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Either Henri Matisse or Pablo Picasso once remarked, “Cezanne is the father of us all.” Although Paul Cezanne exhibited with the impressionists, assimilating their approaches to color and light as well as their freer, bolder brushwork, he ultimately broke from them, resurrecting an interest in form, which they rejected. He developed a fine obsession with simple objects, emphasizing outline while reducing and concentrating them to their essence in repeated studies. This laid the groundwork for modern art, particularly cubism, with its hallmark abstraction of objects into planes and simultaneous multiple perspectives. *Sugar Bowl, Pears and Tablecloth* features the rich color and attention to shape and space that characterize his work. This issue features a paper examining sugar moieties in the Dombrock blood group.

**On Our Cover**

David Moolten, MD
Seroprevalence of unexpected red blood cell antibodies among pregnant women in Uganda

K. Eipl, C. Nakabiito, K. Bwogi, M. Motevalli, A. Roots, L. Blagg, and J.B. Jackson

We conducted a population-based, cross-sectional study among pregnant women in Kampala, Uganda, to determine ABO and D blood types and to determine the percentage who have unexpected red blood cell (RBC) antibodies and their specificities. De-identified blood samples from routine testing of 1001 pregnant women at the Mulago Hospital antenatal clinics in Kampala were typed for ABO and D and screened for the presence of unexpected RBC antibodies with confirmation and subsequent antibody identification. Of the 1001 blood samples tested, 48.9 percent, 26.4 percent, 21.0 percent, and 3.8 percent tested positive for blood groups O, A, B, and AB, respectively. Of these samples, 23 (2.3%) were negative for D, and 55 (5.5%) showed initial reactivity with at least one screening RBC. The RBC antibody screen was repeated on these 55 samples, and antibody identification was performed at the Johns Hopkins Hospital Blood Bank in Baltimore, Maryland. Twenty-one of the 55 samples were confirmed to have evidence of agglutination. Nine of the 21 samples demonstrated the presence of clinically significant RBC antibodies with anti-S being the most common, 8 samples demonstrated the presence of benign or naturally occurring antibodies, and 4 had only inconclusive reactivity. This study revealed a relatively high frequency of D and a low frequency of demonstrable clinically significant alloantibodies that may cause hemolytic disease of the newborn or hemolytic transfusion reactions among pregnant women in Kampala, with anti-S being the most frequent antibody specificity. Immunohematology 2012;28:115–7.

Key Words: pregnant women, red blood cell antibodies, Uganda

Alloimmunization is the development of antibodies to an antigen detected on the cells of another member of the same species. Red blood cell (RBC) alloimmunization among pregnant women is not infrequent given maternal exposure to fetal RBCs having different paternal antigens. The presence of these alloantibodies puts the fetus and newborn at risk of hemolytic disease1 and places the mother and infant at risk for hemolysis if transfusion is needed. D has been found to be the Rh system antigen most commonly associated with hemolytic disease of the fetus and newborn (HDFN).1 Currently, the prevalence of D has not been well studied, nor has the prevalence of other unexpected RBC antibodies in pregnant women in Kampala, Uganda. One recent study in southwestern Uganda found a maternal alloimmunization rate of 2.2 percent,2 which is comparable to that found in a similar study conducted in Zimbabwe, which indicated a maternal alloimmunization rate of 1.7 percent.3 Given the varying prevalence of different RBC antigens throughout the world,4 further examination of this part of the world is important for clinical care. Therefore, we conducted a population-based, cross-sectional study to determine the ABO and D blood types among pregnant women in Kampala and to determine the percentage who have unexpected RBC antibodies and their specificities.

Materials and Methods

Study samples were drawn from 1009 de-identified blood samples collected in EDTA anticoagulant left over from routine testing at two antenatal clinics at Mulago Hospital. Eight of the 1009 total samples were found to have insufficient volumes of plasma for all tests to be carried out and were therefore not included in the analysis. All remaining samples were tested for ABO and D and screened for the presence of unexpected RBC antibodies. All blood typing and initial antibody screening were performed at the Uganda Core Laboratory of the Makerere University-Johns Hopkins University Research Collaboration, which provides diagnostic testing for patient care and clinical research studies at the Infectious Diseases Institute in Kampala. The plasma from all samples showing a positive screen for the presence of unexpected antibodies was frozen and shipped from the Uganda Core Laboratory to the Johns Hopkins Hospital Blood Bank in Baltimore, Maryland, for identification of antibody specificities. Shipment of samples was done in compliance with local and international regulations, by IATA certified laboratory personnel, and under the terms of a Materials Transfer Agreement approved by the Uganda National Council for Science and Technology as required. Both local and Johns Hopkins University institutional review board approvals for the study were obtained.

All leftover EDTA samples were stored at 4°C for a maximum of 30 days after collection. Blood typing and antibody screening were performed using protocols adapted from the Johns Hopkins Hospital Blood Bank. Forward and reverse ABO and D typing were performed on all samples. Forward ABO and D typing was performed using patient
RBCs and commercial reagents according to manufacturer’s instructions (anti-A, anti-B, anti-D, and Rh control; Immucor, Norcross, GA). Reverse ABO grouping was performed using sample plasma and commercial group A cells and group B cells according to manufacturer’s instructions (Referencell2, Immucor). Weak D screening was performed on all samples testing D negative on immediate spin using monoclonal anti-IgG and Coombs control cells according to manufacturer’s instructions (Immucor). Antibody screening was performed on all samples, using a commercial low-ionic-strength saline solution (LISS; Ortho Clinical Diagnostics, Raritan, NJ), commercial Screening Cell I and Screening Cell II (Immucor), and commercial anti-IgG and Coombs control cells by manufacturer’s instructions. The plasma of all samples that showed a positive reaction at any step during the antibody screening procedure was extracted and stored at −20°C for later shipment to the Johns Hopkins Hospital Blood Bank for confirmation of antibody specificities. At Johns Hopkins, samples were retested for unexpected RBC antibody identification by the Capture assay on an automated instrument (Galileo, Immucor) or by gel test (ID-MTS Gel, Ortho Clinical Diagnostics).

**Results**

A total of 1009 maternal blood samples were collected for ABO and D typing, and unexpected RBC antibody testing was performed between June and August 2011. Only sex and presumed pregnancy status were known, as all samples were de-identified before testing. Of the 1001 blood samples tested (after 8 had been excluded because they had insufficient plasma volume), 48.9 percent, 26.4 percent, 21.0 percent, and 3.8 percent tested positive for blood groups O, A, B, and AB, respectively, and 23 (2.3%) were negative for D (Table 1). Fifty-five (5.5%) samples showed initial reactivity with at least one screening RBC. The RBC antibody screen and identification on these 55 samples were performed at the Johns Hopkins Hospital Blood Bank. If sample volume was limited, only an antibody identification panel was performed. Twenty-one of the 55 samples were confirmed to have evidence of reactivity. Nine of the 21 samples demonstrated the presence of potentially clinically significant RBC antibodies with anti-S being the most frequent (see Table 2), 8 samples demonstrated the presence of benign or naturally occurring antibodies (3 anti-Le, 2 anti-M, 1 anti-N, and 2 cold agglutinins), and 4 had inconclusive reactivity.

**Discussion**

Our study revealed that approximately half of 1001 pregnant women tested at Mulago Hospital in Kampala were group O, and 26.4 percent, 21.0 percent, and 3.8 percent were groups A, B, and AB, respectively. Overall, 2.3 percent were D−, which is lower than the 7.1 percent found in black blood donor populations in the United States but comparable to the 3.6 percent recently reported from southwestern Uganda. The percentage of pregnant women with unexpected RBC antibodies was 2.1 percent, which is relatively low given the high fertility rate of 6.7 births per woman in Uganda, and is similar to the 2.2 percent rate found in southwestern Uganda. Pregnant women in Uganda are not routinely typed and screened, yet only 1 of 23 D− women demonstrated an anti-D. Overall 9 (0.9%) women demonstrated a potentially significant RBC antibodies thought to be potentially significant for HDFN. Anti-S was the most frequent RBC alloantibody, detectable in 7 (0.7%) women, followed by 1 each of anti-D and anti-K. Anti-S was also the RBC antibody with the highest prevalence in another study among pregnant women in southwestern Uganda at a rate of 0.6 percent.

| Table 1. ABO and D blood groups among 1001 pregnant women at Mulago Hospital |
|-----------------------------|---|---|---|
| ABO Group | Total (n) (%) | D− (n) (%) | D+ (n) (%) |
| O | 489 (48.9) | 14 (2.9) | 475 (97.1) |
| A | 264 (26.4) | 4 (1.5) | 260 (98.5) |
| B | 210 (21.0) | 5 (2.4) | 205 (97.6) |
| AB | 38 (3.8) | 0 (0) | 38 (100) |

| Table 2. Antibody specificities among 21 pregnant women at Mulago Hospital |
|-----------------------------|---|
| Antibody specificity | n* |
| Anti-S† | 7 |
| Anti-D† | 1 |
| Anti-K§ | 1 |
| Anti-Le*‡ | 3 |
| Anti-M*‡ | 2 |
| Anti-N*‡ | 1 |
| Cold agglutinins*‡ | 2 |
| Inconclusive reactivity | 9* |

*Total greater than 21 because 5 samples had inconclusive reactivity in addition to a specific red blood cell antibody.
† Clinically significant red blood cell alloantibodies.
‡ Clinically benign antibodies.
Maternal hemorrhage associated with childbirth is also significant in Uganda. A recent study in Uganda reported that of 55,803 live births, blood was requested for 185 of 229 women with severe maternal morbidity. However, blood was not available for 34 percent of these women as a result of either lack of blood in the hospital blood bank, lack of transport to the national blood bank, or lack of blood in the national blood bank. Although morbidity was not known to be caused by difficulty in obtaining compatible blood, antenatal screening for RBC antibodies would likely allow timely availability and compatibility of blood for transfusion for those women with identified clinically significant RBC antibodies.

The implications of our findings are limited given the de-identified nature of the specimens tested and lack of follow-up data on the newborns of these women. Consequently, this study only showed the presence of demonstrable alloantibody as opposed to the percentage of pregnant women with a history of alloantibody and those currently demonstrating alloantibody. Nevertheless, this study does give the frequency and specificities of demonstrable clinically significant antibodies that may cause HDFN or require compatible blood for transfusion among pregnant women and their neonates in Kampala and it demonstrates the feasibility of establishing type and screen testing for RBC alloantibody in a health care setting.

Acknowledgments

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Paroxysmal cold hemoglobinuria: a case report


A 15-month-old white male child was admitted to the pediatric intensive care unit with symptoms of upper respiratory tract infection, increased somnolence, pallor, jaundice, fever, and decreased activity level. The purpose of this case study is to report the clinical findings associated with the patient’s clinical symptoms and differential laboratory diagnosis.

**Key Words:** cold agglutinin syndrome (CAS), direct antiglobulin test (DAT), Donath-Landsteiner (D-L), hemolytic anemia (HA), LISS (low-ionic-strength saline), indirect antiglobulin test (IAT), paroxysmal cold hemoglobinuria (PCH)

**Case Report**

A 15-month-old white male child was admitted to the pediatric intensive care unit with a several-day history of upper respiratory tract infection symptoms, followed by increased somnolence, pallor, jaundice, and fever. He was tachycardic with fever and pale, and he had a decreased activity level, jaundice, and bilateral otitis media. There was no past history of transfusion or hemoglobinuria.

