<td>ERIK (from transcript 1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GPZan (M&quot;)</td>
<td>St&quot; (from transcript 2)</td>
</tr>
</tbody>
</table>

Dantu

GP.Dantu is encoded by a GYP(B-A) hybrid. This hybrid gene consists of exons 1, exons 2, pseudoexon 3, exon 4 of GYPB, and exons 5 to 7 of GYPB. There are four types of Dantu phenotype, designated NE, MD, Ph, and JO variants (Table 7). The MD hybrid gene is flanked by GYPB and GYPB, suggesting that this type originated from a single unequal crossing-over event.50 The NE and Ph variants contain a cis GYPB but lack a GYPB.51 In the case of NE, the GYP(B-A) hybrid gene is duplicated; thus NE and Ph variants can be distinguished by the ratio of GP.Dantu [GP(B-A) hybrid] to GPA.52 RBCs with the JO variant have only one-half the normal levels of GPA, thus leading to the assumption that this variant may contain a GPA-B-A hybrid molecule and an unchanged GYPB rather than an unchanged GYPB and GP(B-A) hybrid.53
Table 7. Mechanism, ethnicity, and occurrence of Dantu phenotypes

<table>
<thead>
<tr>
<th>Dantu phenotypes</th>
<th>Mechanism</th>
<th>Ethnicity</th>
<th>Occurrence (relative)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NE</td>
<td>GYP(A-GYP(B-A)-GYP(B-A))</td>
<td>Black</td>
<td>Most (common phenotype)</td>
</tr>
<tr>
<td>MD</td>
<td>GYP(A-GYP(B-A)-GYP(B-A))</td>
<td>White</td>
<td>Rare (1 proband)</td>
</tr>
<tr>
<td>Ph</td>
<td>GYP(A-GYP(B-A))</td>
<td>Black</td>
<td>Rare (1 proband)</td>
</tr>
<tr>
<td>Jo</td>
<td>GYP(B-A)-GYPB</td>
<td>White</td>
<td>Rare (1 proband)</td>
</tr>
</tbody>
</table>

M<sup>e</sup>

The M<sup>e</sup> antigen is associated with a GYP(A-B-A) hybrid; it has the same amino acids at positions 1 (leucine) and 5 (glutamic acid) as GYP<sup>e</sup> but threonine in position 4 is substituted by an asparagine. RBCs with GPM<sup>e</sup> do not react with anti-M and anti-N but do react with anti-M<sup>e</sup>. M<sup>e+</sup> RBCs react with anti-DANE because of the amino acids Asn-Glu-Val at positions 4, 5, and 6 are thought to be part of the DANE epitope.

St<sup>+</sup> (Stones)

The St<sup>e</sup> antigen is most commonly associated with a GYP(B-A) hybrid but also can be associated with GYP(A-B-A) or GYP(A-E-A) hybrid genes.

GPSch (M<sup>Ψ</sup>) is encoded by a GYP(B-A) hybrid. The hybrid gene arose from a single unequal crossing over between misaligned GYP<sup>A</sup> and GYP<sup>B</sup>. This misalignment led to GYP<sup>B</sup> exons 1 to pseudoxon 3 being joined to exons 4 to 7 of GYP<sup>A</sup>. The St<sup>e</sup> epitope is determined by the amino acid sequence of the junction of exon 2 to exon 4 of either GPB or GPA. The molecule is composed of amino acids 1 to 26 of GPB and amino acids 59 to 131 of GPA.

GPZan (M<sup>Ψ</sup>) is encoded by a GYP(A-ΨE-A) hybrid. The GPZan phenotype is characterized by the co-transmission of M and St<sup>e</sup>. The homologous segment of GYPB pseudoxon 3 replaces exon 3 and the 5′ end of intron 3 of GYP<sup>A</sup>, including the defective donor splice site of the pseudoxon. Thus, the encoded glycoporin consists of GPA lacking the amino acids encoded by exon 3.

GPPEBH is another St<sup>e</sup> carrying glycoporin, which arises by a single nucleotide substitution at position 179 in GYP<sup>A</sup>. This variant is caused by a G>A mutation in the 3′ end of exon 3, which creates a Gly>Arg substitution at amino acid position 59. The mutation also affects pre-mRNA splicing because of the partial inactivation of the adjacent 5′ donor splice site. The full-length transcript encodes a variant GPA molecule with the arginine substitution at amino acid position 59 defining the ERIK antigen, whereas the shorter transcript lacks exon 3 and carries the St<sup>e</sup> antigen.

GPMar is encoded by a GYP(A-ΨE-A) hybrid that arose from a homologous DNA transfer from GYPE to GYP<sup>A</sup>. This GYPE segment covers pseudoxon 3 and extends to the defective donor splice site mutated by the G>A transition at +1 position of GT dinucleotide, which abolishes a donor splice site as well as the expression of exon 3 of GPA. GYPE is apparently identical to the GYPZan in the mode of gene conversion and in the resulting glycoporin.

The so-called or obsolete Miltenberger subsystem

A number of low-prevalence antigens in the MNS blood group system were for many years grouped together in the Miltenberger (Mi.) subsystem. Originally, RBCs reactive with the anti-Mi<sup>+</sup> serum were classified into four classes on the basis of their different reactions with four type sera called Verweyst (Vw), Miltenberger (Mi<sup>+</sup>), Murrell (Mur), and Hill (Hil). Some of the classes of Miltenberger did not react with anti-Mi<sup>+</sup> but reacted with one or more of the other three specific antisera, e.g., GPH<sup>Ψ</sup>(Mi.V) RBCs reacted with anti-Mi<sup>+</sup> but did not react with anti-Hil. The Miltenberger subsystem grew to 11 classes, which were defined by one or more determinants reacting with type-specific antisera (Table 8).

As the complexity of the Miltenberger subsystem increased, further expansion no longer seemed feasible, desirable, or relevant. A notation was introduced to replace the classification of MNS variants into the increasingly complicated Miltenberger subsystem. In this notation, the serologically specified phenotypes are defined by characteristic glycoporin variants (GP for the glycoprotein and GYP for the gene) with the abbreviated name of the propositus in whom the variant has been described, e.g., Mi.V becomes GPH<sup>Ψ</sup> and the encoding gene is referred to as GYP<sup>Ψ</sup>. The Miltenberger subsystem will not be expanded further and is now considered obsolete.

The molecular basis of antigens in the obsolete Miltenberger subsystem can be placed into the following categories.

1. Glycoporin A-B hybrids: GYP(A-B)

GPH<sup>Ψ</sup> (Mi.V) and GJP<sup>Ψ</sup> (Mi.XI) are each encoded by a GYP(A-B) hybrid gene (Fig. 3-I). The GYP<sup>A</sup> to GYP<sup>B</sup> junction in the GYP<sup>Ψ</sup>Hil gene is located at the 5′ end of the intron 3 of GYP<sup>A</sup>, whereas in GYP<sup>B</sup> the junction occurs at the 3′ end of intron 3 and includes 7 nucleotides (nts) of exon 4 of GYP<sup>B</sup>. The allele responsible for GPH<sup>Ψ</sup> encodes s antigen, travels with either Ms or Ns, and expresses Hil and MINY.
antigens, whereas GPJL expresses an altered S, a weak M, TSEN, and MINY antigens. No example of GPHil phenotype was found in testing 50,000 English donors, but GPHil was found with a frequency of 1 in 2000 in one survey of Swiss blood donors.

2. Glycophorin A-B-A hybrids: GP(A-B-A)

GPVw (Mi.I), GPHut (Mi.II), GPNob (Mi.VII), GPJoh (Mi.VIII), and GPDane (Mi.IX) are each encoded by a GP(A-B-A) hybrid (Fig. 3-II). In these hybrid genes, inserts of different short portions of the pseudoexon of GYPB replace the same number of nucleotides in exon 3 of GYPB. The small inserts range from 1 to 16 bp and do not alter the open reading frame or disrupt the splice sites. Thus, the short part of the pseudoexon is translated in this hybrid.

The insert encoding GPVw and GPHut variants results in an amino acid polymorphism at position 28; the threonine present in GPA is changed to methionine in the case of GPVw and to lysine in the case of GPHut. GPVw and GPHut phenotype RBCs are recognized by anti-Vw and anti-Hut, respectively. The allele responsible for GPVw usually travels with Ns, NS, or MS whereas GPHut travels with MS or NS. The highest prevalence of GPVw phenotype, 1.43 percent, was found in southeastern Switzerland.

RBCs with GPNob express the Nob antigen. Structural analysis of GPNob showed that it differs from GPA at amino acid positions 49 and 52; the arginine at position 49 in GPA is substituted by threonine and the tyrosine at position 52 is replaced by serine because of ten nucleotides in exon 3 of GYPB (nt 67–76) that have been replaced by the corresponding sequence of the GPB pseudoexon. The GPNob phenotype has been only found in white donors; three positive reactors with the Raddon serum were found in tests on 4929 random group O blood donors at Bristol, England, a frequency of 0.06 percent. GPNob is associated with Ms and MS.

GPJoh closely resembles GPNob but has the Hop antigen as well as the Nob antigen. The altered GPA of
GPJoh differs from GPNob by having only the arginine to threonine substitution at amino acid position 49. The frequency of GPJoh is unknown. The GPJoh traveled with Ns in the families of the two known propositi.

RBCs carrying GPDane express Mur and DANE antigens. In GPDane, exon 3 of GYPA (codons 35–41) is replaced by the corresponding sequence of the GYPB pseudoexon. As a consequence of this gene conversion event, the hepta peptide sequence of GPA, 35Ala-Ala-Thr-Pro-Arg-Ala-His, is changed to the hexapeptide sequence 35Pro-Ala-His-Thr-Ala-Asn. GPDane has a prevalence of 0.43 percent in Danes. In the four Danish propositi, GYPDane was inherited with Ms.


GPMur (Mi.III), GPHop (Mi.IV), GPBun (Mi.VI), and GPHF (Mi.X) are each encoded by a GYP(B-A-B) hybrid (Fig. 3-III).

GPMur RBCs are Mur+, Hil+ and MINY+ and the allele responsible for GPMur always travels with s antigen, either as Ms or Ns. GPBun is almost identical to GPMur but GPBun cells are Hop+ and the allele responsible for GPBun was aligned with Ms. Both GPMur and GPBun are encoded by a GYPB but differ in the length of the GPB pseudoexon insert (55 bp for GPMur and 131 bp for GPBun). Because this segment comprises a portion of both exon 3 and intron 3, which carries a functional 5′ splicing signal, the rearrangement results in the expression of a normally unexpressed GYPB pseudoexon sequence. The GYPBun gene differs from the GYPMur gene by only one nucleotide in the coding sequence. This results in a predicted arginine (GPMur) or threonine (GPBun) at position 48. GPMur and GPBun are rare in Caucasians but GPMur has a prevalence between 5 and 10 percent in some Asian populations, 9.6 percent in Thais, and 7.3 percent in Taiwanese.

GPHop, which expresses TSEN but not Hil, is identical to GPBun. The allele responsible for GPHop always travels with S, whereas the allele for GPBun always carries the s antigen.

GPHF is characterized by M and an unusually strong s antigen as well as by its reactivity with anti-Hil and anti-MINY. This glycophorin hybrid is similar to GPMur and GPBun. In GPHF, a 98-bp insert from exon 3 of GYPA creates a GYP(B-A-B) hybrid, which encodes a peptide differing from GPMur by five amino acid residues and from GPBun by six amino acid residues.

