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On Our Cover

Marion Reid’s talents and passions are not limited to the laboratory. Her sketch of a fish swimming through sea grass, which is featured on the cover, is both elegant and concise. The same could be said about her innovative approach to immunohematology and her contributions over the years to both our knowledge and our methods. Her report on the Scianna blood group, also included in this issue, is no exception, utilizing molecular methods to further characterize the rare Sc null phenotype.

David Moolten, MD
With many thanks: Dedication to Marion E. Reid

This issue of *Immunohematology* is dedicated to Marion E. Reid, PhD, FIBMS, DSc (Hon.), immunohematologist *extraordinaire*. Why? Because, after an amazing career of more than 50 years in the field of immunohematology and transfusion medicine, Marion has decided that it is time for her to retire so that she can pursue her many other interests. To dedicate this, or any other, issue of *Immunohematology* to Marion is a very fitting tribute, as in 1976, when she was working for the American Red Cross (ARC) in California, she founded and became the coeditor of the *Red Cross Reference Laboratory Newsletter*, which morphed into the *Red Cell Free Press*, the precursor to the current *Immunohematology* journal.

Marion often said that hemagglutination is her “first language,” but as her numerous achievements attest, she quickly learned other “languages” and availed herself of new techniques such as Western blotting (immunoblotting) or the application of the polymerase chain reaction (PCR) to the study of blood groups. Her passion to honor unexpected results obtained by hemagglutination and to identify their cause in order to understand why they occurred has resulted in many discoveries and greatly increased our understanding of the nature of the red blood cell antigens we encounter daily. Her work was driven by caring about the patient and the transfusion medicine staff that helps the patient. She had (and still has) a great desire to share knowledge and help others grow; this is reflected by the many reviews, chapters, and books that bear her name. She is a constant contributor to *Immunohematology* (there are two papers in this issue with her name in the list of authors), and she served for many years on the editorial board and as a reviewer.

Marion’s career began in Brentwood, England, at the North East Metropolitan Blood Transfusion Service (NEMBTS), where she learned how to apply hemagglutination to the detection of blood group antigens and antibodies. After her start at the NEMBTS, Marion sought adventure in the United States and accepted a position as chief technologist of the Antibody Laboratory at New York Blood Center. This was followed by a further westward migration to California, initially to work at Spectra Biologicals in Oxnard. During her years in California, she held various reference laboratory positions with the ARC Blood Services in San Jose and with San Francisco General Medical Center. Her time in the reference laboratories honed her skills as an immunohematologist and led to the development of several techniques that are still routinely used, such as the use of sulfhydryl compounds to disperse autoagglutination, a rapid and simple method for freezing small volumes of erythrocytes in liquid nitrogen, and methods for adsorption and elution procedures.