The patient’s laboratory results were a hemoglobin level of 4.4 g/dL, hematocrit of 13.2 percent, white blood cell (WBC) count of 21,000/μL (40 segmented neutrophils, 15 bands, 35 lymphocytes, 7 monocytes, 2 eosinophils), red blood cell (RBC) count of 1.12 million/mm³, and platelet count of 355,000/μL. The reticulocyte count was 1.5 percent (normal 0.5 to 1 percent). Examination of the blood smear revealed poikilocytosis, spherocytosis, polychromasia, and nucleated RBCs. Small clumps of RBCs were also seen on the blood smear with the presence of erythrocytophagocytosis. Chemistry results revealed an indirect bilirubin of 3.8 mg/dL (0.6 to 10.5 mg/dL), blood urea nitrogen of 14 mg/dL (5–30 mg/dL), creatinine of 0.3 mg/dL (0.5 to 1.5 mg/dL), and lactate dehydrogenase (LDH) of 578 IU/L (110–144 IU/L). A stat urine Gram’s stain was ordered, but no evidence of bacteria was reported. His urine was described as amber in color and clear with a trace of WBCs, squamous epithelium, and a small amount of bacteria and was positive for myoglobin. Urine chemistries showed elevated ketones, protein, and a small amount of blood. The patient was admitted with a diagnosis of acute intravascular hemolysis and possible pigment nephropathy and to rule out sepsis. He was started on oxygen by nasal cannula and given intravenous fluids at half maintenance rates with bicarbonate administered to alkalinize the urine to prevent crystallization of myoglobin or hemoglobin. His urine output was monitored, and fluids were given judiciously to prevent congestive heart failure. He was also started empirically on ceftriaxone. Blood and urine cultures were obtained and blood samples were sent to the blood bank for compatibility testing owing to the patient’s low hemoglobin level and symptomatic anemia.

**Results**

Blood bank serologic test results demonstrated the patient to be group O, D+. Results of antibody screening identified reactivity only after incubation at 37°C with low-ionic-strength saline (LISS). Six units were crossmatched, and all were found to be incompatible after incubation at 37°C with LISS. An initial antibody identification panel was performed. Reactions were only observed in the indirect antiglobulin test (IAT) after incubation at 37°C with LISS. There was no discernible specificity for the antibody. The patient’s autocontrol tube was also weakly positive after the incubation phase. At this point it was suspected that the patient might have a cold autoantibody, as the use of LISS can often enhance the reactivity of cold agglutinins. A cold antibody identification panel was performed using group O cells, adult I+, and adult ii cells. An autocontrol tube was also included. All cold panel cell tubes were found to be negative at immediate spin (IS) and after room temperature incubation with the exception of the patient autocontrol tube, which was weakly positive. The panel cells were incubated in the cold at 5°C. All of the cold panel cells were weakly reactive after incubation. A cold agglutinin titer was not performed because of the weak reactivity of the cold panel cells. The initial antibody identification panel was repeated using a polyspecific anti-human globulin (AHG) reagent. Results of the second antibody identification panel demonstrated reactivity only at the AHG phase of testing with a possible P1 specificity. The patient’s RBCs were also antigen tested and found to be P1+. Other clinically significant antibodies were ruled out using known typing antisera. Antigen testing was also performed on the incompatible donor units. Five of the six units were found
to be P1+. Additional crossmatched P1− units were found to be incompatible after incubation at 37°C with LISS. At this point the patient’s physician was contacted about the initial antibody workup results and the need to send specimens to a reference laboratory for further workup.

Given the child’s symptoms and previous medical history (respiratory illness), hematology, blood bank, and chemistry test results, the patient’s physician, based on his own experience in treating pediatric patients with autoimmune hemolytic anemias (AIHA), suspected that the patient might have a rare condition known as paroxysmal cold hemoglobinuria (PCH). The physician requested that a cold direct antiglobulin test (DAT) be performed. Results of the cold DAT testing revealed strongly positive reactions (3+) with the polyspecific and the monospecific anti-C3d reagents but a negative reaction with the monospecific anti-IgG reagent. An elution was not performed as a result of lack of IgG coating the patient’s RBCs. To confirm whether this was actually a case of PCH, the Donath-Landsteiner (D-L) antibody test was performed. Results of the D-L test were positive, demonstrating the presence of a biphasic hemolysin. Units originally crossmatched were also tested by the D-L method, and all exhibited evidence of hemolysis after the incubation phase at 37°C. Introduction of polyspecific AHG reagent into compatibility testing procedures also showed the presence of only complement coating donor cells at the AHG phase of testing. At this point in the workup it was highly suspected that the specificity of the autoantibody was not anti-P1 but anti-P because of the biphasic hemolytic nature of the antibody and the fact that individuals of the P1 phenotype usually do not make anti-P1. The patient’s physician was contacted again about the need to send additional specimens to a reference laboratory for further testing. The patient’s physician declined to draw any more specimens from the child because of the low hemoglobin level.

Focus was then shifted to finding compatible blood for the patient. The blood bank supervisor remembered that there was a large Amish community in Cleveland, Ohio, that would probably have P− blood. At this point, the American Red Cross (ARC) in Cleveland, Ohio, was contacted about the patient’s diagnosis and serologic status and the urgent need for blood transfusions. Three units of frozen P− RBCs were procured by the ARC in Cleveland, Ohio, and shipped to the blood bank. Segments were thawed, deglycerolized, and tested against the patient’s serum. All of the donor units were found to be compatible with the patient’s serum after incubation at 37°C with LISS using polyspecific AHG reagent and also by the D-L test method. Eight aliquots (~60 mL each) of deglycerolized RBCs were transfused within 12 hours of the patient’s admission with no adverse effects reported, and the patient’s hemoglobin level eventually stabilized at 13.0 g/dL.

Discussion

PCH is the rarest form of AIHA. It was first described in 1845 in a patient who experienced an acute hemolytic episode associated with reddish-black urine after exposure to the cold.1 In 1904, Donath and Landsteiner identified what they termed to be a “biphasic hemolysin” in blood that could be demonstrated in the laboratory.2 The antibody was demonstrated to bind to RBCs in vitro at low temperatures, activate complement, and produce RBC lysis after warming to 37°C. Historically, PCH has been linked to syphilis. The majority of cases involved chronic PCH in association with congenital syphilis.3 In 1954, PCH was identified in patients without syphilis and in children with febrile illness.4 In 1962, Dacie described PCH as having three distinct types: (1) chronic, syphilitic PCH (occurring in late-stage syphilis); (2) congenital syphilis (occurring in children); and (3) nonsyphilitic PCH, which appears either as an acute transient form, usually viral related, or a chronic idiopathic or autoimmune form.5 Dacie concluded that the demographics of this disease changed in that the chronic syphilitic form of PCH had experienced a marked decline as a result of successful antibiotic therapy in treating patients with syphilis. Today, PCH occurs primarily in young children after a recent viral illness and is most often acute and transient.

Clinical Presentation

PCH can occur at any age. However, it tends to occur in young children after the onset of a recent respiratory or gastrointestinal tract infection. Typically, patients present with fever, chills, and abdominal, back, or leg pain, as well as hemoglobinuria after cold exposure.6 These symptoms are primarily seen in the chronic form of PCH. In the acute transient form of PCH, signs and symptoms generally include pallor, jaundice, fever, and hemoglobinuria.7,8 The peripheral blood smear typically exhibits evidence of hemolytic anemia (poikilocytosis, spherocytosis, polychromasia, and nucleated RBCs). There may also be significant agglutination as well as erythrophagocytosis. The presence of RBCs that have been engulfed by neutrophils is considered to be a distinguishing finding (not diagnostic) in the peripheral blood smear in patients with PCH.9 This finding has been shown to occur in about 20 percent of cases of PCH and 80 percent of cases of acute transient PCH in children.7 The reticulocyte count is often elevated but can vary with the hemolytic process. Hemoglobin
values tend to range anywhere from 2.5 to 12.5 g/L, with a mean value of 6.6 g/L. Significant laboratory chemistry results include an increased indirect bilirubin, increased LDH, and decreased serum haptoglobin. The color of the urine will depend on the amount of intravascular hemolysis, and it may vary from amber to dark reddish-black. The DAT will usually be positive only with anti-C3. The D-L antibody test will be positive, indicating the presence of a biphasic hemolysin.

Etiology

The exact etiology of PCH is unknown. The disease typically occurs after recent onset of viral or bacterial infection. Viral infections that have been implicated in PCH include varicella, measles, mumps, Epstein-Barr virus, cytomegalovirus, adenovirus, and influenza A. Organisms implicated in bacterial infections include *Mycoplasma pneumoniae*, *Klebsiella pneumoniae*, *Escherichia coli*, and *Haemophilus influenzae*. It has also been reported after vaccination for measles. The actual stimulus for PCH appears to be a form of molecular mimicry; that is, viral or bacterial antigens share a structural similarity with certain blood group system antigens found on human RBCs. As a result, the body mounts an autoimmune response, producing antibodies capable of binding with these blood group system antigens. In classic PCH, the specificity appears to be toward P. Autoantibodies to other blood group system antigens (e.g., A, I, i, and IH) have also been implicated in cases of PCH.

Prevalence and Incidence

Most cases of PCH occur in young children. In 1989, Heddle reviewed previously published case reports on PCH in 42 pediatric patients with acute nonsyphilitic PCH. Heddle found that the mean age of diagnosis was 3.8 years, with a range from 15 months to 13 years. These findings were echoed in 1990 by Götsche et al., who reviewed 22 cases of nonsyphilitic pediatric PCH. Götsche et al. found that patient ages ranged from 8 months to 5.5 years, with a mean age of 31 months. There is a mild male sex predilection with PCH; the male-to-female ratio is approximately 2:1 to 5:1. No racial predisposition has been recognized for PCH. Several studies have been performed to assess the incidence of PCH in adults and children. In 1981, Sokol et al. studied 865 cases of AIHA; 17 (2%) were D-L antibody positive. Of these, 13 occurred in children younger than 10 years of age, 1 in a patient 32 years of age, and only 1 in a patient older than 40 years. All of the cases reviewed by Sokol et al. were described as being of the acute form with the exception of the one case occurring in the 32-year-old. By 1984, Sokol et al. had investigated a total of 42 cases of pediatric AIHA. They found that 17 (40%) were D-L antibody positive. They also reported two cases that occurred in adults having the D-L antibody, an acute form in a 62-year-old and a chronic idiopathic form in a 26-year-old. In 1990, Götsche et al. reviewed 599 cases of AIHA and found that 3.7 percent were D-L antibody positive. Of the 531 adults, none had D-L antibody. Of the 68 children, 22 (32.4%) were D-L antibody positive. All 22 cases were described as being acute and transient. Issitt and Anstee have reported the overall incidence of PCH in the general population (adults and children) at about 1 in 600,000 to 1 in 800,000 per year, which accounts for less than 1 percent of all cases of AIHA.

Pathophysiology

The autoantibody responsible for PCH is a biphasic hemolysin. The autoantibody binds to the patient’s own RBCs at lower temperatures (<30°C) in the body extremities and fixes complement. When the RBCs return to warmer areas of the body (37°C), complement is activated via the classical pathway, resulting in intravascular hemolysis. The antibody then elutes from the RBCs and returns to the circulation where it has the potential to again bind to RBCs at lower temperatures, causing additional paroxysms of hemolysis.

P1PK and Globoside Blood Group Systems

P1PK and Globoside are actually dual blood group systems. The P1PK system contains the P1 and P* antigens and the Globoside system contains the P antigen. Together, the two systems define five different phenotypes. Table 1 summarizes the two blood group systems’ antigens and the antibodies that can be produced on inheritance of a particular phenotype. The LKE phenotype does not appear in this table as it remains in the Globoside Collection. The LKE antigen is formed when the two blood group systems’ antigens and the antibodies that can be produced on inheritance of a particular phenotype. The LKE phenotype does not appear in this table as it remains in the Globoside Collection. The LKE antigen is formed when the two blood group systems’ antigens and the antibodies that can be produced on inheritance of a particular phenotype. The LKE antigen is formed when the two blood group systems’ antigens and the antibodies that can be produced on inheritance of a particular phenotype. The LKE antigen is formed when the two blood group systems’ antigens and the antibodies that can be produced on inheritance of a particular phenotype. The LKE antigen is formed when the two blood group systems’ antigens and the antibodies that can be produced on inheritance of a particular phenotype.