### Conclusion

The MNS system is a complex blood group system consisting of more than 40 antigens on GPA and GPB, or on hybrid glycophorin molecules. It is second only to the Rh blood group system in its complexity. The antigens of the MNS blood group system arise from single nucleotide substitution, unequal crossing over, gene conversion, or both between the glycophorin genes. Some of these molecular mechanisms occur as a consequence of misalignment of the chromosomes carrying the glycophorin genes during meiosis. It is possible because the glycophorin family of genes is homologous and adjacent on the chromosome.

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Discrepancies between blood group genotype and RBC phenotype are important to recognize when implementing DNA-based blood grouping techniques. This report describes two such cases involving the ABO blood group in the Korean population. Propositus #1 was a 22-year-old healthy man undergoing pretransfusion testing for minor surgery. Propositus #2 was a 23-year-old male blood donor. RBCs from both propositi were determined to be group AB and demonstrated unusual agglutination patterns on forward typing, which were inconsistent with their ABO genotype determined by allele-specific (AS) PCR. RBCs from propositus #1 demonstrated mixed field agglutination with both anti-A and -B, while RBCs from propositus #2 demonstrated mixed field only with anti-A reagents. Both had B/O genotypes by AS-PCR. Cloning and sequencing of ABO exons 6 and 7 revealed three alleles in both propositi: propositus #1: A102/B101/O04; propositus #2: A102/B101/O01. A panel of nine short-tandem repeat (STR) loci was tested on DNA extracted from blood, buccal mucosal cells, and hair from the propositi and on DNA isolated from their parents' blood. In all tissues tested from propositus #1, three loci demonstrated a double paternal and a single maternal DNA contribution, indicating that he was a chimera or a mosaic; in those from propositus # 2, one STR locus demonstrated a double paternal DNA contribution, indicating that he was a tetragametic chimera. Chimerism and mosaicism are uncommon but important causes of ABO genotype and phenotype discrepancies. The evaluation of patients and donors with unusual or unexpected serology in pretransfusion testing and consensus ABO alleles may include the evaluation of STR loci to detect these phenomena. 

Key Words: chimerism, mosaicism, ABO, genotype, phenotype, discrepancy

Chimeric individuals result from the fusion of two or more zygotes. They differ from mosaic individuals whose cells carry different complements of DNA derived from a single zygote. Chimeric individuals generally are healthy, but often feature differentially pigmented patches of skin or eyes of different colors. Both of these genetic variants can give rise to ABO discrepancies if RBCs with different ABO groups are produced or if a naturally occurring ABO antibody is unexpectedly absent, as revealed on reverse typing. Resolving an ABO discrepancy caused by weak or unusual agglutination on forward typing can require an investigation into the patient's transfusion history and clinical diagnosis and potentially a molecular evaluation of the ABO gene. With the identification of the ABO alleles by Yamamoto et al., it became possible to determine a blood donor's or recipient's ABO blood group at the DNA level. The molecular bases of numerous ABO subtypes in several populations have been elucidated. In Korea, the overall frequency of A and B subtypes among blood donors was reported to be 0.1 percent with cis-AB01 among the most commonly identified subtype alleles. This allele can produce various phenotypes including A1B3, A1B3, A2B3, and A2B and its presence should be considered when evaluating an ABO discrepancy, especially in Korean and Japanese populations, where this allele is most commonly found. A detailed description of the cis-AB phenotype can be found in reference 6. On the basis of common ABO gene polymorphisms in the Korean population, allele-specific (AS) PCR primers to detect A, B, O, and cis-AB01 alleles were designed and used in resolving ABO discrepancies. ABO genotyping is also used as an initial means of resolving disputed paternity claims, which are not infrequent because of the relatively high prevalence of the cis-AB blood group in Korea. We have recently discovered two instances where the results of AS-PCR have not been in accord with the patients' RBC phenotypes obtained by serologic methods. Both of these cases were shown by further molecular testing to be chimeras or mosaics.
Materials and Methods

Blood group serologic tests
Forward typing was carried out by the manual tube method using murine monoclonal anti-A, anti-B, anti-H, and anti-AB (Biotest AG, Dreieich, Germany) according to standard protocols. Reverse typing was also performed by tube methods using A, and B RBCs (Diamed, Cressier sur Morat, Switzerland). The forward and reverse typings were repeated using gel cards containing monoclonal anti-A and anti-B in cases where ABO subtypes were suspected (Diamed).

Preparation of DNA
EDTA-whole blood, buccal mucosal cells, and hair follicles were obtained from the propositi. Genomic DNA extraction was performed using the Wizard Genomic DNA purification kit (Promega Corporation, Madison, WI) on EDTA-blood and the QIAamp DNA micro kit (Qiagen, Hilden, Germany) on buccal mucosal cells and hair follicles. Informed consent was obtained from the study participants before their samples were drawn.

Sequence based typing for HLA-A, -B, and -DR
DNA sequencing-based typing (SBT) for HLA class I and II alleles was performed with commercially available AlleleSEQR HLA -A,-B, and -DRB1 SBT reagents (Atria Genetics, San Francisco, CA) according to manufacturer’s instructions. The sequence results were analyzed with Assign-SBT (Conexio Genomics, Applecross, Western Australia, Australia) software.

Short-tandem repeat analysis
We studied short tandem repeats (STR) on autosomal chromosomes using commercially available kits for the following loci: vWA, FGA, D3S1358, D5S818, D7S820, D8S1179, D13S317, D18S51, and D21S11 (AmpFISTR Profiler Plus PCR Amplification Kit, ABI, Perkin Elmer/Applied Biosystems, Foster City, CA). Analysis was performed using the ABI PRISM 310 Genetic Analyzer (Perkin Elmer/Applied Biosystems).

Karyotyping
Peripheral blood specimens from both propositi and their parents were studied. Approximately 0.5 mL of whole blood was cultured in 10 mL of Roswell Park Memorial Institute (RPMI) 1640 (Gibco, Invitrogen Corporation, Grand Island, NY) with 10% fetal bovine serum and 50 U of penicillin-streptomycin for 72 hours. The cultures were then stimulated with PHA and 20 metaphase cells stained by G-staining methods were karyotyped. The karyotypes were described according to the International System for Cytogenetic Nomenclature 2005.

Molecular analysis of ABO gene
1. Allele-specific PCR
AS-PCR at nucleotides (nt) 261 (exon 6) and at nt 526 and 803 (exon 7) of the ABO gene to discriminate between the A, B, O, and cis-ABO1 alleles was performed using previously described methods. The primers were designed using a modified amplification refractory mutation system. In this technique, the primers' penultimate base was mismatched to increase the specificity of the PCR. PCR was performed in a reaction mixture of 50 µL with 0.25 µM of each primer pair, 300 ng of genomic DNA, 0.2 µM (each) dNTP, 1X PCR buffer [10mM Tris-HCl (pH 8.8), 1.5mM MgCl2, 50 mM KCl, 0.1% TritonX-100], and 2 U DynaZyme (Finzyme Inc., Finland). Thirty cycles of amplification were performed by manual hot-start PCR in a MJ Research PTC-100 thermocycler (MJ Research INC., Watertown, MA) after an initial denaturation at 95°C for 5 minutes. Each cycle consisted of a denaturation step at 94°C for 30 seconds, an annealing step at 64°C for 30 seconds, and an extension step at 72°C for 1 minute. An additional primer extension at 72°C for 10 minutes followed the last cycle. PCR products were separated at 100v for 40 minutes on 1.8% agarose gel prestained with ethidium bromide (0.5 µg/mL).

2. Cloning and sequencing of exons 6 and 7
Genomic DNA was extracted from peripheral WBCs with a DNA isolation kit (SolGent, Daejeon, Korea). The coding and flanking intronic sequences of exons 6 and 7 were amplified, cloned, and sequenced. For the cloning, PCR products (2080 bp) were directly inserted into the PCR 2.1-TOPO vector and transformed into TOP 10 host cells using the TOPO-TA cloning kit (Invitrogen, Carlsbad, CA). The sequencing reaction was performed in a 10 µL mixture containing 5X sequencing buffer, 4 µL of BigDye, 200 ng of purified DNA, 2 µL of primer (1.6 pmoles/µL). Sequence analysis was performed using SEQUENCHER (Gene Codes Corp, Ann Arbor, MI) software. Alleles were named according to the unofficial nomenclature used in the Blood Group Antigen Gene Mutation Database.
Table 1. Summary of the serologic blood group determinations, sequence-based HLA typing, and karyotypes of both propositi and their parents

<table>
<thead>
<tr>
<th>Propositus</th>
<th>Age, Sex</th>
<th>Antigen system</th>
<th>Father</th>
<th>Mother</th>
<th>Propositus</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>22, M</td>
<td>ABO phenotype</td>
<td>AB</td>
<td>O</td>
<td>ABO&lt;sub&gt;ABr&lt;/sub&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Genotype (sequencing)</td>
<td>A102/B101</td>
<td>002/004</td>
<td>A102/B101/004</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AS-PCR</td>
<td>(A/B)</td>
<td>(O/O)</td>
<td>(B/O)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rh</td>
<td>DcEc</td>
<td>DcEc</td>
<td>DcEc</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HLA-A</td>
<td>A&lt;sup&gt;3&lt;/sup&gt;303</td>
<td>A&lt;sup&gt;3&lt;/sup&gt;303</td>
<td>A&lt;sup&gt;3&lt;/sup&gt;303</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HLA-B</td>
<td>B&lt;sup&gt;4&lt;/sup&gt;403</td>
<td>B&lt;sup&gt;3&lt;/sup&gt;501</td>
<td>B&lt;sup&gt;4&lt;/sup&gt;403</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HLA-DRB1</td>
<td>DRB1&lt;sup&gt;1&lt;/sup&gt;0701</td>
<td>DRB1&lt;sup&gt;1&lt;/sup&gt;0403</td>
<td>DRB1&lt;sup&gt;1&lt;/sup&gt;0701</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Karyotype</td>
<td>46,XY</td>
<td>46,XX</td>
<td>46,XY</td>
</tr>
</tbody>
</table>

| 2          | 23, M    | ABO phenotype  | A      | B      | A<sub>AB</sub> |
|            |          | Genotype (sequencing) | A102/001 | B101/001 | A102/B101/001 |
|            |          | AS-PCR       | (A/O)  | (B/O)  | (B/O)      |
|            |          | Rh           | DcEc   | DcEc   | DcEc       |
|            |          | HLA-A        | A<sup>3</sup>0201 | A<sup>3</sup>2402 | A<sup>3</sup>0201 |
|            |          | HLA-B        | B<sup>2</sup>704 | B<sup>0</sup>701 | B<sup>2</sup>704 |
|            |          | HLA-DRB1     | DRB1<sup>1</sup>0405 | DRB1<sup>1</sup>0101 | DRB1<sup>1</sup>0405 |
|            |          | Karyotype    | 46,XY  | 46,XX  | 46,XY      |

mf = mixed field agglutination

Case Studies and Results

Propositus #1 was a healthy 22-year-old man. Routine pretransfusion testing prior to minor surgery revealed an unusual blood group: approximately one half of his RBCs showed strong (4+) agglutination with anti-A and anti-B sera on forward typing while the remaining RBCs were unagglutinated (mixed field agglutination). His serum did not react with A<sub>1</sub> or B RBCs. Furthermore, his blood group was unusual because his father was group AB and his mother was group O (Table 1, Fig. 1). Propositus #2 was a healthy 23-year-old blood donor whose RBCs demonstrated strong and uncomplicated agglutination on forward typing with anti-B reagents but mixed field agglutination with anti-A reagents (Table 1, Fig. 1). His serum did not react with A<sub>1</sub> or B RBCs.