In the mid-1980s, while at San Francisco General Hospital, she was encouraged to pursue a doctorate degree through a distance-learning program at the University of Bristol, England, with Dr. David Anstee and Dr. Mohandas Narla as her advisors. This fueled her interest in the glycophorins (GP), the structures that carry the MNS blood group antigens (GPA and GPB) and those that carry the Gerbich antigens (GPC and GDP), which became the focus of her doctoral thesis. Biochemical methods and the newly discovered hybridoma technology for making monoclonal antibodies were expanding our knowledge base, and Marion applied these to her studies. She returned to England in 1988 to work at the Blood Group Reference Laboratory in Bristol with David Anstee and his team, where her work further contributed to our understanding of the MNS and Gerbich blood group systems. But England could not hold on to her for long! In 1991 she was persuaded to return to New York Blood Center to take up the position of director of the Immunohematology Laboratory (from which
she retired in 2010) and head of the Immunoochemistry Laboratory, Lindsley F. Kimball Research Institute, a position she held until early 2013. At New York Blood Center, Marion had the opportunity to further pursue her passion, discovering new antigens and, with the development of DNA-based assays, elucidating the molecular basis of the expression of many antigens in several of the blood group systems. Among them were ENEV, ENDA (MNS); DAK, JAL, CEST, CELO (Rh); KALT, KTIM, KUCI, KANT (Kell); DOYA, DOMR (Dombrock); and SERF, ZENA, CROV, CRAM (Cromer). She was a pioneer in the development and application of PCR-based assays for the prediction of blood groups and was involved in the development of a high-throughput platform for testing blood group antigens by PCR-based assays. She quickly realized the power of combining DNA analysis with hemagglutination to resolve complex antibody investigations to find answers for longstanding puzzles. She was a part of the team that cloned ART4, the gene that encodes the Dombrock glycoprotein, and determined the molecular basis of Doa and Do8 antigen expression. Because of this, patients with anti-Doa or -Do8 are now able to be transfused with blood that can be confidently considered to be negative for the corresponding antigen, improving the transfusion outcome for patients with these clinically significant antibodies. Marion also spent many happy months figuring out the molecular basis of the high-prevalence antigens Hy and Joa and along the way discovered that the first two people to have made anti-Joa were actually Hy−, thereby explaining some of the confusion in differentiating Hy and Joa antibodies and antigens. Last year Marion was a part of the group that showed that the high-prevalence antigen Jrb is encoded by the gene ABCG2, which resulted in the creation of the JR blood group system; this system is reviewed by Marion and Lilian Castilho in this issue of the journal. Looking at Marion’s impressive list of publications, there seem to be few blood group systems that have not benefitted from her investigative skills.

Marion was a sought-after speaker for many meetings and by many associations, and she loved sharing knowledge and mentoring. Realizing that medical laboratory technologists were in danger of becoming extinct, she wanted to motivate young people to become interested in a career in transfusion medicine or medical laboratory sciences and also to become blood donors, as metropolitan areas always had blood shortages. To that end she spearheaded a team of friends and colleagues to develop an educational program that consisted of a series of interactive fun events about blood called “Bloodology.” The program, which was a great success, was presented at various museums to children of all ages and their families, and from it a series of informative Bloodology booklets arose; they are still available on the website of New York Blood Center today. She also wrote several larger tomes including three editions of the Blood Group Antigen FactsBook, and lately she turned her attention to the history of blood groups and the pioneers in the field, which resulted in the publication of The Discovery and Significance of the Blood Groups, which she coauthored with Ian Shine.

Marion has received many prestigious awards over the years, including three given by the AABB: the Ivor Dunsford Award in 1995, the Sally Frank Award in 2002, and the Emily Cooley Award in 2012. In 2006 she was honored with the International Women in Transfusion Award, an award that is bestowed jointly by the AABB, the International Society of Blood Transfusion, and the British Blood Transfusion Society. Marion was an enthusiastic collaborator with colleagues in New York and other parts of the United States, and throughout the world. No matter how busy, she was always ready to help, to listen, and to support a colleague, resulting in her worldwide network of friends.

Marion is not only passionate in her search for knowledge; she is just as passionate about enjoying all that life has to offer, from hiking in her favorite places, to painting and drawing, or to just spending time with friends. She impresses all with her boundless energy and her strength and determination to overcome even the toughest challenges that she encounters. Marion would often comment that she cannot believe how fortunate she has been: she was allowed to follow her passion because it has enriched our practice of immunohematology. She will be greatly missed by the community, but her legacy will continue in the people she taught, the staff members that she managed, and her colleagues around the globe. And, thanks to the Internet, wherever she may roam she is only a mouse-click away. It is with our thanks and great admiration that we declare this issue of Immunohematology the “Marion Reid issue,” and we wish her a happy, healthy, long, and fulfilling retirement.