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Antibodies produced</th>
<th>European Prevalence</th>
<th>African Prevalence</th>
</tr>
</thead>
<tbody>
<tr>
<td>P</td>
<td>None</td>
<td>79</td>
<td>94</td>
</tr>
<tr>
<td>P</td>
<td>Anti-P1</td>
<td>21</td>
<td>6</td>
</tr>
<tr>
<td>P*</td>
<td>Anti-PP1P (Tj')</td>
<td>Rare</td>
<td>Rare</td>
</tr>
<tr>
<td>P</td>
<td>Anti-P</td>
<td>Rare</td>
<td>Rare</td>
</tr>
<tr>
<td>P*</td>
<td>Anti-P + Anti-P1</td>
<td>Rare</td>
<td>Rare</td>
</tr>
</tbody>
</table>

Donath-Landsteiner Antibody Test

The confirmatory test for PCH is the D-L antibody test. To perform this test correctly, a serum sample is needed. Plasma cannot be used because the anticoagulant binds calcium, which is important to the complement cascade. Owing to the complement dependency of the autoantibody to demonstrate biphasic hemolysis, the serum sample must be fresh and kept at 37°C, and the serum must be separated from the clot at this temperature. The test is performed based on procedures found in the American Association of Blood Banks Technical Manual as follows:19

1. Three sets of 10 × 75 mm tubes are labeled as follows: A1-A2-A3; B1-B2-B3; C1-C2-C3.
2. Ten volumes of the patient’s serum are added to each tube 1 and 2 of each set.
3. Ten volumes of fresh normal serum are added to each tube 2 and 3 of each set.
4. One volume of 50 percent suspension of P+ RBCs is then added to each tube.
5. The three “A” tubes are then placed in a bath of melting ice for 30 minutes and then at 37°C for 1 hour.
6. The three “B” tubes are placed in a bath of melting ice and kept in the melting ice for 90 minutes.
7. The three “C” tubes are placed at 37°C and kept at 37°C for 90 minutes.
8. At the end of the incubation periods, the tubes are gently mixed, centrifuged, and examined for hemolysis.

The D-L test is considered to be positive when the patient’s serum, with or without added complement, causes hemolysis in the tubes that were incubated first in the melting ice and then at 37°C and there is no hemolysis in any of the tubes maintained at 37°C or in the melting ice.

Results of the D-L testing for the patient in this case study are detailed in Table 2.

The D-L test does have some limitations in that it has a low sensitivity. Results are positive only when the serum antibody titer is high and the test is performed precisely. Falsely negative results may occur if there are low levels of antibody or complement present as a result of consumption during the hemolytic process in vivo. Falsely negative results may also occur if donor serum containing P or Pk is added as a source of complement as any globoside present may neutralize the D-L antibody.20

Differential Diagnosis

PCH can be confused with cold agglutinin syndrome (CAS) and warm autoimmune hemolytic anemia (WAIHA). Careful evaluation of serologic test results is necessary to obtain the correct diagnosis. Table 3 summarizes the serologic findings of all four categories of AIHA.21

Characterization of the immunoglobulin type as well as the thermal amplitude of the autoantibody or autoantibodies is important in obtaining the correct diagnosis. IAT and DAT test results must be carefully evaluated. If PCH is suspected, a D-L assay should be performed. If the D-L assay is positive, a diagnosis of PCH is confirmed. If the D-L antibody test is negative, other forms of AIHA should be investigated. Determination of the autoantibody specificity in AIHA is not always possible. In classic PCH, the autoantibody shows specificity for P. If P− RBCs are available, they can be tested against the patient serum. If the P− RBCs do not react, a specificity of autoanti-P is confirmed. If the P− RBCs react with the patient’s serum, alternative antibody specificities must be considered. Specificities to I, i, H, and IH have been reported in the literature.17

Conclusion and Treatment

The exact nature of the biphasic hemolysin in this case could not be fully determined. The autoantibody did appear to show P antigen specificity in the antibody identification studies that were performed. The patient’s typing results ruled out the possibility that the antibody specificity was directed toward the P1 antigen. P1+ individuals usually do not make antibodies toward P or GLOB blood group system antigens. However, there have been cases documented in the literature of autoanti-P1 causing in vitro hemolysis but not presenting as a biphasic hemolysin. The DAT was only positive with anti-C3, ruling out WAIHA and mixed-type AIHA as possible causes. Reactivity with anti-C3 was also shown to increase significantly after incubation in the cold. Antibody titers were not performed because of the weak reactivity of the cold panel

<table>
<thead>
<tr>
<th>Tube</th>
<th>Melting ice, 30 min + 37°C, 1 hour</th>
<th>Melting ice, 90 min</th>
<th>37°C, 90 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>Positive with hemolysis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A2</td>
<td>Positive with hemolysis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A3</td>
<td>Negative</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B1</td>
<td></td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>B2</td>
<td></td>
<td>Negative</td>
<td></td>
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<tr>
<td>B3</td>
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<td>Negative</td>
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<td>C1</td>
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<td>Negative</td>
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<td>C2</td>
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<tr>
<td>C3</td>
<td></td>
<td>Negative</td>
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</table>

PCH: a case report
cells. An elution was not performed because there was no detectable IgG coating the patient’s RBCs. The positive D-L antibody test coupled with the fact that only P– RBCs were compatible with the patient’s serum supports the explanation that this was a case of classic PCH involving autoanti-P. This D-L antibody was atypical: it displayed a broader thermal range than is usually seen in classic PCH. It was detectable only after incubation at 37°C with LISS and in the AHG phase of testing after the addition of polyspecific AHG reagent. Determining the specificity of autoagglutinins can be very time-consuming as well as expensive. This can be further complicated if the patient has been recently transfused. Most blood banks and transfusion services do not have the resources necessary to resolve such complex serologic problems. If AIHA is suspected and the patient has been recently transfused, samples should be sent to a reference laboratory for further serologic analysis.

The mainstay of treatment of PCH is to provide supportive care. As mentioned previously, this condition is usually acute and transient. If the anemia is mild and there is no renal involvement, hospitalization is usually not necessary. If the degree of anemia is severe, supportive care along with RBC transfusions may be warranted. In this particular case the anemia was quite severe, and the patient did require transfusions with several units of P– RBCs to stabilize his anemia and alleviate clinical symptoms. It was fortunate in this patient’s case that P– blood could be obtained. The prevalence of this phenotype is approximately 1 in 200,000 and it may take several days to find the blood. Most patients with severe anemia cannot wait for this rare blood to be found. The AABB Technical Manual, 17th edition, makes the following recommendation regarding transfusion of suspected PCH patients: “Transfusion of random donor blood should not be withheld from PCH patients whose need is urgent. RBCs negative for P should be considered only for those patients who do not respond adequately to random donor blood.” PCH patients should be kept in a warm environment to minimize the potential for D-L antibody binding and acute hemolysis. Wynn et al. recommends maintaining the patient’s room at 30°C until the hemoglobinuria subsides. RBCs and any other fluids given to the patient should also be warmed before infusion. If the PCH is recurrent or chronic, the patient should be given instructions to avoid cold exposure as much as possible.

The use of steroids in treating patients with PCH is controversial. There have been several large studies that have concluded that steroids are not beneficial and should be discontinued once a diagnosis of PCH is made. Other studies have suggested that steroids are effective in removing RBCs coated only with IgG, less effective in clearing RBCs coated with complement and IgG, and least effective in clearing RBCs coated with both IgM and complement. In 1971, Ries et al. reported a case of PCH in which the D-L antibody had an extremely high thermal range. The patient was initially treated with corticosteroids and the clinical symptoms improved. As soon as the corticosteroids were tapered off, the patient experienced recurrent hemolysis. Studies investigating the use of steroids concluded that their use should be reserved for patients who have recurrent PCH and cannot limit their

| Table 3. Differential diagnosis in autoimmune hemolytic anemia |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| **WAIHA** | **CAS** | **Mixed type** | **PCH** |
| **Autoantibody** | IgG (rarely IgM or IgA) | IgM | IgG and clinically significant IgM | Biphasic IgG (D-L antibody) |
| **Clinical presentation** | Variable; predominantly extravascular hemolysis | Mild anemia; hemagglutination and vascular obstruction; intravascular hemolysis may occur | Combined WAIHA and CAS; or WAIHA | Acute onset, severe anemia, intravascular hemolysis |
| **DAT** | IgG (90% cases), alone or with C3; C3 alone (7% cases) | C3 alone (>90% cases) | IgG and C3 (>70% cases) | C3 alone (>95% cases) |
| **Eluate** | Panreactive | Nonreactive | Panreactive | Nonreactive |
| **Antibody screen** | Panreactive (50% cases) | Negative unless performed in cold | Panreactive | Negative |
| **Special studies** | Extended RBC phenotyping; serum adsorptions to investigate underlying alloantibodies | High titer (≥64), High thermal amplitude (≥30°C) IgM | Low titer (<64), high thermal amplitude (≥30°C) IgM | D-L assay |
| **Typical autoantibody specificity** | Relative specificity for Rh antigens | li antigens | P antigen |

WAIHA = warm autoimmune hemolytic anemia; CAS = cold agglutinin syndrome; PCH = paroxysmal cold hemoglobinuria; DAT = direct antiglobulin test; RBC = red blood cell.
cold exposure. Another drug that has been successfully used to treat PCH is rituximab. Rituximab's mechanism of action targets the CD20 marker on B cells. B cells are removed, thus limiting antibody production. The use of rituximab has been found to be most effective in treating patients with refractory cold and warm AIHAs.

Splenectomy has not been shown to be an effective form of treatment for PCH because the spleen plays no major role in the pathogenesis of intravascular hemolysis.

References


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The production, serologic evaluation, and epitope mapping of ten murine monoclonal Dombrock antibodies

M. Grodecka, K. Wasniowska, G. Halverson, K. Yazdanbakhsh, M.E. Reid, and E. Lisowska

The Dombrock (Do) glycoprotein is a glycosylphosphatidylinositol (GPI)-linked membrane protein carrying Dombrock blood group antigens. There are no standardized typing reagents for Doa or Dob. We have developed ten different monoclonal antibodies (MoAbs) that are specific for Dombrock. The objectives of this study were to characterize these MoAbs serologically and determine the epitopes they recognize. MoAbs were generated by standard fusion methods. Mice were immunized with transfected human embryonic kidney 293T cells expressing high levels of Doa or Dob. The MoAbs were tested serologically with untreated and enzymatically or chemically modified red blood cells (RBCs). Serologic inhibition studies were performed with synthetic peptides corresponding to Doa and Dob amino acid sequences. Pepscan epitope analysis was done on an array of immobilized tridecapeptides corresponding to the full-length polypeptide. All ten antibodies were serologically specific for Dombrock. Eight of the antibodies recognized epitopes that were resistant to treatment with ficin, pronase, α-chymotrypsin, and neuraminidase, but sensitive to trypsin and 0.2 M dithiothreitol (DTT). Five have anti-Doa-like specificity. The epitope recognized by MIMA-52 was neuraminidase sensitive, and MIMA-127 epitope recognized a DTT-resistant, linear epitope 90QKNYFRMWQK99 of the Dombrock polypeptide. MIMA-127 was the only one of the ten Dombrock MoAbs mapped to a specific sequence of the Dombrock glycoprotein; the other nine MoAbs did not provide a specific peptide binding pattern. The other MoAbs could not be mapped as they most likely recognize nonlinear, conformation-dependent epitopes, as is evident by their sensitivity to reduction of disulfide bonds by DTT. The dependence of some epitopes on antigen glycosylation is also a possibility. Immunohematology 2012;28:124–9.