Both propositi were morphologically normal, had no known twins, and denied ever receiving a blood transfusion or a stem cell or solid organ transplant. Samples from these propositi were to detect a cis-AB allele in propositus #1 and to elucidate the molecular basis of the mixed field agglutination in propositus #2. By AS-PCR performed on DNA extracted from peripheral blood, the ABO genotype of both propositi was B/O. Cloning and direct sequencing of ABO exons 6 and 7 revealed three alleles in both propositi: propositus #1:

P1

![P1](image1)

P2

![P2](image2)

Fig. 1. In propositus #1 (P1, top), mixed field agglutination was observed with anti-A and anti-B sera indicating the simultaneous presence of two distinct RBC populations. This propositus' A allele was not detected until exons 6 and 7 were cloned and sequenced. In propositus #2 (P2, bottom), a mixed field agglutination pattern was observed with anti-A because of the presence of A and B RBCs. SSP = sequence-specific primer, SBT = sequence based typing.
A102/B101/O04 (likely genotype: A102/O04 plus B101/O04); propositus #2: A102/B101/O01 (inferred ABO genotype from serological results: A102/B101 and B101/O01) (Table 1). The brother and sister of propositus #1 demonstrated uncomplicated B and A phenotypes and genotypes, respectively. A panel of nine STR loci was tested on DNA extracted from blood, buccal mucosal cells, and hair from both propositi, and on DNA isolated from blood from their parents. In propositus #1, three loci demonstrated a double paternal and a single maternal DNA contribution from all tissue samples studied (Table 2, Fig. 2). The FGA locus was uninformative in the propositus as his parents shared an allele. In propositus #2, one STR locus (D7S820) demonstrated a double parental DNA contribution, while other loci were uninformative in that they revealed either double maternal or paternal DNA contributions with only a single contribution from the other parent, or else it was not possible to determine if the propositus' extra allele was maternal or paternal owing to shared alleles between the parents (Table 2, Fig. 2). Both propositi had normal male (46, XY) karyotypes. DNA-sequence based HLA typing revealed only single parental DNA contributions in both propositi.

**Discussion**

Propositus #1 demonstrated one maternal and two paternal DNA contributions in all tissues tested. There are two embryologic explanations for this finding: he might be a mosaic who arose from one zygote with three pronuclei instead of the normal two, i.e., the zygote was formed from one ovum containing a haploid nucleus fertilized by two haploid sperm nuclei (dispermy) or one ovum plus one diploid sperm nucleus (diplospermy). Dispermy is a frequent event (1% of human conceptions) and results in a triploid zygote. As the zygote divides, some or all daughter cells become diploid. Thus, if one zygote with three pronuclei survived as an embryo, it would be composed of different cell populations. Equally plausible, however, is that propositus #1 is a dispermic chimera resulting from the parthenogenetic division of the ovum and its subsequent fertilization by two spermatozoon carrying different paternal DNA—one with a 23,Y chromosome complement and another spermatozoon with a different 23,Y DNA complement. This would explain the apparent single maternal and double paternal DNA contribution. Propositus #2 is a tetragametic chimera because one STR locus clearly demonstrated the presence of two maternal and two paternal alleles. The finding of only a single parental HLA type in these individuals was unexpected but a similar finding has been previously reported. The detection of only one parental HLA haplotype in this tetragametic chimera probably relates to the relatively lower sensitivity of HLA typing methods compared with STR techniques.

In this study, both propositi were initially found to have ABO genotype and phenotype discrepancies; both had group A and group B RBCs detectable by serologic methods, but only demonstrated B and O alleles by AS-PCR, i.e., an A allele was not initially detected in either

| Table 2. STR analysis of both propositi and their parents. Only the informative loci are shown for each propositus. |
|---|---|---|---|---|---|
| Propositus | DNA polymorphism | Father (blood) | Mother (blood) | Propositus (blood) | Propositus (buccal cells) | Propositus (hair) |
| 1 | D8S1179 | 10,14 | 12,15 | 10,12,14 | 10,12,14 | 10,12,14 |
| | vWA | 16,19 | 15,18 | 15,16,19 | 15,16,19 | 15,16,19 |
| | FGA | 22,23 | 17,22 | 17,22,23 | 17,22,23 | 17,22,23 |
| 2 | D7S820 | 9,10 | 8,12 | 8,9,10,12 | 8,9,10,12 | 8,9,10,12 |

*Italicized* alleles indicate paternally derived alleles. *Bolded* alleles indicate maternally derived alleles. *Underlined* alleles indicate those that are shared by mother and father and are uninformative in the propositus.
propositus using this technique, probably because of the relative insensitivity of this test and the small quantity of DNA containing an A allele present. In fact, the cis-AB phenotype was initially suspected in propositus #1. The failure, by AS-PCR, to detect an allele known to encode the cis-AB phenotype led us to perform the STR testing and gene sequencing which revealed the cause of his unusual forward typing. When propositus #2 was an embryologic explanation for his genotype and phenotype discrepancy was sought. The mixed field agglutination pattern of RBCs was thus the result of the simultaneous presence of separate group A and group B RBCs in propositus #1 and of separate group AB and B RBCs in propositus #2. As these two cases suggest, chimerism and mosaicism can confound ABO genotyping such that if blood group genotyping becomes more commonly used, more cases might be identified in attempts to explain similar genotype and phenotype discrepancies.11,12 Our approach to the evaluation of patients with unusual serology on forward typing and consensus ABO alleles now includes the evaluation of STR loci in the propositus and available relatives to determine if chimerism or mosaicism is responsible for the unusual ABO phenotype.

Acknowledgments
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References
Hemolytic disease of the fetus and newborn (HDFN) results from maternal IgG antibodies that cross the placenta to the fetal circulation during gestation and cause RBC destruction and complications before birth (HDF), or anemia and hyperbilirubinemia after birth (HDN), or both. In its most severe form, HDF produces hydrops fetalis, which is characterized by total body edema, hepatosplenomegaly, and heart failure and can lead to intrauterine death. Before discovery of Rh immunoglobulin (RhIG), HDFN from anti-D was a significant cause of perinatal mortality or long-term disability. Routine administration of RhIG to D− women during pregnancy and shortly after the birth of D+ infants effectively reduced the incidence of HDFN caused by anti-D. Maternal alloimmunization to other RBC antigens in the Rh, Kell, and other blood group systems can not be routinely prevented and these antibodies can also cause HDFN. Advances in prenatal care, noninvasive monitoring, and intrauterine transfusion have improved the outlook for affected pregnancies to the extent that even hydrops fetalis can be reversed and effectively treated in many cases. This review will provide an update on the current issues in prevention and treatment of HDFN, emphasizing recent insights into long-standing controversies regarding maternal weak D phenotypes and D alloimmunization, noninvasive fetal diagnosis and monitoring of affected pregnancies, and recent treatment guidelines. *Immunohematology* 2006;22:188–195.

The incidence of D alloimmunization in pregnancy decreased from 14 percent to between 1 and 2 percent following the introduction of postnatal prophylaxis with Rh immunoglobulin (RhIG) in the late 1960s and, after 1979, was further reduced to 0.1 percent with the addition of routine antenatal RhIG prophylaxis.\(^1\)\(^−\)\(^3\) Smaller family size in recent decades has also contributed to the decline in the number of cases, but hemolytic disease of the fetus and newborn (HDFN) caused by anti-D continues to occur in about 6.7 of 1000 live births in the United States, which likely reflects inadvertent failure to administer RhIG prophylaxis, inadequate prenatal care, or antenatal sensitization prior to RhIG administration at 28 weeks' gestation.\(^4\)\(^5\)

More than 50 different specificities of RBC antibodies have been implicated in HDFN, but most cases of severe fetal anemia that require treatment in utero are caused by anti-D or anti-c (Rh system), or anti-K (Kell system) (Table 1).\(^1\)\(^,\)\(^2\)\(^,\)\(^6\) The epidemiology of HDFN in different ethnic and racial populations directly reflects the frequency of blood group alleles in the population and the likelihood of incompatibility and consequent maternal alloimmunization.\(^6\)\(^,\)\(^7\)

Incompatibility with respect to the D antigen occurs in about 10 percent of all pregnancies among Caucasians and African Americans; in contrast, the D− phenotype is extremely rare among Asian women and HDFN caused by anti-D is seldom encountered in these populations. In a study of 17,568 screened pregnancies, the prevalence of new antibody production was 0.24 percent (95% CI, 0.17–0.32).\(^8\) Anti-D is still one of the most common antibodies found in pregnant women, but other antibodies have surpassed anti-D in some studies. In one large series, anti-K was detected at a rate of 3.2 per 1000 maternal samples compared with anti-D at 2.6 per 1000.\(^9\) In another recent study of 1133 Dutch women with positive antibody screens, anti-E was the most common antibody detected (23%) followed by anti-K (18.8%), anti-D (18.7%), and anti-c (10.4%).\(^10\)

Severe hemolytic disease requiring intrauterine transfusion was caused by anti-D, -K, or -c, in 85 percent, 10 percent, and 3.5 percent of HDF cases, respectively.\(^1\)\(^1\) Rh and Kell antibodies are more likely associated with severe hemolysis than are other antibodies, but HDFN associated with these and other blood group antibodies can demonstrate a broad spectrum of symptoms, ranging from mild anemia and hyperbilirubinemia in an infant to life-threatening complications before birth. About one-half of D+ infants with detectable maternal anti-D in their serum are unaffected or only mildly affected and require no treatment; whereas 20 percent are severely affected in utero.\(^1\)\(^2\) About one-half of these severely affected
fetuses have significant hemolysis before 34 weeks’ gestation and require intrauterine transfusion. A similar spectrum of disease severity is observed with anti-c, anti-K, and anti-Fya, with severe disease affecting as many as 7 percent, 38 percent, and 16 percent of susceptible fetuses, respectively. Subsequent pregnancies are more likely to be severely affected than are first pregnancies because of the anamnestic immune response.

Weak D and RhIG Prophylaxis

To prevent maternal alloimmunization, RhIG should be given to all D– women who do not have detectable anti-D at 28 weeks’ gestation and within 72 hours of delivery or other potentially sensitizing event. Testing for ABO, D, and unexpected antibodies should be performed on samples from all pregnant women in the first trimester, but the need to test D– pregnant women for weak expression of D has been controversial. Subsequent pregnancies are more likely to be severely affected than are first pregnancies because of the anamnestic immune response.

### Table 1. Probability of causing severe HDFN associated with RBC antibodies

<table>
<thead>
<tr>
<th>Blood group</th>
<th>Highest likelihood of severe HDFN</th>
<th>Rare cases of severe HDFN</th>
<th>Usually associated with mild disease</th>
<th>Not a cause of HDFN</th>
</tr>
</thead>
<tbody>
<tr>
<td>MNS</td>
<td>M, S, s, U, M†, Vw, Mur, Mr†, Hut, Hil, M*, Far, s†, En†, MUT</td>
<td>M, S, s, U, Mr†, Mit</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>Rh</td>
<td>D, C</td>
<td>C, E, f, Ce, C*, C*, E*, G, Hr, Hr, Rh29, Go†, Rh52, Be†, Evans, Tar, Rh42, Sec, JAL, STEM</td>
<td>E, e, f, C*, D*, Rh29, Riv, LOC R</td>
<td></td>
</tr>
<tr>
<td>Lutheran</td>
<td>K</td>
<td>Ku, Js†, K11, K22</td>
<td>K23, K24</td>
<td></td>
</tr>
<tr>
<td>Kell</td>
<td>K</td>
<td>Ku, Js†, K11</td>
<td>K23, K24</td>
<td>Le–, Le–a</td>
</tr>
<tr>
<td>Lewis</td>
<td>D</td>
<td>Fy– (rare), Fy3 (rare)</td>
<td></td>
<td>Le–, Le–a</td>
</tr>
<tr>
<td>Duffy</td>
<td>Fy–</td>
<td>Fy– (rare), Fy3 (rare)</td>
<td></td>
<td>Le–, Le–a</td>
</tr>
<tr>
<td>Kidd</td>
<td>Jk–</td>
<td>Jk– (rare), Jk3</td>
<td></td>
<td>Le–, Le–a</td>
</tr>
<tr>
<td>Other</td>
<td>Di†, Wr†, Rd, Co†, Co3, PP1ρ</td>
<td>Di†, Sc3, Co†, Ge2 (rare), P1, Wr†, Yt†, Yt†, Sc1, Sc2, CH/GR, CROM, KN, JMH, I</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Vel, MAM</td>
<td>Ge3, Ls†</td>
<td></td>
<td>Le–, Le–a</td>
</tr>
<tr>
<td></td>
<td>Bi, Kg, JONES, HIJK, REIT</td>
<td>Lan, At†, Jr†</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>JFP, HOFM</td>
<td></td>
<td>Le–, Le–a</td>
</tr>
</tbody>
</table>

* For some of the antibodies listed, the information is based on a very small number of examples, sometimes only one, resulting in overlap between categories.