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New York Blood Center
Long Island City, NY
Thermal amplitude test

C. Hopkins and T.K. Walters

The thermal amplitude test is performed to determine the reactivity of a cold autoantibody at varying temperatures: 4°C, 22°C, 30°C, and 37°C. Cold autoantibodies that are reactive at temperatures greater than 30°C have the potential to be clinically significant regardless of the antibody titer. Cold autoantibodies that are reactive at temperatures less than 30°C are not considered to be clinically significant. Immuno hematology 2013;29:49–50.

Key Words: thermal amplitude, cold agglutinin disease, cold autoantibody, titer, clinical significance

Principle

Cold autoantibodies and agglutinins are naturally occurring in most people, and incidental cold agglutinins are seen in normal healthy blood donors at low titers (<1:64).1 These benign cold autoantibodies are reactive at 4°C and have little or no reactivity at temperatures greater than 30°C. Cold autoantibodies may become clinically significant, resulting in red blood cell (RBC) hemolysis, if their titer increases or if they become reactive at temperatures greater than 30°C.2 Cold agglutinin disease (CAD) is hemolytic anemia secondary to cold autoantibodies and is responsible for approximately 13 to 15 percent of total cases of autoimmune hemolytic anemia (AIHA).3 It is characterized by immunoglobulin (Ig) M (and, rarely, IgG) autoantibodies, which cause agglutination of RBCs in vitro at room temperature and can cause hemolysis in vivo if the antibody is reactive at temperatures greater than 30°C and present at sufficient titers.2,4 These autoantibodies are usually directed against I, i, and related RBC antigens.5 The autoantibodies reactive at temperatures greater than 30°C may cause RBC hemolysis through activation of the complement pathway, leading to formation of membrane attack complex on the RBC membrane and intravascular hemolysis, or they may cause extravascular hemolysis via phagocytosis of C3b-coated RBCs by macrophages.6 Combined cold and warm AIHA (mixed AIHA) occurs when a patient has both warm AIHA and CAD.

Indications

Benign cold autoantibodies may be detected in the serum of many normal individuals. Pathologic cold autoantibodies capable of causing intravascular or extravascular hemolysis and CAD are characterized as having reactivity at cold temperatures (4°C–18°C) and warm temperatures (30°C–37°C) and are of high titer (>1:512 at 4°C).5–7 Both benign and pathologic cold autoantibodies may be seen in patients with warm AIHA. Patients with clinical and laboratory evidence of immune-mediated hemolytic anemia and a detectable cold autoantibody need additional evaluation to determine the significance of this cold autoantibody. The thermal amplitude of the cold autoantibody, defined as the highest temperature at which the autoantibody binds the RBC antigen, most accurately predicts severity of the disease.2,6,7

Reagents/Supplies

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Supplies</th>
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<tbody>
<tr>
<td>Two examples of a 3%–5% saline suspension of Group O (I+) washed RBCs</td>
<td>Water baths at 37°C, 30°C, and 22°C</td>
</tr>
<tr>
<td>RBCs chosen should lack any RBC antigens for which the patient has known antibodies</td>
<td>10 × 75-mm or 12 × 75-mm test tubes</td>
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<tr>
<td>0.9% saline</td>
<td>Tube rack</td>
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<tr>
<td>Calibrated thermometer</td>
<td>Transfer pipettes</td>
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<td>Calibrated centrifuge</td>
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RBC = red blood cell.
Procedure