Key Words: Dombrock, hemagglutination, monoclonal antibodies, Pepscan analysis, synthetic peptides

The Dombrock (Do) blood group glycoprotein (CD297) is an interesting glycosylphosphatidylinositol (GPI)-linked component of human erythrocyte membranes, identified as a polymorphic member of the adenosine diphosphate (ADP)-ribosyltransferase gene family.1,2 The Dombrock glycoprotein carries the polymorphic Doa and Dob antigens and other high-prevalence antigens: Gregory (Gya), Holley (Hy), Joseph (Joa), DOYA, DOMR, and DOLG.3–7 Rare human antibodies to the Dombrock antigens, although only weakly reactive in vitro, have caused acute and delayed hemolytic transfusion reactions.8–10 The poor reactivity of the antibodies and the lack of fully characterized antisera and commercial red blood cells (RBCs) have created problems for resolving serologically difficult patient samples. Considering the importance of using well-defined antibodies in the studies on the Dombrock glycoprotein, we have produced several novel murine monoclonal antibodies (MoAbs) by immunizing mice with human embryonic kidney (HEK) cells that were transiently transfected to express the Dombrock antigens Doa or Dob. These antibodies were evaluated for their serologic specificity and were subjected to Pepscan epitope mapping and to peptide inhibition studies.

Materials and Methods

Transfection and Expression of Dombrock in HEK 293T Cells

The full-length cDNA coding for the Doa and Dob transcripts was cloned in pIRES2-EGFP (pDI2E) vector (Clontech, Mountain View, CA), allowing the simultaneous expression of green fluorescent protein (GFP) and the Dombrock gene.2 HEK 293T cells were transiently transfected with 10 μg of Doa and Dob cDNA expression constructs by the calcium phosphate transfection method and analyzed for surface expression using anti-Gya by flow cytometry as described previously.11 Briefly, 10⁶ transfected HEK 293T cells were incubated for 30 minutes at 37°C with anti-Gya and, after several washes, were stained with phycoerythrin (PE)-conjugated horse anti-mouse IgG (H+L) (Vector Laboratories, Burlingame, CA), washed, and analyzed by flow cytometry (Canto, Becton Dickinson, San Jose, CA). On average, Dombrock expression was detected by flow cytometry on approximately 50 percent of transfected cells, consistent with a transfection efficiency of 50 percent (data not shown).

Hybridoma Production

HEK cells transiently transfected with Dombrock cDNA corresponding to Doa or Dob were used to immunize BALB/c
mice three times during a 6-week period by intravenous (IV) injection of $2 \times 10^6$ tHEK cells plus a murine CpG adjuvant.\textsuperscript{12} Once adequate immunization was achieved, as demonstrated by hemagglutination of antigen-positive human RBCs, polyethylene glycol (PEG; Sigma Chemical Company, St. Louis, MO) fusions were done between the mouse splenocytes and X63-Ag8.653 mouse myeloma fusion partner cells. Briefly, the mouse splenocytes were mixed with the fusion partner X63.Ag8.653 cells at a ratio of roughly 3:1 and washed with phosphate-buffered saline (PBS) without calcium ($\text{Ca}^{2+}$) or magnesium ($\text{Mg}^{2+}$) (GIBCO/Invitrogen, Grand Island, NY). Fusions were achieved by the addition of 1 mL of PEG (with 5% DMSO; Sigma) dropwise slowly for 60 seconds with constant mixing followed by constant mixing at 37°C for 90 seconds. Then 1 mL of PBS (without $\text{Ca}^{2+}$ and $\text{Mg}^{2+}$) was added in a dropwise fashion for 60 seconds, repeated once, and then 1 mL of PBS was added for 30 seconds, repeated once, and then topped off to a volume of 15 mL with PBS. The cells were then allowed to rest at 37°C, followed by gentle centrifugation at 1000 rpm for 5 minutes and washed in warmed HAMS/DF-12 culture medium (Sigma) with 10 percent fetal bovine serum added (Hyclone Laboratories, Inc., Logan, UT). The cells were then plated onto sterile 96-well tissue culture trays (BD Falcon, Franklin Lakes, NJ) at 1.5 million cells per well in HAMS/DF-12 with hypoxanthine, aminopterin, and thymidine added (HAT; Sigma). After 7 days, 100 µL of additional culture medium with supplemental growth factors (StemCell Technologies, Vancouver, BC) was added to each well.

**Screening Fusion Trays for Antibody Secretion**

The plates were analyzed 14 days after fusion for the presence of antibodies by hemagglutination as follows: a 50-µL aliquot of supernatant fluid from each well was added to the corresponding wells of a 96-well v-bottom assay tray (Greiner Bio-One, Longwood, FL), 25 µL of a 1 percent RBC suspension of screening cells was added, and the trays were mixed on a titer plate shaker (Lab-Line Instruments, Melrose Park, IL). After 30 minutes the trays were observed for direct agglutination. The wells were washed by adding 100 µL PBS, mixing, and centrifuging at 1000 rpm for 30 seconds. The wash PBS was flicked off by inverting the tray over the sink and blotting dry on a paper towel. For indirect agglutination, 50 µL of anti-mouse IgG (The Binding Site, San Diego, CA) that had been diluted 1:100 in 6 percent albumin in PBS was added to each well, mixed, and incubated for 30 minutes, then observed for agglutination. The desired hybrids were then cloned three times by limiting dilution to assure monoclonality.

**Serologic Evaluation and Synthetic Peptide Inhibition Studies**

The final culture of each hybrid was grown in vitro, and the supernatant fluid was tested for reactivity by standard tube and gel hemagglutination methods using normal and chemically or enzymatically treated RBCs. Enzyme treatments with papain, trypsin, α-chymotrypsin, pronase, and neuraminidase and chemical treatment with 0.2 M DTT were done using standard methods described elsewhere.\textsuperscript{13} Hemagglutination inhibitions were performed in two stages: first incubating equal volumes (50 µL) of antibody with the synthetic peptide at a concentration of 1 µg/µL, mixing and incubating 30 minutes. Next, one drop of 3 percent RBCs suspended in PBS was added to each tube and hemagglutination was performed as described earlier to complete each test.

**Epitope Mapping Using Immobilized Peptides**

Eighty-one tridecapeptides (peptides consisting of 13 amino acids) synthesized on pins, which covered the sequence corresponding to the full-length membrane-bound Dombrock protein (i.e., amino acid residues 45–297, which lacks the leader sequence and phosphatidylinositol glycan-anchoring sequences), were obtained from Mimotopes (Clayton, Victoria, Australia). The selected decapeptides were synthesized using the epitope scanning kit purchased from Mimotopes. The decapeptides were synthesized on activated plastic pins fixed to the plate corresponding to a 96-well microtiter plate. The syntheses were done by stepwise elongation of the peptides from C- to N-terminus, following the manufacturer’s instructions.

Binding of the antibodies to tridecapeptides and decapeptides on pins was tested as described.\textsuperscript{14–17} The pins were consecutively immersed (with washing between the incubations) in the wells containing (1) the test MoAbs, incubated overnight at 4°C, (2) alkaline phosphatase–conjugated goat anti-mouse IgG (Dako, Copenhagen, Denmark), incubated for 1 hour at ambient room temperature, and (3) phosphatase substrate tablets (Sigma). The absorbance of the substrate solution samples was read at 405 nm in a microtiter plate reader (PerkinElmer 2300 multiplate reader, Melrose Park, IL). The test antibodies were used at dilutions of 1:20, if not stated otherwise.

**Results**

**Serologic Characterization of the Antibodies**

The MoAbs from ten hybridomas were Dombrock-specific as they reacted with all Dombrock-positive RBCs
but not with Gy(a–) and Hy– RBCs. Five were anti-Do<sup>b</sup>-like by hemagglutination as they reacted, after selected dilution in 6 percent albumin in PBS, with Do(a–b+) or Do(a+b+) RBCs, but not with several examples of Do(a+b–) RBCs. The remaining five recognized epitopes independent of the Do<sup>a</sup>/Do<sup>b</sup> polymorphism. The MoAbs, all isotype IgG2a, had similar reactivity patterns with enzymatically treated or chemically modified RBCs, with a few exceptions (Table 1).

### Table 1. Serologic reactivity of Dombrock monoclonal antibodies

<table>
<thead>
<tr>
<th>Monoclonal antibody</th>
<th>Clone ID</th>
<th>Specificity</th>
<th>Ficin</th>
<th>Papain</th>
<th>Trypsin</th>
<th>Chymotrypsin</th>
<th>Neuraminidase</th>
<th>DTT</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIMA-52</td>
<td>10B5</td>
<td>Do protein</td>
<td>NT</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>W</td>
</tr>
<tr>
<td>MIMA-53</td>
<td>8E9</td>
<td>Do protein</td>
<td>NT</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>S</td>
</tr>
<tr>
<td>MIMA-55</td>
<td>7H6/F7</td>
<td>Do&lt;sup&gt;b&lt;/sup&gt;</td>
<td>NT</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>S</td>
</tr>
<tr>
<td>MIMA-64</td>
<td>7H6/B3</td>
<td>Do&lt;sup&gt;b&lt;/sup&gt;</td>
<td>NT</td>
<td>R</td>
<td>S</td>
<td>W</td>
<td>R</td>
<td>S</td>
</tr>
<tr>
<td>MIMA-73</td>
<td>15D6</td>
<td>Do&lt;sup&gt;b&lt;/sup&gt;</td>
<td>NT</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>S</td>
</tr>
<tr>
<td>MIMA-98</td>
<td>7D10</td>
<td>Do protein</td>
<td>R</td>
<td>NT</td>
<td>S</td>
<td>W</td>
<td>R</td>
<td>S</td>
</tr>
<tr>
<td>MIMA-123</td>
<td>6B5</td>
<td>Do&lt;sup&gt;b&lt;/sup&gt;</td>
<td>NT</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>S</td>
</tr>
<tr>
<td>MIMA-125</td>
<td>5A11</td>
<td>Do protein</td>
<td>NT</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>S</td>
</tr>
<tr>
<td>MIMA-127</td>
<td>7C8</td>
<td>Do protein</td>
<td>R</td>
<td>NT</td>
<td>S</td>
<td>S</td>
<td>NT</td>
<td>R</td>
</tr>
<tr>
<td>MIMA-143</td>
<td>1G8</td>
<td>Do&lt;sup&gt;b&lt;/sup&gt;</td>
<td>NT</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>S</td>
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ID = identification; DTT = dithiothreitol; NT = not tested; R = resistant; S = sensitive; W = weaker reactivity after treatment. Bold font highlights the significant differences in MoAb reactivity.