Alloimmunization to the D antigen and HDFN caused by anti-D can occur in women with partial D antigen expression. Denomme et al. evaluated partial D, weak D types, and novel RHD alleles among 33,864 screened multiethnic patients and found 54 typing discrepancies which represented mutated RHD alleles. Among these cases, 10 of 25 (35%) obstetric patients were assigned D+ status, but expressed a D variant known to permit anti-D alloimmunization (e.g., DAR, DFR, and DAU, and categories DIIIa, DVa, and DVI).

The frequency with which partial D individuals develop anti-D in the general population is not known, but it may be higher than previously recognized, as demonstrated in a recent CAP survey that found that one-third of transfusion services reported at least one patient whose RBCs were of a weak D phenotype with anti-D formation in a 12-month period. Although severe HDF in women whose RBCs are of a weak D phenotype is rare, antibodies have been more frequently reported in women with a partial DVI phenotype, and fatal HDF has occurred in pregnancies of such alloimmunized women.

AABB does not require testing for weak D in pregnancy, and, if an IAT is not performed, most women with partial D will be classified as D– and will be candidates for RhIG. In the CAP survey, however, 58.2 percent of transfusion services routinely performed testing for weak D in patients whose RBCs were negative in direct testing with anti-D reagents. ABB Standards require designating the RBCs of individuals with positive tests for weak D as D+, and the ACOG advises against administering RhIG to women with...
RBCs of known weak D phenotypes. However, the CAP survey found that only 50 percent of transfusion services reported weak D as D positive, and 71.1 percent of transfusion services that routinely test for weak D give RhIG to these pregnant women. 

On the basis of the demonstrated risk of D alloimmunization in women with a partial D phenotype, some experts have cautioned that if pregnant women are tested for weak D, an appropriate anti-D reagent or technique should be used that will not detect DVI so that these women are assigned D– status for transfusion and Rh prophylaxis. The alternative is to not test pregnant women (or transfusion recipients) for weak D, which would result in most of these individuals being classified as D– and candidates for RhIG prophylaxis. This presents a challenge to countries that do not have a sufficient supply of RhIG and need to more accurately distinguish those individuals with D variants who are not at risk of anti-D alloimmunization from those who are at risk and should receive RhIG prophylaxis. Not surprisingly, a recent survey showed that international practices on testing for weak D and RhIG administration diverge from practice in the United States, and 8 of 10 countries perform further testing for weak D or D variants if the woman’s RBCs type as D– or if the typing results are anomalous, to limit administration of RhIG to women with partial D at risk for developing anti-D. The relative effectiveness of RhIG in preventing the sensitization of partial D women compared with D– women is not yet known.

Infants born to D– women should have their blood type determined using a reagent and a method that detects DVI and weak D, an event that would result in most of these individuals being classified as D– and candidates for RhIG prophylaxis. This presents a challenge to countries that do not have a sufficient supply of RhIG and need to more accurately distinguish those individuals with D variants who are not at risk of anti-D alloimmunization from those who are at risk and should receive RhIG prophylaxis. Not surprisingly, a recent survey showed that international practices on testing for weak D and RhIG administration diverge from practice in the United States, and 8 of 10 countries perform further testing for weak D or D variants if the woman’s RBCs type as D– or if the typing results are anomalous, to limit administration of RhIG to women with partial D at risk for developing anti-D. The relative effectiveness of RhIG in preventing the sensitization of partial D women compared with D– women is not yet known.

Noninvasive Fetal Rh Genotyping

The initial type and antibody screen that is performed for all pregnant women not only identifies D– women who are candidates for RhIG, but also identifies alloimmunized women that require further monitoring for HDF during the pregnancy. If a clinically significant maternal antibody is present, the blood type of the biologic father should be determined to assess whether the antigen could be present on the RBCs of the fetus. Paternity must be certain, however, to draw meaningful conclusions. Serologic studies of RBC antigen expression may be informative. If the biologic father’s RBCs lack the antigen, the infant is not at risk for HDF during pregnancy and no further fetal testing is necessary. If the father’s RBCs have double-dose expression of the implicated antigen, the trait will be inherited and the fetus should be monitored for HDF; if they have single-dose expression, his offspring have a 50 percent chance of inheriting the blood group antigen allele. Because no antithetic allele for D exists, paternal zygosity cannot be definitively predicted by serologic means. The most probable combination of haplotypes can be predicted, because RHD inheritance is closely linked to RHCE, and the probability that the father is heterozygous for the D allele can be estimated from a model that takes into account the serologic results as well as the ethnic background and the number of previous D+ children. Tables of gene frequencies in Caucasians, African Americans, and Mexican Americans have been published to estimate the likelihood of paternal heterozygosity for the D allele.

If prenatal testing indicates that the father may carry an antigen to which a clinically significant antibody could be made, fetal testing should be performed to determine if the allele is present. Most RBC polymorphisms can now be tested for by molecular analysis at the DNA level, including the complex RHD and RHCE loci (for D, C, c, E, and e antigens), as well as the Kell (for K and k antigens), Duffy (for Fy and Fy antigens), and Kidd (for Jk and Jk antigens) loci. The Rh system is by far the most complex of the human blood group systems, and considerable genetic diversity underlies the aberrant RH alleles associated with the D– phenotype. The most prevalent D– genotype in Caucasian populations is the complete deletion of RHD; in African blacks and African Americans, other variant RHD genes are more likely to be found. The RHD pseudogene, which contains a 37-bp insertion in exon 4 and results in no
detectable transcription of the gene, is found in 66 percent of D– black Africans and 24 percent of D– African Americans. 23

Similarly, a variant RHD-CE-D gene encodes the r’s (dCcε) haplotype (phenotype) that underlies serologic D negativity in 22 percent of D– African Americans. These variant RHD alleles have important implications for prenatal diagnosis of fetal blood type in different populations because the presence of one of these genes in the fetus can lead to a false positive result (i.e., the fetus is predicted to be D+ by molecular methods but is found to be D– by serology after birth) and unnecessary prenatal intervention. A maternal blood sample should be analyzed in parallel with the fetal sample. False negative results (i.e., the fetus is predicted to be D– but is found to be D+ by serology at birth) have been attributed to erroneous paternity or rearrangement at the paternal RHD gene locus. If no paternal sample is available, a predicted D– fetal blood type determined by molecular methods should be treated with caution, and the pregnancy should be monitored to ensure that the titer of maternal anti-D does not increase. 2

The most common method to obtain fetal DNA for molecular testing is still amniocentesis in the United States because noninvasive alternatives are not yet available. Amniocentesis is performed at 24 weeks’ gestation and is relatively safe but is associated with a pregnancy loss rate of about 0.3 percent. 25 This risk is avoided with noninvasive methods to obtain fetal cells or fetal cell-free DNA from the maternal circulation and maternal blood has been used routinely for determination of fetal RHD status in Europe. 10, 26 Fetal DNA can be isolated from maternal plasma as early as 32 days’ gestation and constitutes 3 to 6 percent of the plasma DNA pool in the second and third trimesters. 10 Cell-free fetal DNA is rapidly cleared and does not persist into subsequent pregnancies in contrast to the potential for long-term persistence of fetal leukocytes that can complicate analysis of cellular fetal DNA. 27 Several groups have demonstrated 96 to 100 percent accuracy in predicting the RhD phenotype with over 200 pregnancies tested. 10, 19, 26 False positives were the result of the presence of a pseudogene or D variant; false negatives resulted from the failure to isolate sufficient fetal DNA. When negative results are obtained (e.g., no RhD-specific signal is detected), the presence of fetal DNA in the plasma should be confirmed by another fetus-specific DNA sequence from a highly polymorphic paternal antigen or from the Y chromosome for male fetuses (e.g., SRY). There are no reported strategies for Kell testing or Rh testing other than for RHD using a sample of maternal blood, which likely reflects the difficulty of developing a sensitive assay for subtle allelic differences that will still be specific in the presence of an excess of maternal DNA. 10, 26

Monitoring Affected Pregnancies

When a clinically significant antibody is detected in a woman’s first pregnancy, maternal antibody titers are typically determined each month until approximately 24 weeks’ gestation. Although the concept of a critical titer has been challenged, most laboratories consider the titer of anti-D that is associated with a significant risk of severe HDF to be 32. Maternal antibody titers are only useful in a first pregnancy; not in assessing subsequent antigen-positive pregnancies. The utility of antibody titers in monitoring Kell-sensitized pregnancies is limited because the severity of intrauterine disease may not correlate with maternal antibody titers, and severe HDF has occurred with low anti-K titers. 2 Conversely, most other RBC antibodies are less likely to cause severe disease than anti-D and anti-K, and higher thresholds for antibody titers during pregnancy have been used. 7 Because the antibody titer will depend on laboratory technique, there can be considerable variability in titers when the same sample is analyzed by different institutions; however, serial assessment of titer by an individual institution should reliably reflect trends when careful attention is given to consistent and appropriate laboratory methods. For all RBC antibodies implicated in HDF, a fourfold increase in antibody titer is considered a significant change that warrants further diagnostic investigation.

The most significant advance in monitoring alloimmunized pregnancies has been the recent demonstration that the severity of fetal anemia can be predicted by a noninvasive method, Doppler ultrasound, which obviates the need for serial amniocentesis to measure bilirubin concentration (ΔOD550) in most cases. 28 In the presence of significant fetal anemia, the velocity of blood flow through the middle cerebral artery (MCA) increases and the change can be detected by Doppler ultrasound. In a prospective study of 165 fetuses with maternal RBC alloimmunization (anti-D, -c, -E, or -Fya), an increase in peak velocity in the MCA expressed as more than 1.5 multiples of the median (MoM) had a sensitivity of 88 percent and a specificity of 82 percent for severe fetal
anemia. The performance of Doppler ultrasound was better than that of amniocentesis using the Liley curve, but similar to that of amniocentesis with Queenan's method. Doppler ultrasound has also been used to manage Kell-sensitized pregnancies and is preferred to amniocentesis which is often unreliable when HDF is caused by anti-K because these antibodies not only cause hemolysis but also suppress erythropoiesis as reflected by falsely reassuring ∆OD450 values in the setting of profound fetal anemia. Fetal blood sampling to directly measure fetal hematologic parameters is generally undertaken after Doppler MCA ultrasound suggests the presence of severe or worsening fetal anemia.