Obtain an appropriate sample for testing (Tables 1, 2). The patient's sample should be collected and maintained at 37°C until the serum and the RBCs can be separated. This is done to avoid in vitro autoadsorption. Alternatively, an EDTA plasma sample can be used if it is warmed to 37°C with repeated mixing and then separated for testing. Before performing the thermal amplitude test, the patient's sample should be evaluated for antibodies to clinically significant antigens, and appropriate antibody identification or exclusion should be performed. Prepare two different examples of washed 3 to 5 percent saline suspensions of group O adult (I+) RBCs. If the patient has an identified antibody to an RBC antigen, the cells selected should lack this antigen to prevent falsely positive results. Label two tubes appropriately. Warm the tubes, serum, and RBCs separately until they reach 37°C (about 5–10 minutes). For each RBC suspension to be tested, place one drop of RBCs and three drops of serum into each of the warmed test tubes. Mix well and incubate at 37°C for 1 hour. Centrifuge according to the appropriate time and speed based on current calibrations for serologic agglutination testing for the centrifuge. Return the tubes to the 37°C water bath for 5 minutes before reading. Examine and grade the tubes for agglutination and record results. Transfer the tubes to a 30°C water bath and incubate for 1 hour. Remove the tubes and centrifuge. Place the centrifuged tubes back into the 30°C water bath for 5 minutes before reading the results. Examine and grade the tubes for agglutination and record results. Last, transfer the tubes to a 22°C water bath and allow them to incubate for 1 hour. Centrifuge the tubes and return them to 22°C for 5 minutes. After the tubes have incubated, examine and grade them for agglutination. Record the results.

An alternative to using centrifugation is to use the settling method in which the 37°C and 30°C incubations are extended to 2 hours each and the tubes are then examined for agglutination without centrifugation. The tubes undergoing room temperature incubation can be centrifuged after 30 minutes or 1 hour at 22°C. Interpret the results. If agglutination is present at 22°C but not at 30°C, then the antibody has limited thermal amplitude. If agglutination is observed at 30°C but not 37°C, then the antibody may have the potential to be clinically significant. If agglutination is observed at 37°C, then the antibody should be considered clinically significant.

In addition, the literature suggests that if no reactivity is observed at 30°C and the patient has hemolytic anemia, then an additional test at 30°C using 30 percent albumin may be helpful to further distinguish a clinically significant antibody capable of causing RBC destruction from a clinically insignificant cold autoantibody.

Limitations

Proper sample collection is critical to obtain valid results for this test. Improper sample collection can lead to in vitro autoadsorption of the cold autoantibody and falsely negative results. Choosing antigen-negative RBCs for patients with known antibodies is critical to preventing falsely positive results.

References


Courtney Hopkins, DO (corresponding author), Medical Director, and Tiffany K. Walters, MT(ASCP)SBBSTM, Immunohematology Reference Lab Manager, South Carolina Blood Services, American Red Cross, 2751 Bull Street, Columbia, SC 29201.
Hemolytic disease of the fetus and newborn (HDFN) owing to anti-U has rarely been reported. U is part of the MNS system. M and N glycoproteins are located on glycophorin A (GPA); S and s antigens are on glycophorin B (GPB). Individuals who lack GPB are S– and s– and also lack U. The U– phenotype occurs almost exclusively in the African population and has a very low frequency (0.25%). Anti-U is of immunoglobulin G class and can cause hemolytic transfusion reaction and HDFN. In this report we present the use of a noninvasive method to detect anemia in the fetus and the subsequent use of intrauterine transfusion (IUT) with blood of a very rare phenotype. For the first time, we used deglycerolized and 3-week-old red blood cell units for IUT without signs of adverse reactions and with the expected effect on the hemoglobin value. We conclude that this transfusion strategy could be applied safely. *Immunohematology* 2013;29:51–54.

**Key Words:** anti-U, hemolytic disease of the fetus and newborn, intrauterine transfusion

We found no detailed reports of possible deviations from routine blood requirements and transfusion practices for intrauterine transfusion (IUT) of blood components in the literature. We therefore propose that this case report could be of value when decisions concerning transfusion strategy in similar clinical situations are needed.