The results of hemagglutination of protease-treated RBCs did not reveal any information about the epitopes that each MoAb recognized on the Dombrock molecule because it is not known which peptide bonds of the Dombrock polypeptide chain are hydrolyzed by the proteases used. For initial epitope mapping a panel of 81 tridecapeptides, covering the entire membrane-bound sequence of the Dombrock glycoprotein, was used (Fig. 1). Each tridecapeptide differed by three

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**Fig. 1** Amino acid sequence of the Dombrock glycoprotein. The blood group Do<sup>a</sup>/Do<sup>b</sup> polymorphism at amino acid residue 265 is shown. Five potential N-glycosylation sites are underlined. Epitope for the MIMA-127 antibody is highlighted in gray.
amino acids sequentially from the next and corresponded to amino acid residues 45–297 of the Dombrock glycoprotein (UniProt entry Q93070, Fig. 1). Of these ten MoAbs, only one antibody, MIMA-127, displayed a specific and strong binding to the peptides 87–100 (IEAQKNYFRMWQK) and 90–103 (QKNYFRMWQKAHL; Fig. 2A). To verify this observation, a set of nine decapeptides (shown in Fig. 2B), corresponding to the sequence of amino acids 86
\[ \text{DIEAQKNYFRMWQKAHL} \]

of Dombrock, was synthesized on pins. MIMA-127 bound most strongly to the decapeptide 90–99 and more weakly to the peptide 91–100 (Fig. 2B). The binding to the decapeptides was weaker than to the respective tridecapeptides (at 20-fold antibody dilution) and was increased when the more concentrated dilution of the antibody was used. Thus, our results confirmed that MIMA-127 recognizes the sequence 90
\[ \text{QKNYFRMWQK} \]

owing to conformational changes in the protein. Testing other antibodies at fivefold dilution did not increase their binding to any peptides.

The Do\(^\text{a}\) and Do\(^\text{b}\) blood group antigens differ by the presence of asparagine (Asn) and aspartic acid (Asp), respectively, at position 265 of the Dombrock glycoprotein. Our set of tridecapeptides contained Asn at this position, which contributed to a lack of binding of antibodies identified serologically as anti-Do\(^\text{b}\)-like. To test these antibodies, two sets of six decapeptides, covering the amino acid sequence 258–272 of Do\(^\text{b}\) and differing by the presence of Asn265 or Asp265, were used. However, no Do\(^\text{b}\)-like antibodies bound to these peptides (data not shown).

**Inhibition of Antibodies With Soluble Peptides**

Synthetic peptides corresponding to the amino acid sequence surrounding the Do\(^\text{a}\)/Do\(^\text{b}\) polymorphism at position 265 were used in inhibition assays. We used Do\(^\text{a}\)-specific peptides that were either 15
\[ \text{HPRGDWLQLRSTGNL} \]

Fig. 2 Mapping the MIMA-127. Pins made with the peptides listed were incubated with MIMA-127 followed by mouse IgG, then alkaline phosphatase, and the absorbance was measured at 405 nm. Binding to the tridecapeptides (A) and decapeptides (B) is shown. Dilution of the antibody solution used in the experiments is indicated. The sequence of the most active tridecapeptides and a decapeptide is shown and their common sequence fragment (epitope) is boxed.
16 (255VINMSYHRGDLWQLR270), or 21 (255VINMSYHRG
DVLQRLSTGNL275) amino acids in length. None of the
Dombrock MoAbs were inhibited by these synthetic peptides.

Discussion

We have produced ten different murine hybridoma cell
lines that secreted monoclonal antibodies specific to the
Dombrock glycoprotein, and five of them were anti-Do\(^b\)-like by
serologic testing. An attempt to identify the epitopes detected
by the MoAbs on the Dombrock glycoprotein was successful
only for MIMA-127. Our results showed that MIMA-127
recognizes a relatively long linear epitope formed by amino
cid residues 90–99 of the Dombrock glycoprotein (Fig.
2). The results indicated that peptide 89–98, which did not
contain Lys99, and peptide 91–100 without Glu90, were less
active than peptide 90–99. Lack of specific reactivity of other
antibodies with synthetic peptides suggests that the MoAbs
recognize conformation-dependent nonlinear epitopes. The
Dombrock glycoprotein contains five cysteine residues that
can form two disulfide bonds, resulting in the formation of
nonlinear epitopes.3 This conclusion is strongly supported by
our finding that all epitopes recognized by the MoAbs were
destroyed by treatment of RBCs with DTT (reduction of
disulfide bonds), except the epitope for MIMA-127, which was
DTT-resistant.

On the other hand, the lack of reactivity of anti-Do\(^b\)-like
MoAbs with immobilized and soluble peptides covering the
blood group polymorphism region may be attributable to the
absence of glycosylation on the synthetic peptides. There are
two potential N-glycosylation sites, at Asn257 and Asn274
residues flanking the Do\(^b\)/Do\(^b\) (Asn265Asp) polymorphism
site. The effect of glycosylation on peptidic epitope reactivity
can differ: an antibody can recognize both amino acids and
carbohydrates (glycopeptidic epitope), or an oligosaccharide
chain may interact with amino acid residues in a manner
that changes the local conformation of the polypeptide chain
and may result in exposing or burying the adjacent peptidic
epitope.18 One of these possibilities can also apply to the
reactivity of MIMA-52, which recognized a sialic acid–
dependent epitope.

A lack of interaction of other antibodies either with
immobilized or with soluble synthetic linear peptides and
sensitivity of the epitopes to DTT suggests that most Dombrock
monoclonal antibodies recognize nonlinear, conformation-
dependent epitopes. For some of the antibodies the effect of
antigen glycosylation on the reactivity of epitopes cannot be
ruled out. However, all antibodies obtained were shown to
be specific for the Dombrock protein and can be used for its
identification on the erythrocyte surface.

Despite several attempts using similar methods of
immunization with transfected cells, we were not successful
in producing a monoclonal antibody with Do\(^b\)-like specificity.
It may be worth attempting immunization with synthetic
peptides that are specific for Do\(^b\); however, conformation
and glycosylation appear to play important roles in antigen
recognition to stimulate the immune system for antibodies
with Do\(^b\) specificity.

Antibodies to Dombrock blood group antigens are typically
weakly reactive, and thus they are often misidentified. The
monoclonal antibodies reported in this manuscript are typical
in that they are not strongly reactive, and their use in serologic
testing must be carefully controlled. Testing in gel cards with
anti-mouse IgG has proved to be the best method for antigen
typing to identify antigen-negative donor units and to be
helpful in resolving serologically difficult patient samples by
phenotyping their RBCs.39 Though the mouse IgG gel cards
are currently not available in the United States, we developed
a method of converting neutral gel cards into mouse IgG gel
cards for this purpose.

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K.Y. in the transfection and expression studies of transiently
transfected HEK 293T cells. Hemagglutination and
hemagglutination/inhibition assays were performed by G.H.
Epitope mapping and Pepscan analysis were performed by
M.G., K.W., and E.L. The manuscript was drafted and revised
by all authors. The final approval of the manuscript was by
G.H., M.R., and E.L. The authors thank Robert Ratner for
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Dombrock blood group system, DOYA, ablates expression
of Do\(^a\) and weakens expression of Hy, Jo\(^a\) and Gy\(^b\) antigens.


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The molecular basis of the LU:7 and LU:–7 phenotypes

K. Hue-Roye and M. E. Reid

The Lutheran blood group system currently consists of 20 antigens that have been assigned ISBT numbers. Of these, all but LU7 have been associated with one or more nucleotide changes in \( LU \). The purpose of this study was to determine the molecular basis associated with the LU:–7 phenotype. We obtained a stored sample from one proband with this phenotype and sequenced \( LU \). Using genomic DNA, exons 1 through 15, and their flanking intronic regions, of \( LU \) were amplified by polymerase-chain reaction, and the products were sequenced. A homozygous novel missense nucleotide change of \( c.1274A>C \) in exon 10 of \( LU \) was observed. This change is predicted to encode Ala at position 425 in place of Glu of the consensus Lu glycoprotein. Based on these results, and an absence of a record of this change in the Single Nucleotide Polymorphism database, Glu425 in the Lu glycoprotein is required for expression of Lu7, and Ala425 is associated with the LU:–7 phenotype. This completes the molecular basis associated with all antigens known to be in the Lutheran blood group system. Immunohematology 2012;28:130–1.

Key Words: blood group antigen, blood group system, Lutheran

The first Lutheran antigen was described in 1945 and named after the donor whose blood stimulated the production of anti-Lu in a patient with systemic lupus erythematosus who had received multiple transfusions. It should have been named Lutteran, the donor’s name, but the writing on the label of the blood sample was misread as Lutheran. The Lutheran blood group system (ISBT 005) consists of 20 antigens carried on a single-pass type 1 membrane glycoprotein (CD239) with five disulfide-bonded, extracellular, immunoglobulin superfamily (IgSF) domains, which has adhesion properties and may mediate intracellular signalling. The Lutheran glycoprotein (597 amino acids) is encoded by \( LU \), which consists of 15 exons and is located on chromosome 19q13.32. \( LU \) also encodes, by alternative splicing of exon 13, the basal-cell adhesion molecule (B-CAM; 557 amino acids).\(^1,2\) Lutheran glycoprotein is found predominantly in the basal layer of the epithelium and endothelium of blood vessels and in a broad range of cells and tissues, namely brain, heart, kidney glomeruli, liver, lung, pancreas, placenta, skeletal muscle, arterial wall, tongue, trachea, skin, esophagus, cervix, ileum, colon, stomach, and gall bladder.\(^3\)

Two antigens that were originally placed in the Lutheran blood group system, LU10 and LU15, were declared obsolete, and there is no evidence that LU11 is inherited or carried on the Lu glycoprotein.\(^2\) LU3 is absent for red blood cells (RBCs) with the Lu null phenotype. Of the remaining 18 antigens, all but LU7 have been associated with one or more nucleotide changes in \( LU \). The LU:–7 phenotype was identified in 1972 and given the next number in the series of Lu(a–b+) people who had made an antibody compatible only with Lu(a–b–) RBCs. Only two people with this phenotype have been reported,\(^4,5\) and both are lost to follow-up. However, we obtained a stored sample from one of them. The race and ethnicity of the patient are unknown, but her plasma was reported to have an IgG anti-Lu7.\(^5\) The purpose of this study was to determine the molecular basis associated with the LU:–7 phenotype.

Materials and Methods

A blood sample from the only available sample obtained from an LU:–7 subject was recovered from liquid nitrogen storage, and genomic DNA was prepared using the QIAamp DNA Blood Mini Kit (QIAGEN, Inc., Valencia, CA). Exons 1 through 15 and their flanking intronic regions of \( LU \) were amplified by polymerase chain reaction (PCR), and the amplicons were sequenced by GENEWIZ, Inc. (South Plainfield, NJ).

Results

Analysis of the sequencing electropherograms for the LU:–7 sample showed a homozygous novel missense nucleotide change in exon 10 of \( LU \): c.1274A>C. This change is predicted to encode Ala at position 425 in place of Glu of the consensus Lu glycoprotein. This nucleotide change was confirmed by extracting DNA a second time, amplifying exon 10, and sequencing the amplicon.
Discussion

Based on these results, Glu425 in the Lu glycoprotein is required for expression of LU7, and Ala at position 425 is associated with the LU:–7 phenotype. This completes the molecular basis associated with all antigens known to be in the Lutheran blood group system (see Table 1).

To support the association of a variant with the phenotype, it is general practice to show the presence of the genetic variant in more than one unrelated individual with the same phenotype. Unfortunately for LU:7/LU:–7, this is currently not possible. However, the location of amino acid 425 in the second constant region in the extracellular domain of the Lutheran glycoprotein is consistent with the location of a detectable antigen. Furthermore, the c.1274A>c nucleotide change in LU has not been recorded (as of August 2012) in the Single Nucleotide Polymorphism database, showing that it is not found in homozygosity, or indeed heterozygosity, in the general population. These data support the claim that c.1274A>c underlies the LU7/LU:–7 polymorphism. As the nucleotide is on the LU*02 background, the provisional ISBT variant allele name is LU*02.-07.

Acknowledgments

We thank Sunitha Vege of the Laboratory of Immunohematology and Genomics, New York Blood Center, for sharing primer sequences that were used in this study and Robert Ratner for assistance in preparing the manuscript.