**Intrauterine Transfusion for HDF**

Prenatal monitoring of maternal antibody titers and fetal MCA velocity with Doppler ultrasound may indicate the need for fetal blood sampling and intrauterine transfusion. If a woman with a rare null phenotype has corresponding RBC antibodies, the identification of antigen-negative blood for transfusion is a major challenge facing the transfusion service. Washed maternal RBCs are a potential source of antigen-negative RBCs that can be collected and used for intrauterine transfusion to the fetus. Blood donation during pregnancy may risk premature labor and fetal intrauterine growth restriction but is usually well tolerated and must be weighed against the need for antigen-negative blood for the fetus. Recently, ABO-incompatible maternal blood was successfully used for intrauterine transfusion when ABO-compatible RBCs which lacked the implicated antigen were not available. A pregnant woman with the rare homozygous type D– (RH:-17), characterized by the complete absence of C, c, E, and e antigens and the elevated expression of D on the surface of RBCs, demonstrated antibodies against the high-incidence antigen Rh17. Her second infant was severely anemic at birth, and a hydrosopic fetus was identified early in the course of her third pregnancy. ABO-compatible, Rh17–RBCs were not available; consequently, washed maternal RBCs (group B) were used for seven intrauterine transfusions to the group O fetus prior to 36 weeks’ gestation. At birth, the infant’s RBCs typed group B, D+ and had a negative DAT with no evidence of mixed field agglutination. A Kleihauer-Betke test on cord blood indicated the presence of adult Hb and no detectable fetal Hb. Phototherapy was initiated at birth but was discontinued after the first day because the indirect bilirubin was 55 µmol/L at birth and remained stable, while the initial Hb (143 g/L) improved by day 3 (209 g/L). Exchange transfusion was not required; the infant was reported to be healthy at 2 weeks of age with no evidence of neurologic impairment. The case demonstrates that ABO incompatibility is not a deterrent to intrauterine transfusion of maternal blood because anti-A and anti-B are not present during gestation and are usually absent or only weakly detectable at birth. ABO-mismatched transfusion is an option for rare cases when antigen compatible or group O RBCs cannot be obtained for intrauterine transfusion. Maternal blood should be washed to remove antibody, leukoreduced to lower the risk of CMV transmission, and irradiated to prevent GVHD.

**Evaluation of Infants at Risk for HDN**

Immunohematologic testing of infants born to women with potentially significant RBC antibodies should include ABO and D typing as well as a DAT. If HDN is suspected on clinical grounds, but the DAT and maternal antibody screen are negative, the possibility of incompatibility should be investigated by testing the mother’s serum or an eluate prepared from the infant’s RBCs against the biologic father’s RBCs. A negative DAT does not rule out the possibility of immune-mediated hemolytic anemia and may reflect a low-antigen density on fetal RBCs or low avidity of the offending antibody under the reaction conditions. However, when the infant’s DAT is negative, neonatal hyperbilirubinemia should not be attributed to ABO incompatibility; other possible causes of hemolysis should be evaluated.

Routine immunohematologic testing of infants born to women with negative antibody screens is not necessary, except to determine the need for RhIG for D– women. Regardless, many institutions continue to perform ABO and D typing and a DAT on all newborns; others selectively test infants born to group O, D+ mothers. The latter strategy, which is intended to identify infants at risk of developing ABO HDN, has an extremely poor predictive value and is not recommended because it will miss cases of hemolysis resulting from nonimmune causes. In one study, the positive predictive value of a routine cord DAT was only 23 percent and the sensitivity only 86 percent. Functional assays that evaluate antibody-mediated RBC destruction, such as the erythrophagocytosis assay, may be useful adjuncts to predict the severity of HDN but are technically demanding and suffer from
limitations similar to those of the DAT. Instead of reliance on laboratory screening for HDN or tests to predict its course, all newborns should be followed for jaundice in the first week of life with directed use of serum bilirubin and other laboratory testing, as appropriate.

**Treatment of HDN**

Hyperbilirubinemia does not occur before birth because the bilirubin that results from the immune-mediated destruction of fetal RBCs is transported across the placenta and eliminated by the maternal liver. After birth, however, serum bilirubin can accumulate to dangerous levels with ongoing hemolysis that poses a direct threat of brain damage because the infant’s liver function is not fully developed. Hyperbilirubinemia also occurs in otherwise healthy infants, resulting in neonatal jaundice which is the most common indication for treatment in newborn infants. The degree and duration of hyperbilirubinemia that place an infant at risk for bilirubin encephalopathy and kernicterus have been debated for years. Recently, a prospective, blinded study compared 140 term and near-term infants with total serum bilirubin levels of 25 mg/dL (428 µmol/L) or more to 419 control subjects and found no significant differences between the two groups in neurodevelopmental outcomes. However, the subgroup of infants with hyperbilirubinemia resulting from immune-mediated hemolytic disease had lower IQ scores than the control group. This observation corroborates previous studies that suggested that hemolysis enhances the risk of bilirubin-induced central nervous system injury, although it is not clear why. Factors potentiating bilirubin toxicity in the setting of hemolytic disease may include acid-base disturbances, asphyxia, free heme groups, and other byproducts of hemolysis or drugs that displace bilirubin from albumin and other plasma-binding protein. The recent clinical practice guidelines from the American Academy of Pediatrics treat infants with jaundice caused by immune-mediated hemolysis more aggressively than infants with physiologic jaundice, for every given serum unconjugated bilirubin concentration. Phototherapy is the mainstay of treatment for neonatal jaundice, but exchange transfusion will be necessary when phototherapy fails to adequately decrease bilirubin concentration or when the initial serum bilirubin concentration places the infant at high risk for kernicterus. Treatment of jaundiced preterm infants and jaundice on the first day of life in any infant requires individualized treatment decisions. Treatment decisions after the first 24 hours are guided by gestational age, bilirubin concentration, and the rate of its increase (> 0.5 mg/dL/hr), as well as the presence of comorbid factors such as hemolysis, asphyxia, significant lethargy, temperature instability, sepsis, or acidosis. Immediate exchange transfusion is recommended if an infant shows signs of acute bilirubin encephalopathy (e.g., hypertonia, arching, fever, high pitched cry) or if total serum bilirubin is 25 mg/dL (428 µmol/L) or more. Severe reactions related to the procedure have been reported in about 5 percent of infants and include citrate-related arrhythmias, bleeding caused by dilutional coagulopathy or thrombocytopenia, catheter-related infection, and bacterial sepsis. The mortality rate among term infants within six hours of exchange transfusion was estimated as 3 to 4 per 1000.

Infants who respond to phototherapy alone or those who receive intrauterine transfusion may not require exchange transfusion. However, these infants may need straight RBC transfusions (also referred to by some clinicians as booster or top-off transfusions) during the first 1 to 3 months of life for late-onset anemia resulting from ongoing low-grade hemolysis or erythropoietic suppression. In general, the transfusion decision should be guided not only by the Hb concentration but also by the reticulocyte count and, most importantly, by the infant’s condition, particularly when the infant is lethargic, feeding poorly, or not thriving.

**Conclusion**

The advent of RhIG prophylaxis to prevent D alloimmunization in pregnancy represents one of the most significant medical advances in modern times, reducing the risk of D alloimmunization from 14 percent in the late 1960s to 0.1 percent with the routine use of antenatal and postpartum RhIG administration. Further improvements in prenatal care, noninvasive monitoring, and intrauterine transfusion have provided effective treatment to even the most severely affected infants with hydrops fetalis caused by anti-D or other clinically significant, maternal RBC antibodies. Women with RBCs of known partial D phenotypes may be at significant risk of alloimmunization and may benefit from RhIG administration. Noninvasive fetal diagnosis using maternal DNA is possible for predicting the presence of RHD, although
not yet routine in the United States. Doppler ultrasound, now in widespread use, allows for monitoring of affected pregnancies without the potential complications associated with amniocentesis or fetal blood sampling. Infants with hyperbilirubinemia associated with HDN are at greater risk of neurologic complications than infants without hemolysis, and the current practice guidelines from the American Academy of Pediatrics identify HDN as a significant risk factor that requires more aggressive treatment at any given level of hyperbilirubinemia than in the absence of hemolysis.

References
23. Singleton BK, Green CA, Avent ND, Martin PG, et al. The presence of an RHD pseudogene containing a 37 base pair duplication and a nonsense mutation in Africans with the Rh D-


Anne F. Eder, MD, Executive Medical Officer, American Red Cross, National Headquarters, Biomedical Services, 2025 E St NW, Washington DC 20006.

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Currently, the goal of testing for RBC antibodies is to use a method that will detect, if possible, all antibodies that are considered clinically significant and yet not detect antibodies of little clinical importance in transfusion or pregnancy. The focus of test method development has been on the more controllable variables of the first and second stages of agglutination. Tube test methods have been developed over the years to achieve shorter turnaround times for quicker test results and improved sensitivity, with the occasional negative impact on relevant results. The focus on improving efficiency through automation, and personnel resourcing challenges of the transfusion service, have led laboratories to select methods tailored to meet their needs. This review compares the newer methods used in the gel test and solid phase test with commonly used tube methods. Both of the newer methods were developed with future adaptation to automation in mind. Further literature reviews about antibodies detected in only one or two methods and their general lack of clinical relevance as well as the occasional rare examples that produce significant clinical effects on transfused patients are also discussed.

Table 1. Wanted and unwanted antibodies

<table>
<thead>
<tr>
<th>Wanted</th>
<th>Unwanted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rh</td>
<td>Cold-reactive alloantibody</td>
</tr>
<tr>
<td>Kell</td>
<td>Cold-reactive autoantibody</td>
</tr>
<tr>
<td>Duffy</td>
<td>Warm-reactive autoantibody</td>
</tr>
<tr>
<td>Kidd</td>
<td>Inconclusive</td>
</tr>
<tr>
<td>S/s</td>
<td>HTLA</td>
</tr>
<tr>
<td>Other blood group antibodies</td>
<td>Anti-D (passively acquired)</td>
</tr>
<tr>
<td>that have caused hemolytic transfusion reactions</td>
<td></td>
</tr>
</tbody>
</table>

Approaches to improving antibody screen test sensitivity and specificity relate to the ability to manipulate and control important variables in the first stage of agglutination (antigen concentration, temperature, incubation time, ionic strength, and pH) and, if possible, to affect certain aspects of the second stage of agglutination. The out-of-our-control variables are the antibody equilibrium constant (affinity) and the chemical bonding characteristics of the antigen and antibody.

Over time, new methods and reagents introduced with the intent to increase sensitivity have sometimes resulted in decreased specificity.

Methods of Testing

Tube test methods

Tube test methods have been the long-standing method for antibody screening since before the introduction of newer technologies for antibody detection, such as the gel test and solid phase testing. Various methods, such as the use of saline with two drops of serum, saline with increased serum-to-cell ratios (4–8 drops of serum to 1 drop of 2-4% reagent RBCs); bovine serum albumin (BSA) (22%, 30%, and polymerized); LISS RBC suspension and LISS-additive methods; polybrene; PEG and PEG-LISS additive
methods; and proteolytic enzymes (ficin, papain, bromelin, etc.) have been used for antibody detection, some more effectively than others. Because of the effect of enzymes on certain RBC antigens, an enzyme method cannot be solely used for antibody screening and identification.

The saline tube test is easy to use and low in cost, but has poor sensitivity, particularly when used in protocols with shorter incubation times. Because the long incubation required to achieve good sensitivity conflicts with the need to provide blood and components in faster timeframes, the routine use of saline test methods has fallen to about 2 percent of laboratories in the United States.3,4 BSA methods contributed to an improvement in detection of antibodies; mainly those that are direct agglutinating, but only minimally improved the sensitivity of indirect agglutinating antibodies. The BSA method continued to be challenged because of the longer incubation times needed to detect antibodies of the weaker variety and its ability to detect unwanted reactivity.