**Case Report**

Anti-U was identified at the International Blood Group Reference Laboratory (IBGRL) in Bristol, United Kingdom, in blood samples from a group A, D+ woman of Somali origin during her third pregnancy. The anti-U titer increased from 1 to 256, and the monocyte monolayer assay (MMA) result increased from 0 percent to 50 percent during this pregnancy. MMA is a qualitative test routinely used to establish the clinical significance of the antibody, in either transfusion or hemolytic disease of the fetus and newborn (HDFN).1 Low-ionic-strength saline (LISS), indirect antiglobulin, and gel methods from Diamed, Switzerland, were used for antibody detection, antibody titration, and crossmatching and for the direct antiglobulin test (DAT). The newborn needed two exchange transfusions.

In gestational week 15 of the woman’s fourth pregnancy, the anti-U titer was 256, and the MMA was 50 percent without fresh complement. Genomic typing in the Centre National de Référence pour les Groupes Sanguins in Paris revealed a deletion of the *GYPB* gene and no glycophorin B on the red blood cell (RBC) surface.

The anti-U titer was monitored regularly and remained stable at 256. RBC antibody screening was performed to detect the presence of additional antibodies throughout the course of the pregnancy. Alloadsorption was performed on two of these occasions, and at gestational week 28 a blood sample was analyzed at the IBGRL in Bristol. No additional antibodies were detected.

When obstetric history and antibody levels indicate risk for fetal anemia, the pregnancy is monitored with Doppler assessment of the peak systolic velocity (PSV) in the fetal middle cerebral artery (MCA). Values greater than 1.5 times the median of the MCA PSV were used as the threshold for fetal intervention with IUT.

The MCA PSV was significantly increased at gestational weeks 26 and 27, indicating signs of anemia (Fig. 1). A sample from the umbilical vein showed the fetus to be group A, D+ and U+, and DAT positive with a hemoglobin (Hb) of 7.2 g/dL. Only anti-U was detected in an eluate prepared from the umbilical sample.

The need for blood for the mother during delivery, for the IUT of the fetus, and for exchange transfusion of the newborn was assessed. Autologous transfusions were excluded because the Hb of the mother was slightly less than 11.0 g/dL. The National Frozen Blood Bank in Liverpool, French Rare Donor Registry, and Blood Center Skane, Sweden, were able to provide fresh and frozen U– blood. All blood components given to fetuses and newborns are irradiated.

IUT was performed under aseptic conditions and with ultrasound guidance; a 22-gauge needle was inserted into the intravascular space of the fetus using free-hand technique.
The choice of the fetal vessel to be used was dependent on the appearance of the fetus, umbilical cord, and placenta at the time of each transfusion. Usually the cord insertion was preferred, but alternatively the intrahepatic part of the umbilical vein was the vessel of choice after fetal analgesia and paralysis. Premedication of the mother was not used.

The first IUT with a deglycerolized O, D+, U– RBC unit was performed at 28 weeks’ gestation. Hb was 11.8 g/dL after the transfusion. The second IUT was performed at 30 weeks’ gestation with a group O, D+, U– fresh washed RBC unit; Hb was 7.5 g/dL before and 13.5 g/dL after. The third IUT was performed at 32 weeks’ gestation with a 3-week-old washed U– RBC unit; Hb was 7.9 g/dL before, but no sample was tested after. The fourth IUT was performed at 34 weeks’ gestation with a group A, D+, U– deglycerolized RBC unit. Hb was 8.6 g/dL before, and no sample was tested after. All four units had a hematocrit between 70 and 75 percent. We regularly use fresh blood, less than 5 days old, for IUT, but in the present case we had to transfuse both deglycerolized RBCs and units 1 to 3 weeks of age. All units were transfused with no sign of adverse events and with the expected effect on Hb. There was no need for blood transfusion for the mother during delivery.