References


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Table 1. Lu phenotypes and their associated molecular bases²,⁸

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Exon</th>
<th>Nucleotide change*</th>
<th>Predicted amino acid change</th>
</tr>
</thead>
<tbody>
<tr>
<td>LU:2/LU:1</td>
<td>3</td>
<td>230G&gt;A</td>
<td>Arg77His</td>
</tr>
<tr>
<td>LU:2/LU:1</td>
<td>5</td>
<td>686G&gt;A</td>
<td></td>
</tr>
<tr>
<td>LU:4/LU:–4</td>
<td>5</td>
<td>524G&gt;A or 524G&gt;T</td>
<td>Arg175Gln or Arg175Leu</td>
</tr>
<tr>
<td>LU:5/LU:–5</td>
<td>3</td>
<td>326G&gt;A</td>
<td>Arg109His</td>
</tr>
<tr>
<td>LU:7/LU:–7</td>
<td>10</td>
<td>1274A&gt;C</td>
<td>Glu425Ala</td>
</tr>
<tr>
<td>LU:6/LU:9</td>
<td>7</td>
<td>824C&gt;T</td>
<td>Ser275Phe</td>
</tr>
<tr>
<td>LU:8/LU:14</td>
<td>6</td>
<td>611T&gt;A</td>
<td>Met204Lys</td>
</tr>
<tr>
<td>LU:12/LU:–12 (1)</td>
<td>2</td>
<td>99-104del GCGCTT</td>
<td>delArg34, Leu35</td>
</tr>
<tr>
<td>LU:12/LU:–12 (2)</td>
<td>3</td>
<td>419G&gt;A</td>
<td>Arg140Gln</td>
</tr>
<tr>
<td>LU:13/LU:–13</td>
<td>11</td>
<td>1340C&gt;T</td>
<td>Ser447Leu or Gln581Leu</td>
</tr>
<tr>
<td>LU:16/LU:–16</td>
<td>6</td>
<td>679C&gt;T</td>
<td>Arg227Cys</td>
</tr>
<tr>
<td>LU:17/LU:–17</td>
<td>3</td>
<td>340G&gt;A</td>
<td>Gli114Lys</td>
</tr>
<tr>
<td>LU:18/LU:19</td>
<td>12</td>
<td>1615A&gt;G</td>
<td>Thr539Ala</td>
</tr>
<tr>
<td>LU:20/LU:–20</td>
<td>7</td>
<td>905C&gt;T</td>
<td>Thr302Met</td>
</tr>
<tr>
<td>LU:21/LU:–21</td>
<td>3</td>
<td>282C&gt;G</td>
<td>Asp94Glu</td>
</tr>
<tr>
<td>LU:22/LU:–22†</td>
<td>2</td>
<td>223C&gt;T</td>
<td>Arg75Cys</td>
</tr>
</tbody>
</table>

*The ISBT allele names can be found at www.isbt-web.org.
†Expression of Lu22 is dependent on presence of Arg77.⁸
Knowledge of blood group distribution is important for blood transfusion management. A search of published data clearly demonstrates that blood group antigens can be distributed differently within different nationalities. Therefore, information about the antigen distribution of different blood group systems in the Lao population will be useful for providing better blood transfusion services in the Lao People's Democratic Republic (PDR), a country in Southeast Asia.

The Lao Red Cross National Blood Transfusion Centre is a government organization that controls policies and standards of blood transfusion services in the Lao PDR. Basically, the donated blood is typed for ABO and D and screened for four infectious markers: human immunodeficiency virus (HIV), hepatitis B virus (HBV), hepatitis C virus (HCV), and syphilis. Therefore, except for ABO and D, there is little known information about blood group antigen distribution in the Lao population. Lack of knowledge about antigen prevalence makes it challenging to predict finding compatible blood for transfusion in sensitized patients. Information about the distribution of blood group antigens in blood donors is helpful for estimation of the availability of compatible blood for patients who have developed antibodies through previous transfusions or as the result of pregnancy. In this study, RBC samples from volunteer Lao national blood donors of Lao Blood Centre were typed for A, A₁, B, M, N, S, s, Mi, P₁, D, C, E, c, k, Le⁺, Le⁻, Fy⁺, Fy⁻, Jk⁺, and Jk⁻.

Materials and Methods

Sample Size

Calculation of sample size was based on the prevalence of common negative antigens reported in Northeast Thai blood donors. The number of blood units collected at Lao Blood Centre was approximately 500 per month. The sample size estimation was calculated by the following formula:

\[ n = \frac{NZ^2}{\alpha^2 P(1-P)/e^2} \]

where \( n \) is sample size, \( N \) is population size, \( Z \) is units of the standard normal distribution, \( P \) is population proportion, \( \alpha \) is probability of type 1 error or significance level, and \( e \) is random error. The calculated sample sizes for donors negative for D, E, c, Le⁺, P₁, Mi, and Jk⁺ were 1324, 136, 142, 693, 107, 64, and 561, respectively. In considering budget and time limitations, this study proposed to perform antigen typing in 30 percent of blood donors available in a 3 month period, which was about 450 individual donors.

Specimens

RBC samples collected from Lao national (by interview) volunteer donors who donated between September 13 and November 12, 2010, and between January 2 and February 18, 2011, were used for blood group antigen typing.
### Typing Antisera

Anti-A,-A1, -B, -Ab, -D, -C, -E, -c, -e, -M, -N, -s, -S, -Fy, -Fy, -Jk, -Jk, -K, and -k and anti-human globulin (AHG) were obtained from commercial sources (Thai Red Cross National Blood Center for Anti-A,-A1, -B, -Ab, -D, -C, -E, -m, -N and AHG; Ortho Clinical Diagnostics, Raritan, New Jersey, for Anti-C, -e, -s, -s, -Jk, -Fy, -Fy, -k, and -k). Anti-Le, anti-Le, anti-Le, and anti-M, and anti-P1 obtained from blood donors’ plasma were kindly provided by Blood Transfusion Centre, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand.

Monoclonal typing antisera included Anti-A, -A1, -B, -A, -B, -D, -C, -E, -c, -e, -M, -N, -Jk, and -Jk. Anti-S, -s, -Fy, -Fy, -K, and -k were manufactured from pooled human sera (Ortho Clinical Diagnostics). Anti-M and anti-N were rabbit polyclonal immunized antisera.

### Methods

The antigen typing was performed by standard tube test following the providers’ instructions. Antigens C, c, D, E, e, M, N, Le, Le, P1, Mi, Jk, and Jk were typed by saline room temperature phase. Antigens S, s, Fy, Fy, K, and k were typed by saline antiglobulin phase. For each test of antigen typing, positive and negative RBC controls were included. The positive control cells were donors with single dose antigen expression, selected from panel cells (products of Thai Red Cross National Blood Center).

### Data Analysis

Data analysis for antigen frequencies and probability values was performed using STATA software version 10.0 (StataCorp LP, College Station, Texas). The prevalence among populations was compared using the t test. A probability value of less than 0.05 was considered significant.

This study was approved by the Lao National Ethics Committee for Health Research, dated September 10, 2010, No.325/NECHR and the Thai Human Research Ethics Committees, dated February 22, 2011, No.HE532464.

### Results

There were 464 blood samples included in this study. As shown in Table 1, the O blood group was the highest prevalence at 37.72 percent, followed by B at 35.56 percent, A at 19.83 percent, A, B at 6.47 percent, and A, B at 0.43 percent. The MNS system demonstrated two major phenotypes: M+N+S–s+, and M+N+S–s+ at 46.55 percent and 43.53 percent, respectively. Other phenotypes were found as follows: 5.17 percent M–N+S–s+, 2.59 percent M+N–S+s+, and 2.16 percent M+N+S+s+. In addition, Mi was also tested and found at 31.25 percent Mi(a+) and 68.75 percent Mi(a–). Regarding the P1PK system, it was discovered that most blood donors in this study were P1–, at 81.03 percent, and 18.97 percent were P1+. The phenotype prevalence in the Rh system was D+C+E–c+e+ at 60.43 percent, D+C+E+c+e+ at 19.13 percent, D+C+E+c+e+ at 10.22 percent, D+C+E+c+e+ at 6.09 percent, D+C–E+c+e+ at 1.30 percent, D+C–E+c+e+ at 0.43 percent. There were only two phenotypes in the Kell system, kk and Kk, with 99.72 percent kk and only 0.28 percent Kk. Prevalence of the Lewis blood group system was 50.43 percent Le(a–b–), 28.45 percent Le(ab–), and 21.55 percent Le(ab+). There were three phenotypes in the Duffy blood group system: Fy(a+b–), Fy(ab+b+), Fy(a–b+), with prevalence of 80.82 percent, 19.18 percent, and 0.22 percent, respectively. Four phenotypes were found in the Kidd system, Jk(a+b–), Jk(ab–), Jk(ab–), and Jk(b–), at 39.44 percent, 31.03 percent, 25.22 percent, and 4.31 percent, respectively. Table 2 presents the results of the typing, demonstrating the distribution of blood group antigens in Lao blood donors. Phenotype comparison with Thais and Taiwanese is illustrated in Table 3. Table 4 presents the prevalence of individual antigens of the MNS and Rh systems in several additional populations for comparison.

### Discussion

In the ABO system, the O blood group had the highest prevalence at 37.72 percent followed by 35.56 percent B, 19.83 percent A, 6.47 percent A and B, and 0.43 percent A and B.
The single antigens in the MNS system and Mi<sup>a</sup> also showed similar hierarchy for prevalence to that in Thai studies (Table 4).<sup>2,3,6,9,10</sup> The M antigen was the most common (84.4%–95%) followed by N (51%–63%). The s antigen was found in 100 percent, whereas antigen S was found in only 4.74 percent of Lao compared with Northeast Thais at 17.75 percent,<sup>2</sup> and 15.5 percent in Bangkok Thais.<sup>3</sup> In addition, when considering antigen Mi<sup>b</sup>, it is interesting to note that it was found at a higher prevalence in Lao than in Thais, 31.25 percent in Lao, but only 13.92 percent in Northeast Thais<sup>2</sup> and only 9.1 percent in Bangkok Thais (Table 3).<sup>3,10</sup> All three studies used anti-Mi<sup>b</sup> typing antisera from blood donor plasma. We also performed Mi<sup>a</sup> antigen typing in another 100 random samples and found 23 percent Mi<sup>(a+)</sup>. The data from this study indicate that there