Low and Messeter's introduction of the low-ionic strength saline method and the resultant LISS-based additives that came to market contributed the first significant improvement in antibody detection by allowing an increased rate of antibody uptake in a shorter timeframe.5 Additionally, these additives increased the amount of antibody uptake. The specificity of both BSA and LISS tests was made difficult by the use of polyspecific antihuman globulin (AHG) reagents that contributed to the detection of unwanted reactivity. Switching to a monospecific antiglobulin reagent such as anti-IgG improved specificity but produced a decrease in sensitivity of the BSA test.2 A less frequently used enhancement, polybrene, although quite popular with the few laboratories that used it routinely, offered speed and sensitivity but took some significant technique adjustment for laboratorians to become proficient in its use; therefore the test method never enjoyed much popularity. During the 1980s, LISS-based methods became increasingly popular; they remain today the most frequently used tube test method for antibody detection.

In 1987, Nance and Garratty introduced PEG as a new potentiator for the detection of RBC antigen-antibody reactions.6 A PEG/LISS method using this additive gained popularity as a routine antibody screen method in some institutions and as a routine or adjunct method for antibody identification in many others.

The use of proteolytic enzymes combined with another method able to detect enzyme-sensitive antibody specificities as a test for routine antibody screen test never gained much favor in the United States but could be found in routine use in some provinces of Canada, some European and some Asian-Pacific countries.7 The routine use of enzymes for antibody screening has significantly decreased globally with suggested changes in practice8,9; however, enzymes have gained popularity for antibody identification.

**Gel test**

The gel test (ID-Micro Typing System Gel Test, Ortho-Clinical Diagnostics) was developed by Lapierre and commercialized into a plastic card with six microtubes containing dextran acrylamide gel with antisera (specific antibody or AHG) or neutral buffer.10,11 The principle of this test is based on the use of gel to trap agglutinates. Measured volumes of RBCs in a low-ionic environment are added to plasma in the upper chamber of the microtubes. The gel cards are incubated, if necessary, and centrifuged. Anti-IgG contained in the gel microtube reacts with RBCs sensitized with antibody, producing agglutination. The gel traps the agglutinates, producing reactions that can be graded from 1+ to 4+. The reactions are stable and allow for the review of results for up to 24 hours after testing is completed. RBCs can be prepared for testing using a special RBC diluent, making the gel test flexible for use with any selected RBC. The lack of a washing step contributes to the sensitivity of the test. Ultimately, these features made the gel test ideal for automation.

Other column agglutination-based tests designed on similar test principles are available outside of the United States but are not discussed here.

**Solid phase RBC adherence test**

The solid phase RBC adherence test (SPRCA) was developed in the early 1980s through the work of Plapp et al.12 The principle of this test is based on the ability of an antigen or antibody to be bound to the solid matrix of a plastic microplate well. When the appropriate reactant (antigen or antibody) is added to the well, an antigen-antibody reaction occurs, resulting in adherence of RBCs or tagged RBCs to the well. Two different principles are used in the products commercially available in the United States.

The Capture-R Ready test (ImmucorGamma, Atlanta, GA) consists of a monolayer of RBC membranes bound to the surface of a polystyrene
microplate well. When serum or plasma and a LISS additive are added to the well with the monolayer of RBCs and incubated at 37°C, antibodies, if present, can interact with the bound membrane antigens. After washing to remove any unbound antibody, anti-IgG-coated indicator RBCs are added to the microwell. After centrifugation to allow interaction and binding of indicator RBCs with antibody bound to the membrane, a positive reaction is indicated by RBC adherence to the well surface. A negative test is indicated by the formation of a button of RBCs in the center of the microwell.

The principle of the other SPRCA test (Solidscreen, Biotest, Dreieich, Germany) follows the more traditional approach of the standard IAT. Antibody screen RBCs and serum or plasma are combined in a low-ionic environment and allowed to incubate. After washing to remove unbound antibody, anti-IgG is added. The specially activated (treated) bottom of the microwell allows RBCs sensitized with antibody and anti-IgG to adhere, once centrifuged, to the microwell via the Fc portion of the anti-IgG molecule.

The SPRCA tests are available in a microplate format; the plate frame will hold from 1 to 12 strips of 8 microwells or individual strips of 8 microwells, depending on the test system and automation used.

Both the gel test and the SPRCA test produce easily defined endpoints that allowed for their transition to an automated platform.

Transition to newer technologies

Overall, tube testing is considered to be the most intensive of the procedures used for antibody detection from the perspective of the number of steps and trained personnel required to perform the test. Generally, depending on the tube test method selected and the phases of reactivity that a facility chooses to perform, the number of procedural steps can range from 14 to 19. For SPRCA testing, the number of steps varies from 13 to 15 among the methods. Gel testing can be completed in 8 steps using commercially prepared RBCs and in up to 12 steps if RBC suspensions need to be manually prepared.

Tube test reagents are generally lower in cost as compared with the newer technologies. However, overall costs associated with the skill required, hands-on time, and non-value-added activity time are higher with tube test methods. The transformation of antibody detection test methods into newer technologies and the innovation of automation capability with these technologies have provided transfusion services with a pathway to efficiency and standardization with a well-balanced capability in antibody detection testing. This new-found capability along with the difficulty in finding experienced staff to fill the ever-increasing vacancies have propelled transfusion services to select tests and automation to fill the gaps created by these challenges. The automation of these methods has eliminated costs linked to the human intervention and manipulation required of tube tests.

Methods in Use

Table 2 shows the distribution of methods in use for antibody screening based on the College of American Pathologists Transfusion Medicine Survey for antibody screen test method reporting. The data clearly demonstrate the continued downward trend in the use of saline and albumin tube test methods, with LISS-based methods having the highest percentage of usage at 37 percent among tube methods. Methods that use newer technologies in manual-based systems have increased substantially from 30 percent in 2001 to 47 percent in 2005. Additional information concerning the usage of these technologies on automated platforms can be obtained from the aforementioned survey.

Performance Characteristics of Test Methods

As the antibody screen test became part of routine pretransfusion testing, the approach was to detect all antibodies with little regard to their relevance to clinical importance. Eventually, it was recognized that detecting all antibodies meant that significant amounts of effort and time were expended, with little additional benefit to the patient when providing timely, safe RBC components. Methods were then adapted, reagents developed, and practices changed that produced improvement to the balance of detection of wanted
and unwanted antibodies. Questions still remain today about the true relevance of some results that are found in testing. Deciding the relevance of what is found relies on the reputation of the serologic specificity without foundation or costly, time-consuming testing to prove the prediction of in vivo RBC destruction for the example of the specificity identified in the patient.

The comparison of methods for sensitivity and for specificity generally do not start from the same point. Many studies are done retrospectively comparing two different time periods. Other studies compare the new test method with what has been previously detected by the predicate method. These types of studies are not ideal because the cohort of stored samples is inherently biased toward the method of original detection. Ideally, prospective studies using consecutive samples are best. Obtaining sufficient numbers of samples is usually achievable for the specificity (true antibody negative population) aspect of the study, but obtaining enough samples to be able to demonstrate statistical significance and breadth of reactivity of specific antibodies for understanding the sensitivity of the new test is another matter. Therefore, laboratories and the manufacturers are left with the usual choice of selecting previously positive, stored samples as the way to evaluate sensitivity of the new test. This is a “one-sided look at the coin” as there is no way to go back to see if there was anything the existing method missed that the new method would have caught. Unfortunately, the ultimate correlation of these studies to in vivo transfusion experience is rare.

This review focused on the comparison of the newer technologies being used by both manual and automated systems with each other and with current commonly used tube methods. The studies that follow show the variation in the sensitivity and specificity of the various comparisons of test method performance as well as the uniqueness of antibody reactivity identified by specific methods.

In a study by Issitt et al. in 1997, 1184 samples were compared concurrently by PEG tube test and SPRCA (Capture-R Ready Screen, ImmucorGamma) methods. The data are presented in Table 3. Six percent (67/1184) of the samples tested demonstrated reactivity by the PEG tube test method while 11 percent (126/1184) demonstrated reactivity by SPRCA. The PEG tube test detected 61 percent of the wanted antibodies and 39 percent of the unwanted specificities while SPRCA found 35 percent of wanted and 67 percent of unwanted specificities.

<table>
<thead>
<tr>
<th>Method</th>
<th>Number of samples</th>
<th>Total number samples w/Abs</th>
<th>% Positive by method</th>
<th>Specificity of antibody detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG</td>
<td>37,832</td>
<td>3085</td>
<td>8%</td>
<td>71%</td>
</tr>
<tr>
<td>Gel</td>
<td>43,405</td>
<td>2715</td>
<td>6%</td>
<td>80%</td>
</tr>
</tbody>
</table>

In a study conducted in the same laboratory several years later, a retrospective review of two different years of testing, one in which the PEG test was used and one in which the gel test (ID-MTS Gel Test, Ortho-Clinical Diagnostics) was used, revealed the results shown in Table 4. In this study, during the year in which the PEG tube test was used, there were four transfusion-related hemolytic events; one mechanical or thermal-related, two with no assignable immune cause, and one delayed hemolytic transfusion reaction caused by anti-S. During that same time period, delayed serologic transfusion reactions (DSTR) were also evaluated. There were 21 missed antibodies (4 anti-E, 3 anti-c, 3 anti-Jk$, 3 anti-Fya, 2 anti-Jkb, 2 anti-D, 1 anti-c, 1 anti-Jka) in 19 patients with DSTR. In the year of testing using the gel test, there were no transfusion-related hemolytic events; however 9 patients had DSTR with 11 missed antibodies (3 anti-K, 2 anti-c, 2 anti-E, -Fya, 1 anti-D, and 1 anti-Jka).

A more recent study (Table 5) comparing gel (ID-MTS Gel, Ortho-Clinical Diagnostics) and SPRCA (Capture-R Ready Screen, ImmucorGamma) with a new indicator RBC coated with a different source of antibody directed at the Fc portion of anti-IgG compared two study time periods. There was a 2.3 percent higher reactive rate in SPRCA testing as compared with that using gel. The wanted antibody detection rates were similar. Overall, 4.2 percent of

<table>
<thead>
<tr>
<th>Method</th>
<th>Total number samples w/Abs</th>
<th>% Positive by method</th>
<th>Wanted</th>
<th>Unwanted</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG</td>
<td>3085</td>
<td>8%</td>
<td>71%</td>
<td>29%</td>
</tr>
<tr>
<td>Gel</td>
<td>2715</td>
<td>6%</td>
<td>80%</td>
<td>20%</td>
</tr>
</tbody>
</table>
patient samples tested by SPRCA had unwanted antibodies detected while 2.0 percent of patient samples tested by gel had unwanted antibodies detected. Fourteen percent of samples from D–obstetrical patients demonstrated passive anti-D by the gel test while 42 percent were reactive by SPRCA. Eliminating the detection of antenatal Rh immunoglobulin as an unwanted antibody reduced the unwanted rate to 0.5 percent for gel and 1.4 percent for SPRCA.

In a study comparing SPRCA from different manufacturers, 934 samples were tested using Capture-R Ready Screen (ImmucorGamma) and Solidscreen II (Biotest). This comparison demonstrates similarity in sensitivity and only minor differences in specificity (Table 6).

In a study that evaluated quantitative concentration of antibody using various test methods, known concentrations of Anti-D were tested using the same RBC (R2R2) by LISS-additive, PEG, and gel test methods. The lowest detectable level for both PEG and gel test methods was 5 ng/mL, while the lowest for the LISS-additive method was 11 ng/mL.