**Results**

Delivery was induced at 36 weeks’ gestation. A healthy boy was born with normal weight; his Apgar score was 10 at 1 minute, Hb was 11.5 g/dL (normal range 13.0–20.0 g/dL), bilirubin was 76 μmol/L (normal range <200 μmol/L), and DAT was negative. His RBCs tested as S– s– (owing to the transfused cells), and anti-U with a titer of 16 was identified in the plasma. The newborn was treated with UV light from 3 hours of age until 32 hours and from 48 hours until 78 hours, a total of 59 hours. Hemoglobin, bilirubin, and reticulocytes were measured daily (see Fig. 2).
The newborn’s Hb was between 11.5 g/dL and 11.9 g/dL for the first 12 days. Sample taken on the 16th day showed an Hb of 8.5 g/dL and an anti-U titer of 2 and was DAT positive but still S–s–. At the age of 3 weeks (October 14, 2010) his Hb was 7.7 g/dL, bilirubin was 61 μmol/L, reticulocytes were $101 \times 10^9/L$, and anti-U titer was 1. The newborn showed symptoms of anemia with fatigue and drowsiness, and he was transfused with a 5-week-old U– RBC unit. His potassium level was 5.5 mmol/L after transfusion (normal range 3.4–6.0 mmol/L). At 2 months of age, the Hb was 10.4 g/dL (normal range 10.0–16.0 g/dL), and the boy was healthy with normal growth.

**Discussion**

Anti-U is extremely unusual; there are very few case reports of it in pregnancy and HDFN,$^{2,3,4}$ and none of them report IUT, only exchange transfusion and UV light treatment. To provide blood for IUT, units were collected at the Skane Blood Center in Sweden as well as in England and France. The fetus exhibited anemia and needed IUT four times; after birth the newborn was treated with intensive UV light treatment for 3 days, but did not require exchange transfusion. At 3 weeks of age, symptoms of anemia occurred and a single unit of RBCs was needed. The RBCs were 5 weeks old, and produced the expected effect on Hb. We usually provide blood less than 1 week old to newborns.
Conclusion

The impact of the age of transfused units in IUT is not generally discussed. We had to perform IUT using both deglycerolized RBC units and units of 3 to 5 weeks of age; they were transfused with the expected effect on Hb and without adverse events. We could also use units of group A as both mother and fetus were group A. We had the opportunity to follow the effect of an unusual immunization on the fetus and the development of anemia in the neonatal period. Early detection and treatment of anemia in the fetus is of great value, and it reduces the need for exchange transfusion in the neonatal period. Our report also shows that well-organized cooperation among international blood centers is a prerequisite for the successful outcome for the child.

References


Johanna Strindberg, MD (corresponding author), Transfusion Medicine C2:66, Joachim Lundahl, MD, PhD, Transfusion Medicine and Clinical Immunology, and Gunilla Ajne, MD, PhD, Clinic of Obstetrics and Gynaecology, Karolinska University Hospital, Huddinge, S-141 86 Stockholm, Sweden.
Molecular analysis of patients with weak D and serologic analysis of those with anti-D (excluding type 1 and type 2)

B.-N. Pham, M. Roussel, D. Gien, C. Andre-Botte, M. Ripaux, C. Auxerre, and P.-Y. Le Pennec

Whether or not patients whose red blood cells (RBCs) carry certain weak D types produce anti-D, and if they do whether it is allo- or autoanti-D, remains controversial. The aim of this study was to determine the serologic features of anti-D in individuals expressing a weak D other than type 1 or type 2 and to assess whether the anti-D was an allo- or autoantibody. Serologic D typing and molecular analyses were performed on 748 individuals. Serologic characterization of anti-D included autologous controls, direct antiglobulin test, elution, and titration of anti-D before and after adsorption of serum onto autologous RBCs. From molecular analyses, 459 individuals exhibited a weak D type. We described seven novel RHD variant alleles. The most frequent types of weak D were type 1 (30.1%), type 2 (23.7%), type 4.0 (10.2%), type 4.2.2 (20.3%), type 11 (3.9%), and type 15 (3.7%). Anti-D was identified in the sera of 9 of 47 individuals with weak D type 4.0, in 14 of 93 with weak D type 4.2.2, in 1 of 18 with weak D type 11, in 1 of 17 with weak D type 15, and in 1 weak D type 33 individual. Anti-D was demonstrated to be an alloantibody in weak D type 4.0, type 4.2.2, and type 15 individuals, but an autoantibody in weak D type 11 and type 33 individuals. In conclusion, only a complete serologic investigation of individuals with a given weak D type identified by molecular analysis allows concluding on the nature of the antibody. Transfusing weak D type 4.2.2 and type 15 patients with D– RBC units and proposing anti-D immunoprophylaxis to women with these weak D types should be considered. *Immunohematology* 2013;29:55–62.