### Table 2. Blood group antigen distribution in Lao blood donors

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Number</th>
<th>%</th>
<th>Number</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>24/464</td>
<td>5.17</td>
<td>440/464</td>
<td>94.83</td>
</tr>
<tr>
<td>N</td>
<td>228/464</td>
<td>49.14</td>
<td>236/464</td>
<td>50.86</td>
</tr>
<tr>
<td>S</td>
<td>442/464</td>
<td>95.26</td>
<td>22/464</td>
<td>4.74</td>
</tr>
<tr>
<td>s</td>
<td>0/464</td>
<td>0.00</td>
<td>464/464</td>
<td>100.00</td>
</tr>
<tr>
<td>Mi&lt;sup&gt;a&lt;/sup&gt;</td>
<td>319/464</td>
<td>68.75</td>
<td>145/464</td>
<td>31.25</td>
</tr>
<tr>
<td>P1</td>
<td>376/464</td>
<td>81.03</td>
<td>88/464</td>
<td>18.97</td>
</tr>
<tr>
<td>D</td>
<td>0/460</td>
<td>0.00</td>
<td>460/460</td>
<td>100.00</td>
</tr>
<tr>
<td>C</td>
<td>14/460</td>
<td>3.04</td>
<td>446/460</td>
<td>96.96</td>
</tr>
<tr>
<td>E</td>
<td>327/460</td>
<td>71.19</td>
<td>133/460</td>
<td>28.91</td>
</tr>
<tr>
<td>c</td>
<td>306/460</td>
<td>66.52</td>
<td>154/460</td>
<td>33.48</td>
</tr>
<tr>
<td>e</td>
<td>11/460</td>
<td>2.39</td>
<td>449/460</td>
<td>97.61</td>
</tr>
<tr>
<td>K</td>
<td>357/358</td>
<td>99.72</td>
<td>1/358</td>
<td>0.28</td>
</tr>
<tr>
<td>k</td>
<td>0/358</td>
<td>0.00</td>
<td>358/358</td>
<td>100.00</td>
</tr>
<tr>
<td>Le&lt;sup&gt;a&lt;/sup&gt;</td>
<td>332/464</td>
<td>71.55</td>
<td>132/464</td>
<td>28.45</td>
</tr>
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<td>Le&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>78.45</td>
<td>100/464</td>
<td>21.55</td>
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<td>Fy&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>0.22</td>
<td>463/464</td>
<td>99.78</td>
</tr>
<tr>
<td>Fy&lt;sup&gt;b&lt;/sup&gt;</td>
<td>375/464</td>
<td>80.82</td>
<td>89/464</td>
<td>19.18</td>
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<tr>
<td>Jk&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>300/464</td>
<td>64.66</td>
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<td>137/464</td>
<td>29.53</td>
<td>327/464</td>
<td>70.47</td>
</tr>
</tbody>
</table>

percent A<sub>1</sub>, 6.47 percent A<sub>1</sub>B, and 0.43 percent A<sub>2</sub>B. The data from this study confirmed the findings of previous studies in Thai blood donors.<sup>2,3</sup> However, these findings are quite different from data on Taiwanese, in whom the A and B blood groups were almost equal, 25.6 percent and 25.8 percent, respectively.<sup>4</sup> This study also confirmed that the second highest ABO blood group among Asians is B;<sup>2,6</sup> whereas in Whites it is A<sup>7</sup> (Table 3). The rare A<sub>1</sub>B subgroup was also found at 0.43 percent, confirming previously published data by Dean,<sup>6</sup> who stated that it is rare to find this phenotype in Asians. The presence of the A<sub>1</sub>B subgroup indicates that the A<sub>1</sub> gene is present among Lao, but this study did not find any A<sub>2</sub> blood group, which might be owing to the small sample size.

Regarding the MNS system, there were five phenotypes found: M+N–S–s+, M+N+S–s+, M–N+S–s+, M+N+S+s+, and M+N–S+s+. This is different from the study in Thai blood donors reported by Fongsarun et al.,<sup>3</sup> who discovered four more phenotypes: M–N+S++s++, M+N–S+s++, M+N+S+s++, and M–N+S+s+, which were also found in other Thai studies.<sup>2,9</sup> In addition, when comparing Lao and Northeast Thais, the percentages of the five phenotypes were close to each other. The difference was that whereas there were 1.87 percent M+N–S–s– and 3.27 percent M+N+S+s– found in Northeast Thais, these phenotypes were not found in Lao (Table 3).<sup>2</sup>
Blood group antigens in Lao

A high probability that anti-Mi^a may be stimulated in Mi(a–) multiply-transfused patients receiving blood from Lao donors compared with those receiving blood from Whites and Blacks because the prevalence of Mi(a+) in those populations is only 0.01 percent. This was confirmed by the Taiwan report, which indicated anti-Mi^a caused transfusion reaction and also hemolytic disease of the fetus and newborn. Therefore, antibody screening cells routinely used in Lao must include Mi(a+) cells.

In the P1PK system, P1+ was less frequently found at 18.97 percent, given the high prevalence of P1− at 81.03 percent. This prevalence is opposite to the prevalence of these antigens in Whites and Blacks, which are mostly P1, 79 percent in Whites and 94 percent in Blacks. The prevalence of the P1 antigen in the Lao population is not different from the prevalence found in Northeast Thais (p > 0.05), but it is significantly different from the prevalence among Bangkok donors and Taiwanese (p < 0.0001; Table 3). With the high prevalence of P1−, anti-P1 would be commonly detected and can be clinically significant if reacting at 37°C, but it would not be a problem for providing negative blood for transfusion.

This study did not find the D− phenotype in Lao blood donors (Table 4). This is different from studies in Northeast and Bangkok Thais, which found about 0.02 percent D− phenotype. This may be related to the small sample size, as D− in Lao blood donors is usually found to be about 0.01 percent. Other antigens in the Rh system, C, E, c, and e, have a similar distribution to that in Northeast Thais, and slightly different from the Bangkok reports. For example, the E antigen is found in 28.91 percent of Lao and 28.00 percent of Northeast Thais, but approximately 38 percent of Bangkok blood donors were E+. It is also interesting to note that the c antigen is found in fewer Asians, 31.67 to 51.6 percent, but found much more often in Whites and Blacks, 80 percent and 96 percent, respectively. With c and E antigens, based on the prevalence of negative and positive phenotypes found in Lao, alloimmunization of anti-E and anti-c may be commonly found in transfusion-dependent patients, but it would not be difficult to provide negative blood for transfusion. In terms of phenotype comparison, this study found that Rh phenotypes in Lao and Northeast Thais were similar. As shown in Table 3, phenotype D+C+E–c–e+ was found in 60.43 percent of Lao and 61.00 percent in Northeast Thais, and D+C+E+c+e− was found in 19.13 percent of Lao and 14.67 percent in Northeast Thais. In addition, some similarity and differences were observed in Bangkok Thais. Specifically, phenotype D+C+E–c–e+ was the one with the highest prevalence (60.43% in Lao, 55.6% in Bangkok Thais), whereas D+C–E+c+e− was found more often in Bangkok Thais than in Lao, 11.21 percent and 0.43 percent, respectively, and D+C–E+c+e− was found at 3.90 percent in Bangkok Thais, but only 1.30 percent in Lao.

In the Kell system, K was found at only 0.28 percent (Table 3). This prevalence is similar to those found in Thais and Taiwanese, but different from those in Whites and Blacks. Because the K antigen is rarely found, anti-K would be expected to be an uncommon antibody among Lao.

This study's data on the Lewis blood group are interesting (Table 3). First, the prevalence of Le(a−b+) was lower than the finding in Thais and Taiwanese, which was only 21.55 percent in Lao, but 40.65 percent in Thais and 67.30 percent in Taiwanese. Second, the phenotype Le(a+b−) at 28.45 percent in Lao was different from that in Taiwanese, which was only 10.3 percent (p < 0.0001). Theoretically, the low prevalence of Le(a−b+) indicates the possibly lower prevalence of the Se gene than in Thais. Lastly, Le(a−b−) at 50.43 percent in Lao was significantly higher than in Thais (p < 0.0001). With the high prevalence of Le(a−b−), naturally occurring antibodies of Lewis would be commonly found in Lao.

Table 3 illustrates that this study found there were three main phenotypes in the Duffy system, Fy(a+b−), Fy(a−b+), and Fy(a+b+), and the prevalence was close to the finding in Northeast Thais, but slightly different from that in Bangkok Thais. In addition, phenotype Fy(a−b−) was not found in Lao as well as being missing in Thais and Taiwanese, confirming the lack of this phenotype in Asians. In terms of individual antigens, Fy(a−) is very rare, but Fy(b−) is high in Lao, 0.22 percent and 80.82 percent, respectively. For the Kidd system, which is shown in Table 3, the prevalence of the Jk(a+b−) phenotype in Lao was 25.22 percent, which is close to those in Bangkok Thais, Taiwanese, and Whites. However, phenotype Jk(a+b+) had a prevalence of only 39.44 percent in Lao, but was found in 50.30 percent of Thais, 48.00 percent of Taiwanese, and 49

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Table 4. Comparison of prevalence of MNS and Rh blood group antigens in different populations

<table>
<thead>
<tr>
<th>Antigen</th>
<th>This study (%)</th>
<th>Northeast Thais (%)</th>
<th>Bangkok (%)</th>
<th>Taiwan (%)</th>
<th>Whites (%)</th>
<th>Blacks (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>94.83</td>
<td>94.39</td>
<td>83.90</td>
<td>79.70</td>
<td>79.00</td>
<td>74.00</td>
</tr>
<tr>
<td>N</td>
<td>50.86</td>
<td>56.06</td>
<td>62.80</td>
<td>67.40</td>
<td>70.00</td>
<td>75.00</td>
</tr>
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<td>S</td>
<td>4.74</td>
<td>10.75</td>
<td>15.50</td>
<td>8.70</td>
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<td>30.00</td>
</tr>
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<td>100.00</td>
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<td>90.00</td>
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<td>99.78</td>
<td>99.40</td>
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<td>92.00</td>
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<td>C</td>
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<td>91.60</td>
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<td>E</td>
<td>28.91</td>
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<td>38.60</td>
<td>43.50</td>
<td>29.00</td>
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<td>48.47</td>
<td>51.60</td>
<td>80.00</td>
<td>96.00</td>
</tr>
<tr>
<td>e</td>
<td>97.81</td>
<td>97.00</td>
<td>87.58</td>
<td>93.80</td>
<td>85.00</td>
<td>89.00</td>
</tr>
</tbody>
</table>
percent of Whites.\textsuperscript{1} The rare phenotype Jk(a−b−) was also found in 4.31 percent of Lao, but found in only 0.06 percent of Bangkok Thais,\textsuperscript{3} not found in Taiwanese,\textsuperscript{4} and found in less than 0.01 percent of Whites and Blacks.\textsuperscript{1}

The rare phenotypes found in Lao donors are those lacking any of the following antigens: C, e, and Fy\textsuperscript{a}, which were found at 3.04 percent, 2.39 percent, and 0.22 percent respectively. It is possible that C, e, and Fy\textsuperscript{a} with their immunogenicity may induce alloimmunization in transfusion-dependent patients and contribute to difficulties providing blood from Lao donors.

The study of blood group antigen distribution in Lao blood donors of the National Blood Transfusion Centre, Vientiane, the Capital City of the Lao PDR, had some limitations. First, there were problems associated with the availability of samples. At the time of this study, the number of blood donors was quite small, so it took time to get enough blood samples. Second, some antisera were expensive and time-consuming to obtain from a foreign country. The expense associated with purchasing of the antisera led to a reduction in the number of some antigens typed, especially K and k.

Notwithstanding these limitations, our recommendation would be to carry out further studies in the near future to build on the benefits from this study. Additional studies about the distribution of blood group antigens in other provinces might be needed. Moreover, further studies about secretor status and prevalence of unexpected antibodies in blood donors and transfusion-dependent patients would be beneficial for transfusion therapy in the country.

In conclusion, this study is the first report of blood group antigen distribution among Lao blood donors. The information obtained will be useful for improving transfusion therapy in the country, especially for estimation of the availability of compatible blood for patients who have developed unexpected antibodies.

**Acknowledgments**

This study was supported by the Centre for Research and Development of Medical Diagnostic Laboratories, Faculty of Associated Medical Sciences, Graduate School, Khon Kaen University, Thailand, and the Thai International Cooperation Agency.

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**References**


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Performance characteristics of two automated solid-phase red cell adherence systems for pretransfusion antibody screening: a cautionary tale

K. Quillen, J. Caron, and K. Murphy

Our institution has implemented two instruments, the Galileo and the Echo, that use different solid-phase red cell adherence assays for antibody screening in pretransfusion compatibility testing. During the initial implementation of these two instruments, we noticed very different problems: falsely positive results on the Galileo, and falsely negative results and lack of reproducibility on the Echo. Comparison of falsely positive antibody screen results from approximately equivalent numbers of samples run on the Galileo and samples tested by standard manual tube technique using low-ionic-strength saline enhancement showed a false-positive rate of 1.4 percent on the Galileo (defined as a positive screen with a negative panel). Testing using the Echo identified four cases of falsely negative antibody screens, (defined as a negative screen on a patient sample subsequently shown to be positive by the same method). In addition, we note a lack of reproducibility on the Echo, which emphasizes the importance of replicate testing during validation of automated antibody screening platforms. *Immunohematology* 2012;28:137–9.