### Method-Dependent Antibodies

All methods of antibody detection have found some antibodies that fall into the category of being method-dependent antibodies. Method-dependent in this author's opinion indicates antibodies that are usually found by only one method. With the introduction of the newer methods, these method-dependent antibodies might be detectable with a second method but are undetectable by all other methods.

In early studies of the effects of low-ionic solution versus normal ionic solution, 195 serums that contained an example of anti-K were studied. Of these, 189 were detected by both normal and low-ionic strength solution test methods, 2 were detected only by the normal ionic strength solution test, and 4 examples were detected only by the low-ionic strength solution test.

Issitt et al. reported on the testing of 10,000 patient samples that were all initially determined to be negative for antibodies using a LISS-based test method. These patients were transfused, if required, with blood found to be compatible by the immediate spin crossmatch method used in their transfusion service. All 10,000 samples were subsequently tested using an enzyme-treated antibody screen test method. Thirty-six examples of enzyme-dependent antibodies in the Rh blood group system were found. Retrospective antigen typing of each of the units transfused to these patients was performed to determine which units were given to patients with the corresponding antibody identified by the enzyme test method. Of 19 patients receiving antigen positive units, 18 had no evidence of a transfusion reaction and had no change in their antibody's serologic reactivity. One patient whose sample demonstrated an anti-c had a delayed hemolytic transfusion reaction. In addition to the Rh antibodies of potential significance, 28 examples of clinically insignificant antibodies based on specificity and 216 cold benign autoantibodies were detected using the enzyme antibody screen test.

Additional studies of 29 LISS-negative, ficin-positive Rh antibodies demonstrated discordant results with other test methods. Nine of 25 of these antibodies tested by polybrene showed reactivity while 11 of 27 reacted by the PEG test method.

In studies by Contreras et al., 14 percent of 218 examples of anti-E were only detected using enzyme-treated screen RBCs; 21 percent of the 218 examples were from patients who were never transfused. In the same study, 61 examples of anti-D were detected in 23,000 obstetrical patient samples by the manual polybrene method only. The titer of these antibodies did not change throughout the pregnancy despite the fetuses being D+.
These examples of method-dependent antibodies appear for the most part to be of no clinical importance; however there is another “side of the coin” to consider. The following represent a few of the sporadic, more recent reports of such antibodies found in the literature or through anecdotal reports.

Callahan et al. had a patient sample in which they found only the presence of anti-E, despite testing with PEG and LISS-additive methods. Chronic transfusion of this patient with E- units found repetitive transfusion reactions with clinical hemolysis, yet with no additional detectable antibody. Upon encountering a severe reaction, further studies using SPRCA found anti-Jk. This antibody was not detectable in LISS, PEG, saline tube methods, or gel. Days later this antibody was still only detectable by SPRCA.

Barker et al. reported on a patient whose serum was negative when tested by LISS tube method, but who, when transfused, had a delayed transfusion reaction. The pretransfusion sample was retrospectively tested by LISS, PEG, and gel tests, all of which were negative. The posttransfusion sample demonstrated reactivity by PEG only and anti-Fya was identified. Approximately one week later, all three methods demonstrated anti-Fya reactivity.

Discussion

Generally, most of the tube methods and automated test methods currently in use have comparable sensitivity and specificity. The selection of the test method for routine antibody screening and identification should be based on the balance of sensitivity and specificity (relevance). One should consider the time, resources, and cost of those test methods that can detect high numbers of unwanted antibodies along with the potential impact of these factors on patient treatment. One must consider alternative approaches to routine methods or alternative methods of testing when clinical manifestations of problems occur that point to transfusion-related hemolytic events. The other consideration is that any routine method could produce unique unexpected reactivity that may have no clinical relevance; an alternate method may be required to avoid this type of reactivity.

The old adage that “no one method will detect all antibodies of clinical importance” is no different today in antibody detection methods using newer technologies in manual and automated systems than it was in tube test methods that transfusion services have used for the past 50 years. The uncontrollable variables that affect antigen-antibody reaction have not changed. The consideration that a test is “perfect” will leave one with the false sense of security that by using a single test method, all clinically significant antibodies will be detected. It is likely that every transfusion medicine professional will encounter at least one unpreventable delayed hemolytic transfusion reaction of significant consequences and several delayed serologic transfusion reactions in his or her career.

As Peter Issitt so eloquently articulated in a previous article in this journal, “It is apparent that the next major advance for in vitro testing will be related to a forecast of the antibody’s in vivo behavior and not the in vitro sensitivity of the method.”

The search for the “Holy Grail” of RBC antibody screening continues . . .

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Attention SBB and BB Students: You are eligible for a free 1-year subscription to *Immunohematology*. Ask your education supervisor to submit the name and complete address for each student and the inclusive dates of the training period to *Immunohematology*, P.O. Box 40325, Philadelphia, PA 19106.
Letter to the Editors

Anti-Cr\textsuperscript{a}: pregnancy and transfusion support

The management of a patient with anti-Cr\textsuperscript{a} in the UK hospitals presents a special challenge as it is impossible to locate compatible Cr(a–) RBCs from the UK donor population. The data regarding hemolytic transfusion reactions (HTR) associated with anti-Cr\textsuperscript{a} are very scanty.

Patients with anti-Cr\textsuperscript{a} have been transfused with antigen-positive RBCs uneventfully.\textsuperscript{1,2} Some transfusion facilities have provided Cr(a–) RBCs for patients with anti-Cr\textsuperscript{a} in which in vivo RBC survival studies demonstrated shortened RBC survival.\textsuperscript{3} Based on functional assay studies, evidence of the clinical significance of Cromer system antibodies is equivocal.\textsuperscript{4} Guidelines of the UK National Blood Service, based on the review by Daniels et al., recommended that weakly reactive Cr system antibodies are not considered clinically significant and Cr(a–) RBCs are not required for transfusion.\textsuperscript{4} Although there is no firm evidence that anti-Cr\textsuperscript{a} causes HTR, it has been advised that consideration should be given to provide antigen-negative RBCs for those with strong antibodies.\textsuperscript{4} There are only a few cases reported of anti-Cr\textsuperscript{a} during pregnancy, with no reported cases of HDN.\textsuperscript{5,6} Antibodies to Cr\textsuperscript{a} antigens are unique as the antibody titer may diminish, or become nondetectable during the course of the pregnancy. Weber et al. have demonstrated that the decrease in anti-Cr\textsuperscript{a} during pregnancy is caused by sequestration of anti-Cr\textsuperscript{a} by the placenta.\textsuperscript{6}

Transfusion management of a patient with anti-Cr\textsuperscript{a} in the European hospital presents a special challenge as Cr(a–) RBCs are exceedingly rare. We report our experience in planning transfusion support for a pregnant patient with anti-Cr\textsuperscript{a}.

A 31-year-old Somalian woman (gravida 5, parity 4) presented at 14 weeks' gestation. Her RBCs typed as group O, R, R, and anti-Cr\textsuperscript{a} (IAT titer = 32) was identified in her serum. The patient was seen again at 28 weeks' gestation, and at that time the titer of the anti-Cr\textsuperscript{a} had dropped to 4. We discussed transfusion support for the mother with the obstetricians. Review of the case records showed that the previous pregnancies were uneventful and the patient had not required any transfusion support. It was planned to deliver the baby by vaginal route. The patient was seen again at the clinic at 35 weeks' gestation. By that time, the IAT titer had further dropped to 1. With the drop in antibody level to a very low titer, we advised transfusing group O, R, R, K– RBCs instead of searching for Cr(a–) RBCs, should the patient require a transfusion. A healthy baby was delivered by vaginal route during the weekend at 39 weeks' gestation. There was no clinical evidence of HDN. Delivery was uneventful and the mother did not require RBC transfusion. Both the mother and the baby were discharged the next day. Attempts to obtain post delivery infant's sample were unsuccessful.

Anti-Cr\textsuperscript{a} does not cause HDN\textsuperscript{5,6} and it has been suggested that monitoring the mother throughout pregnancy is not necessary.\textsuperscript{5} The purpose of the serial antibody titration study in our case was to demonstrate and document the drop or disappearance of antibody. Confirmation of the drop in antibody to a very low titer allowed us to make a final decision not to search for rare Cr(a–) RBCs for transfusion support for this patient.

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Nay Win, MBBS, FRCP, FRCPath, CTM (Edin)
Consultant Haematologist
National Blood Service
75 Cranmer Terrace
London
SW17 ORB
United Kingdom

Malcolm Needs, CSci, FIBMS
Red Cell Immunohaematology
National Blood Service
75 Cranmer Terrace
London
SW17 ORB
United Kingdom

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If you are planning a state meeting and would like copies of Immunohaematology for distribution, please contact Cindy Flickinger, Managing Editor, 4 months in advance, by fax or e-mail at (215) 451-2538 or flickingerc@usa.redcross.org.
Letter to the Editors

A reminder that ZZAP reagent removes complement in addition to IgG from coated RBCs

A case was submitted to our laboratory for serologic classification of an autoimmune hemolytic anemia (AIHA). The patient's RBCs were spontaneously agglutinated (i.e., the 6% albumin control was reactive) after washing with room temperature and 37°C saline in preparation for the DAT. Thus, the RBCs needed to be treated with a sulphydryl reagent (DTT or 2-ME) to disperse the IgM-mediated autoagglutination. The patient's DTT-treated RBCs reacted strongly with anti-C3 and did not react with anti-IgG and a 6% albumin control; they reacted microscopically with anti-IgM. The patient's plasma strongly agglutinated (4+, titer = 4) adult group O RBCs at 37°C (prewarmed), but did not agglutinate cord or DTT-treated autologous RBCs. At 30°C, 18°C, and 4°C, the titer was 16, 128, and 128 with adult RBCs and 4, 32, and 64 with cord RBCs, respectively. The titer with autologous RBCs was consistently one tube less than that obtained with cord RBCs. In our AIHA serum screen, the DTT-treated autologous RBCs incubated at 37°C with the patient's serum did not react while autologous RBCs incubated at 18°C or DTT-treated autologous RBCs that were subsequently enzyme-treated were strongly agglutinated. Additional testing confirmed that the 37°C reactive agglutinin was IgM autoanti-I, not an alloantibody to an antigen not expressed or weakly expressed on cord RBCs. In this patient with hemolytic anemia, the serology was consistent with cold agglutinin syndrome (CAS) associated with moderate titer, high thermal amplitude antibodies.

As Dacie noted in 1962, cold agglutinin titers in patients with CAS are often lower with autologous RBCs than with allogeneic RBCs. This appears to be due to steric hindrance by complement (mainly C3dg) accumulated on the RBCs following exposure to anti-I. Typically, for our serologic classification of CAS, autologous RBCs after washing with 37°C saline give an accurate DAT result; i.e., the cold agglutinin is eluted from the RBCs and the 6% albumin control does not react. On occasion, as was noted in this case, the albumin control is still reactive washing with 37°C saline and sulphydryl treatment is used to disperse the spontaneous agglutination. When 37°C washing or sulphydryl treatment is required for the DAT, that same preparation of the patient's RBCs is used for other tests requiring autologous RBCs, e.g., titration studies.

Removing immunoglobulin from coated RBCs with ZZAP (DTT plus enzyme) is an alternative method to sulphydryl reagents alone for obtaining unagglutinated RBCs suitable for testing other than the DAT. ZZAP was originally reported as an effective reagent for dissociating IgG from RBCs of patients with warm AIHA but immunohematologists often forget that the reagent also removes IgM and complement from coated RBCs. Sulphydryl reagents when used alone dissociate pentameric IgM through cleavage of disulfide bonds connecting the monomeric subunits but do not remove immunoglobulin or complement.