**Key Words:** anti-D, weak D, partial D, D variant, RHD molecular analysis

The D (RH1) antigen is of clinical importance with regard to hemolytic disease of the fetus and newborn (HDFN) and transfusion medicine. This antigen is still the leading cause of HDFN. Furthermore, anti-D has the potential to cause severe hemolytic transfusion reactions. The D antigen carried by the RhD protein is the most immunogenic of the Rh antigens. It has been described as a mosaic composed of multiple epitopes thought to be highly conformational. The high immunogenicity of the D antigen is related to the fact that the entire RhD protein is absent from the red blood cell (RBC) membrane of individuals expressing a D– phenotype. Issitt and Anstee reported that approximately 80 percent of D– healthy volunteers transfused with one or more D+ blood units produce anti-D. However, more recent data show that only 20 to 30 percent of D– patients transfused with one or more D+ units produce anti-D.

The RhD protein is exclusively expressed on RBCs. From structural models, this protein has been predicted to consist of 12 transmembranous helixes with six extracellular regions. The Rh proteins are encoded by two homologous genes, *RHD* and *RHCE*. *RHD* encodes the RhD protein, whereas *RHCE* encodes the RhCE protein, carrying the C/c and E/e polymorphisms. Each gene consists of ten exons. The opposite orientation of the *RHD* and *RHCE* genes on chromosome 1 favors great diversity of these genes as a result of genomic rearrangements. A large number of *RHD* alleles that result in D variants have been identified. The term *D variant* refers to RhD proteins associated with a quantitative or qualitative change of D expression. Classically, weak D, related to a quantitative change of D expression, has been defined as RBCs giving a weaker reaction than RBCs of the same Rh phenotype as reference, according to a defined anti-D reagent and a defined technique. Weak D differs from partial D, as the latter is associated with a qualitative change of D expression. This difference is of clinical importance because patients with a partial D phenotype have the potential to produce alloanti-D against the part of D they lack. More recently, D variants have been classified at the molecular level. Based on *RHD* sequence variations, genetic variants changing the amino acid sequence predicted to be in the membrane-spanning or intracellular regions of the RhD protein were considered to be a feature of weak D, whereas genetic variants changing the amino acid sequence predicted to be in the extracellular regions were considered to be a feature of partial D. Until now, assignment of variant D type resulting from molecular analysis has reported more than 70 different weak D types.

The weak D phenotype has been a subject of controversy since it was described in 1946. Stratton first described RBCs reacting in an atypical manner with anti-D, introducing the term "Dw" phenotype corresponding to a weakened form of D. Considering the evolution of the anti-D reagents (mostly
monoclonal antibodies or MoAbs) and techniques over the years, no definitive serologic variation has been established in the majority of weak D types. For some time, it was generally accepted that patients with a weak D phenotype express a weak but normal entire D antigen. Consequently, the possibility of D immunization related to weak D types was disregarded by many. However, some authors showed that patients with weak D phenotype may produce alloanti-D, suggesting that most weak D types carry altered D antigen. Recently, by performing a complete serologic investigation, we demonstrated that anti-D in weak D type 1 or weak D type 2 individuals were autoantibodies. The aim of the present study was to determine the serologic features of anti-D in individuals expressing a weak D type, excluding type 1 or type 2, and to assess the clinically relevant potential for anti-D immunization in individuals with some weak D types.