**Key Words:** antibody screen, automated solid phase, specificity, reproducibility

Our institution was one of the early adopters of blood bank automation. In 2005, we implemented a large throughput, walkaway instrument (Galileo, Immucor, Norcross, GA), which uses a solid-phase red cell adherence (SPRCA) assay for antibody screening. Before that, we used manual tube method with low-ionic-strength saline (LISS) enhancement for routine pretransfusion antibody screening, reserving ficin and polyethylene glycol (PEG) for individualized workups. We continue to use tube testing with LISS for stat samples and during low-volume shifts.

The Galileo instrument offers walkaway automation with random access capability. In our validation testing, it was more sensitive than LISS, consistent with other reports. More recently, we added a second SPRCA assay instrument (Echo, Immucor), after an abbreviated validation that demonstrated 100 percent concordance between Echo and Galileo results on almost 150 specimens. During the initial implementation of these two instruments, we noticed very different problems: falsely positive results on the Galileo, and falsely negative results and lack of reproducibility on the Echo. These issues form the basis of this report.

**Materials and Methods**

**Galileo Testing**

For a 7-week phase-in period in 2006, samples for antibody screening (ABSC) were tested by manual tube method with LISS (tube, three-cell screen) or on the Galileo (two-cell screen), depending on the urgency of the transfusion or availability of a trained operator. Positive ABSC results on the Galileo were investigated with a panel on the instrument. Inconclusive Galileo panels, defined as those in which no antibody specificity was identified, were followed by a manual PEG panel. The Galileo ABSC result is considered falsely positive if the Galileo panel is negative or if the PEG panel is negative. Positive LISS ABSC results were followed up with LISS panels.

**Echo Testing**

Within the first few months of implementation of the Echo, in 2011, using the latest software (v 1.2.2) and a three-cell screen (Capture-R Ready-Screen [3], Immucor), we noticed four cases of negative antibody screens in patients known to have positive screens on recent prior specimens. Manual visual review of these negative results was equivocal. We noticed that one of the index samples was interpreted as equivocal on one run, which was manually interpreted as negative, then 3+ on a subsequent run of the same instrument using the same lot of reagents. We selected 29 additional samples with positive antibody screens (by Galileo testing), and reran each specimen multiple times on the Echo. The replicate testing was performed with the same reagent lot, typically within the same day. These samples were all less than 14 days old, and had been stored at 1° to 4°C, in accordance with the storage specifications of all patient samples at our institution.
**Results**

**Galileo Testing**

A total of 3297 ABSCs were performed, of which 1362 were done with Galileo testing. Of the 1362 Galileo ABSCs, 67 (4.9%) were positive. In 13 cases, LISS panels had been completed within the prior 7 days or the specimen was not sufficient in quantity for further evaluation. Evaluating the remaining 54 positive ABSCs on the Galileo, 35 (64.8%) were true positives and 19 (35.2%) were false positives (Table 1). Of the 19 falsely positive Galileo ABSCs (19/1349 = 1.4% false-positive rate), 14 had negative Galileo panels and 5 had inconclusive Galileo panels (the latter 5 cases had negative PEG panels). Of the 1935 manual ABSCs, 27 LISS panels were performed of which 100 percent were true positives. We did not observe any patient-specific factors such as pregnancy or medications common to the falsely positive Galileo results, but the reaction values typically were low, just above the numeric cutoff threshold. These falsely positive Galileo ABSC results were reproducible on repeat automated testing (data not shown).

**Echo Testing**

During the period of live testing, 4 missed positive antibody screens were discovered in a total of 3838 tests performed (4/3838 = 0.1%). These samples tested negative on initial Echo ABSC, but raised suspicion because of records showing recent prior positive ABSCs. They are listed in Table 2. Only 1 patient had received any red blood cell (RBC) transfusions between tests (the second patient, who is a chronically transfused sickle cell patient). The true incidence of missing a positive antibody screen is likely much higher, in cases when the patient does not have any recent positive ABSC result for comparison.

Of the 29 samples with known positive antibody screens on the Galileo selected for replicate testing, 5 were discovered to have inconsistent results. These are detailed in Table 3. The reaction strengths varied from 3+ to negative on a representative sample with anti-Fy⁺.

**Discussion**

The goal of pretransfusion compatibility testing is to optimize the detection of clinically significant RBC antibodies in a timely and cost-effective algorithm. Automated SPRCA platforms have been extensively used in antibody screening of blood donors, a population in which the prevalence of alloimmunization is expected to be low. PEG is generally considered to be the most sensitive method for tube indirect antiglobulin testing. Manual SPRCA was found to rival PEG in sensitivity and specificity; automated SPRCA on an earlier instrument (ABS2000, Immucor) had a lower sensitivity than PEG and manual SPRCA but better specificity.2,3 We found that one third of positive ABSC results on Galileo may be falsely positive, with no identifiable specificity on Galileo or PEG panel testing.4 In our anecdotal experience during the past 6 years, falsely positive Galileo ABSC results have recurred in the same patient on subsequent samples over time and have not been associated with the subsequent detection of true alloantibodies.

---

**Table 1. Positive antibody screen results on Galileo and using tube low-ionic-strength saline method**

<table>
<thead>
<tr>
<th></th>
<th>Galileo</th>
<th>Tube LISS</th>
<th>Total</th>
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<tbody>
<tr>
<td>ABSC</td>
<td>1362</td>
<td>1935</td>
<td>3297</td>
</tr>
<tr>
<td>Positive ABSC</td>
<td>67 (4.9%)</td>
<td>27 (1.4%)</td>
<td>94 (2.9%)</td>
</tr>
<tr>
<td>Panels completed</td>
<td>54</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>True positive</td>
<td>35</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>False positive</td>
<td>19</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

ABSC = antibody screen; LISS = low-ionic-strength saline.

**Table 2. Falsely negative antibody screen results on Echo**

<table>
<thead>
<tr>
<th>Known antibody specificities</th>
<th>Number of days from last positive screen</th>
<th>Result on prior ABSC (test method)</th>
<th>Result of repeat ABSC testing on Echo</th>
<th>Echo panel result (reaction strength)</th>
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<tbody>
<tr>
<td>E</td>
<td>2</td>
<td>2+ (Galileo)</td>
<td>3+</td>
<td>anti-E (3+)</td>
</tr>
<tr>
<td>E</td>
<td>60</td>
<td>2+ (Galileo)</td>
<td>2+</td>
<td>anti-E (2+)</td>
</tr>
<tr>
<td>E</td>
<td>4</td>
<td>2+ (Echo)*</td>
<td>2+</td>
<td>anti-E (3+)</td>
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<tr>
<td>K</td>
<td>19</td>
<td>1+ (Galileo)</td>
<td>3+</td>
<td>anti-K (3+)</td>
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</tbody>
</table>

ABSC = antibody screen
*Same reagent lot

**Table 3. Inconsistent antibody screen results on replicates of known positive specimens tested on the Echo**

<table>
<thead>
<tr>
<th>Antibody specificity</th>
<th>Number of replicates</th>
<th>Sequential results (number of replicates)</th>
<th>Antigen dosage on implicated cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fy⁺</td>
<td>2</td>
<td>3+, ?</td>
<td>Single dose</td>
</tr>
<tr>
<td>E, K, M</td>
<td>10</td>
<td>3+(x4), ?(x3), 1+(x2), 2+</td>
<td>Double dose</td>
</tr>
<tr>
<td>Jk⁺</td>
<td>11</td>
<td>3+(x9), ?, 3+</td>
<td>Double dose</td>
</tr>
<tr>
<td>Fy⁺</td>
<td>11</td>
<td>3+, 1+, ?(x2), 2+, neg(6)</td>
<td>Single dose</td>
</tr>
<tr>
<td>Jk⁺</td>
<td>11</td>
<td>3+(x8), ?(x2), 3+</td>
<td>Double dose</td>
</tr>
</tbody>
</table>

*Same patient, 2 different specimens.
? indicates an equivocal result reported on the instrument.
They have not been detected at all in populations at high risk of alloimmunization such as chronically transfused sickle cell patients.

Falsely negative antibody screen results are of greater clinical concern; they have been reported with the Echo and attributed to a defective image analysis algorithm. The incidence of falsely negative antibody screens depends on how cases are identified and the prevalence of alloimmunization in the population studied. It varies from 0.02 percent in a single-institution retrospective study to 0.5 percent based on proficiency testing results in the United Kingdom.

The lack of reproducibility on the Echo that we discovered has not been previously reported. Preanalytic factors such as lipemia and hemolysis were not implicated in the cases, and depletion of sample volume is unlikely because weaker (or even negative) results occurred in the middle of a sequence of replicates, rather than being skewed during the last run. Automation is a relatively recent innovation in the immunohematology testing arena compared with other areas of clinical laboratory testing, and the importance of replicate testing may not be widely appreciated. We submit this report as a cautionary tale that automated blood bank solid-phase platforms from the same manufacturer may have very different performance characteristics and to emphasize the importance of replicate testing during validation. Heightened alertness during the initial implementation of a new test method is required to correlate current results with patients’ prior antibody screen results, despite the well-known problem of antibody evanescence.

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Letter from the Editors

To Contributors to the 2012 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 2012. They are listed below, our thanks to each.

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We also want to thank Marge Manigly, our production assistant, and Sheetal Patel, our editorial assistant, for their help in preparing the journal for press. We also thank Christine Lomas-Francis and Dawn Rumsey, our technical editors; Mary Tod, our copy editor; Lucy Oppenheim, our proofreader; and Paul Duquette, our electronic publisher.

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

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</tr>
</tbody>
</table>

How does one become an SBB?

- Attend a CAAHEP-accredited SBB Technology program **OR**
- Sit for the examination based on criteria established by ASCP for education and experience.

However: In recent years, a greater percentage of individuals 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:
I. GENERAL INSTRUCTIONS
Before submitting a manuscript, consult current issues of Immunohematology for style. Number the pages consecutively, beginning with the title page.

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

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

III. EDUCATIONAL FORUM
A. All submitted manuscripts should be approximately 2000 to 2500 words with pertinent references. Submissions may include:
   1. An immunohematologic case that illustrates a sound investigative approach with clinical correlation, reflecting appropriate collaboration to sharpen problem solving skills
   2. Annotated conference proceedings
B. Preparation of manuscript
   1. Title page
      a. Capitalize first word of title.
      b. Initials and last name of each author (no degrees; all CAPs)
   2. Text
      a. Case should be written as progressive disclosure and may include the following headings, as appropriate
         i. Clinical Case Presentation: Clinical information and differential diagnosis
         ii. Immunohematologic Evaluation and Results: Serology and molecular testing
         iii. Interpretation: Include interpretation of laboratory results, correlating with clinical findings
         iv. Recommended Therapy: Include both transfusion and nontransfusion-based therapies
         v. Discussion: Brief review of literature with unique features of this case
         vi. Reference: Limited to those directly pertinent
         vii. Author information (see II.B.9.)
         viii. Tables (see II.B.7.)
IV. LETTER TO THE EDITOR
A. Preparation
   1. Heading (To the Editor)
   2. Title (first word capitalized)
   3. Text (written in letter [paragraph] format)
   4. Author(s) (type flush right; for first author: name, degree, institution, address [including city, state, Zip code and country]; for other authors: name, degree, institution, city and state)
   5. References (limited to ten)
   6. Table or figure (limited to one)

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