To check our suspicions that the large amount of complement coating the autologous RBCs was blocking agglutination by the anti-I at 37°C, we treated the autologous RBCs with ZZAP reagent and retested with the patient's plasma at 37°C. The ZZAP-treated autologous RBCs were strongly agglutinated (3+) whereas the DTT-treated RBCs were not agglutinated. When tested with anti-C3, the ZZAP-treated autologous RBCs demonstrated a decrease of reactivity to w+ compared with 3+ reactivity with the DTT-treated autologous RBCs tested in parallel. The results of testing of RBCs from four other patients with either warm or cold serum autoantibodies or both confirmed abolished or decreased reactivity with anti-C3 and anti-IgG after the RBCs were treated with ZZAP.

In this case, the pattern of reactivity of the agglutinin in the initial titer and thermal amplitude studies had the appearance of an alloantibody at 37°C. DTT treatment alone appeared to have disassociated the IgM molecules that caused the spontaneous agglutination detected in the DAT but left C3 and at least some IgM monomers on the autologous RBCs. ZZAP treatment of the RBCs confirmed the autoantibody reactivity at 37°C, presumably because of the removal of C3 from the RBCs and enzyme enhancement of the anti-I reactivity. Enzyme treatment
alone would not have enabled testing of the autologous RBCs as enzymes do not abolish spontaneous agglutination.

References

Regina M. Leger, MSQA,MT(ASCP)SBB
American Red Cross Blood Services
Southern California Region
Pomona, CA

George Garratty, PhD, FRCPath
American Red Cross Blood Services
Southern California Region
Pomona, CA
To Contributors to the 2006 Issues

The journal depends on readers, authors, editorial board, peer reviewers, and our Penn-Jersey staff. We wish we could thank all of you personally, but doing so is not practical. Instead, we thank each of you as members of an honored group.

First and foremost, we thank the authors for their reviews, scientific articles, case reports, book reviews, and letters to the editors that come not only from the United States but from many countries of the world. This has given the journal an international flavor.

Our editorial board is a prestigious one and we depend on them, not only for peer reviews, but for guidance in policy and suggestions for improvements. Special thanks go to our medical editors, who review every article for medical content, and to our technical editors, who read every article for technical content. The current board is listed by name in the front of each issue of the journal.

Our peer reviewers did a wonderful job in 2006. In each December issue we list them by name with thanks to each.

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We also want to thank the office staff at Penn-Jersey, Marge Manigly and Judy Abrams, for their help in preparing the journal for press. They manage the manuscript submissions, keep up with subscriptions, and many other behind-the-scenes tasks. We also thank Lucy Oppenheim, our copy editor; and Paul Duquette, our electronic publisher.

Finally, thanks go to our readers, whose enthusiasm and interest in the journal make it all worthwhile.

Sandra Nance
Connie M. Westhoff
Editors-in-Chief

Cindy Flickinger
Managing Editor
Letter from the Editors

Changes in the journal

As 2007 begins, the editors take this opportunity to share some exciting changes that will occur in the journal. First of all, there will be a “changing of the guard” on the editorial board. We thank those board members who have served the journal so admirably over the years. We hope that they will continue to contribute to the journal as authors and peer reviewers. And we welcome new members to the editorial board.

Board members recognized for their vision and special contributions to making the journal possible will become members of the “Emeritus Editorial Board.” Without those individuals, the journal would not exist as we know it today.

We are introducing a new category of submissions, titled “Educational Forum.” The editors are soliciting submissions that illustrate serologic and clinical case histories with a focus on the resolution process to include the progressive steps from serology to clinical care, from bench to bedside. This category will provide and promote continuing education. Examples include an immunohematologic case that illustrates a sound investigative approach with clinical correlation, reflecting appropriate collaboration to sharpen problem solving skills. Annotated conference proceedings will also be accepted in this category. Submissions should be approximately 2000 to 2500 words and written in a progressive disclosure format. Headings may include:

• Clinical Case Presentation
• Immunohematologic Evaluation and Results
• Interpretation
• Recommended Therapy
• Discussion
• References—only pertinent
• Table(s), if applicable

We look forward to receiving submissions to this new category, to celebrating our emeritus board members, and to working with our new board members in the year ahead.

Sandra Nance
Connie M. Westhoff
Editors-in-Chief
ANNOUNCEMENTS

Meetings!

The AABB Immunohematology Reference Laboratory (IRL) Conference 2007 will be held March 23 through 25, 2007, at the Hyatt Regency in Albuquerque, New Mexico. Continuing education credits will be provided. Registration will begin in January 2007. For more information, contact the AABB Department of Meetings and Programs at (301) 215-6480 or, beginning in October 2006, visit the Web site at http://www.aabb.org >meetings and events >national and regional conferences.

June 7–8     Heart of America Association of Blood Banks (HAABB)
The spring meeting of the Heart of America Association of Blood Banks (HAABB) will be held June 7 and 8, 2007, at the Embassy Suites in Kansas City, Missouri. For more information, refer to the Web site at http://www.haabb.org.

Manuscripts: The editorial staff of Immunohematology welcomes manuscripts pertaining to blood group serology and education for consideration for publication. We are especially interested in case reports, papers on platelet and white cell serology, scientific articles covering original investigations, and papers on new methods for use in the blood bank. Deadlines for receipt of manuscripts for consideration for the March, June, September, and December issues are the first weeks in November, February, May, and August, respectively. For instructions for scientific articles, case reports, and review articles, see “Instructions for Authors” in every issue of Immunohematology or on the Web. Include fax and phone numbers and e-mail address with your manuscript.
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For further details visit:

http://www.blood.co.uk/ibgrl/MSc/MScHome.htm

or contact:

Dr Tricia Denning-Kendall,
University of Bristol, Geoffrey Tovey Suite,
TEL 0117 9912093, E-MAIL P.A.Denning-Kendall@bristol.ac.uk
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“Virginia Commonwealth University is an equal opportunity/affirmative action employer. Women, minorities and persons with disabilities are encouraged to apply.”

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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
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   a. One paragraph, no longer than 300 words
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   a. Introduction
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   c. Materials and Methods
      - Selection and number of subjects, samples, items, etc. studied and 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's names or hospital numbers.
   d. Results
      - Presentation of concise and sequential results, referring to pertinent tables and/or figures, if applicable
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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
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      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
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   c. Author information (see II.B.9.)
   d. Tables (see II.B.7.)

IV. LETTER TO THE EDITOR

A. Preparation

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2. Title (first word capitalized)
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What is a certified Specialist in Blood Banking (SBB)?

- Someone with educational and work experience qualifications who successfully passes the American Society for Clinical Pathology (ASCP) board of registry (BOR) examination for the Specialist in Blood Banking.
- This person will have advanced knowledge, skills, and abilities in the field of transfusion medicine and blood banking.

Individuals who have an SBB certification serve in many areas of transfusion medicine:

- Serve as regulatory, technical, procedural, and research advisors
- Perform and direct administrative functions
- Develop, validate, implement, and perform laboratory procedures
- Analyze quality issues, preparing and implementing corrective actions to prevent and document issues
- Design and present educational programs
- Provide technical and scientific training in blood transfusion medicine
- Conduct research in transfusion medicine

Who are SBBs?

- Supervisors of Transfusion Services
- Managers of Blood Centers
- LIS Coordinators
- Educators
- Supervisors of Reference Laboratories
- Research Scientists
- Consumer Safety Officers
- Quality Assurance Officers
- Technical Representatives
- Reference Lab Specialist

Why be an SBB?

- Professional growth
- Job placement
- Job satisfaction
- Career advancement

How does one become an SBB?

- Attend a CAAHEP-accredited Specialist in Blood Bank Technology Program OR
- Sit for the examination based on criteria established by ASCP for education and experience

Fact #1: In recent years, the average SBB exam pass rate is only 38%.
Fact #2: In recent years, greater than 73% of people who graduate from CAAHEP-accredited programs pass the SBB exam.

Conclusion:

The BEST route for obtaining an SBB certification is to attend a CAAHEP-accredited Specialist in Blood Bank Technology Program.

Contact the following programs for more information:

<table>
<thead>
<tr>
<th>Program</th>
<th>Contact Name</th>
<th>Contact Information</th>
</tr>
</thead>
<tbody>
<tr>
<td>Walter Reed Army Medical Center</td>
<td>William Turcan</td>
<td>620-782-6210; <a href="mailto:William.Turcan@NA.AMEDD.ARMY.MIL">William.Turcan@NA.AMEDD.ARMY.MIL</a></td>
</tr>
<tr>
<td>Transfusion Medicine Center at Florida Blood Services</td>
<td>Marjorie Doty</td>
<td>727-568-5433 x 1514; <a href="mailto:mdoty@fbsblood.org">mdoty@fbsblood.org</a></td>
</tr>
<tr>
<td>Univ. of Illinois at Chicago</td>
<td>Veronica Lewis</td>
<td>312-996-6721; <a href="mailto:veronica@uic.edu">veronica@uic.edu</a></td>
</tr>
<tr>
<td>Medical Center of Louisiana</td>
<td>Karen Kirkley</td>
<td>504-903-2466; <a href="mailto:kkirkl@lsuhsc.edu">kkirkl@lsuhsc.edu</a></td>
</tr>
<tr>
<td>NIH Clinical Center Department of Transfusion Medicine</td>
<td>Karen Byrne</td>
<td>301-496-8335; <a href="mailto:Kbyrne@mail.cc.nih.gov">Kbyrne@mail.cc.nih.gov</a></td>
</tr>
<tr>
<td>Johns Hopkins Hospital</td>
<td>Christine Beritela</td>
<td>410-955-6580; cberител@jhmi.edu</td>
</tr>
<tr>
<td>ARC-Central OH Region, OSU Medical Center</td>
<td>Joanne Kosanke</td>
<td>614-253-2740 x 2270; <a href="mailto:kosankej@usa.redcross.org">kosankej@usa.redcross.org</a></td>
</tr>
<tr>
<td>Hoxworth Blood Center/Univ. of Cincinnati Medical Center</td>
<td>Catherine Beiting</td>
<td>513-558-1275; <a href="mailto:Catherine.Being7@uc.edu">Catherine.Being7@uc.edu</a></td>
</tr>
<tr>
<td>Gulf Coast School of Blood Bank Technology</td>
<td>Clare Wong</td>
<td>713-791-6201; <a href="mailto:cwong@giveblood.org">cwong@giveblood.org</a></td>
</tr>
<tr>
<td>Univ. of Texas SW Medical Center</td>
<td>Barbara Laird-Fryer</td>
<td>214-648-1785; <a href="mailto:Barbara.Fryer@UTSouthwestern.edu">Barbara.Fryer@UTSouthwestern.edu</a></td>
</tr>
<tr>
<td>Univ. of Texas Medical Branch at Galveston</td>
<td>Janet Vincent</td>
<td>409-772-4866; <a href="mailto:jvincent@utmb.edu">jvincent@utmb.edu</a></td>
</tr>
<tr>
<td>Univ. of Texas Health Science Center at San Antonio</td>
<td>Bonnie Fodermaier; Linda Smith</td>
<td>SBB Program: 210-358-2807; <a href="mailto:bfodermaier@university-health-sys.com">bfodermaier@university-health-sys.com</a>; MS Program: 210-567-8869; <a href="mailto:smithla@uthscsa.edu">smithla@uthscsa.edu</a></td>
</tr>
<tr>
<td>Blood Center of Southeastern Wisconsin</td>
<td>Lynne LeMense</td>
<td>414-937-6403; <a href="mailto:Irlemense@bcsew.edu">Irlemense@bcsew.edu</a></td>
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