**Materials and Methods**

**Samples**

Samples were obtained from 748 individuals referred to the Centre National de Référence pour les Groupes Sanguins (CNRGS) between 2007 and 2010 for different reasons, namely, depressed D phenotype, discordant results between two anti-D reagents, or anti-D in individuals with a D+ phenotype. EDTA blood (15 mL) and serum (15 mL) were obtained from these individuals for serologic D typing and molecular RHD testing.

**Serology**

D antigen status, together with C, E, c, and e status, was evaluated using two commercially available reagents: one monoclonal reagent (Ortho BioVue System, Ortho Clinical Diagnostics, Raritan, NJ), and one polyclonal reagent (DiaMed, Cressier/Morat, Switzerland), with gel testing according to the manufacturers’ recommendations. These reagents are “CE marked,” and they are licensed according to the European Community Standards.

D antigen reactivity was further analyzed using immunoglobulin (Ig) G MoAbs and an IgM MoAb. The IgG MoAbs were HIRO3 and HIRO7 from the Japanese Red Cross (Dr. Uchikawa), D7 from Dr. Sondag-Thüll (Liège, Belgium), 415-1E4 from Dominion Biologicals Ltd. (Dartmouth, Canada), P3x249, P3x35, and HM16 from Diagast (Loos, France), FEF3 from the International Blood Group Reference Laboratory, United Kingdom (Dr. Anstee), and RD7C2 from Institut Pasteur de Paris, France (Dr. Edelman). The IgM MoAb was P3BROU7 from Etablissement Français du Sang, Rennes, France (Dr. Martin).

The direct antiglobulin test (DAT) using gel method (anti-IgG and anti-C3d separately) was performed using the commercial kit DC-Screening II (DiaMed), according to the manufacturer’s recommendations.

Elution was performed on the RBCs of individuals producing anti-D using an acid elution method (Gamma Elu-Kit II, Immucor Gamma, Norcross, GA). The kit was used according to the manufacturer’s recommendations. The eluate was tested using an indirect antiglobulin test (IAT) gel method (DiaMed), according to the manufacturer’s recommendations, with native and papain-treated RBCs.

Anti-D was identified by testing the serum against a panel of RBCs (Reference National Panel, Paris, France). The IAT gel method was performed according to the manufacturer’s instructions (DiaMed), with native and papain-treated RBCs.

Adsorption with autologous RBCs was performed when samples were obtained in sufficient quantity and if there was no history of transfusion in the past 4 months. Adsorption of serum onto an equal volume of washed papain-treated autologous RBCs was performed at 37°C for 40 minutes, followed by centrifugation at 3100g for 10 minutes. The serum was adsorbed a minimum of three times. The remaining serum was tested against papain-treated RBCs in an IAT gel test.

Anti-D was titrated against a pool of papain-treated D+C–E+c+e– RBCs by using IAT at 37°C. The titration end point of anti-D was determined before and after adsorptions onto autologous RBCs.

**Molecular Testing**

Genomic DNA was extracted from whole-blood samples by using the MagNA Pure Compact Instrument (Roche Molecular Biochemicals, Mannheim, Germany) with the MagNA Pure Compact Nucleic Acid Isolation Kit I (Roche Molecular Biochemicals), according to the manufacturer’s recommendations.

**Allele-Specific Polymerase Chain Reactions**

RHD allele-specific primer amplification assays, primer sequences, and polymerase chain reaction (PCR) conditions to detect the RHD*weak D type 1 (RHD*01W1) and the RHD*weak D type 2 (RHD*01W2) alleles were previously described.
Exon Amplification for DNA Sequence Analysis

PCR exon amplification was performed on genomic DNA for sequence analysis. RHD primer sequences were previously described. The ten RHD exon PCRs were performed in a thermal cycler on 100 ng of genomic DNA in a total reaction volume of 50 µL. Reaction mixtures contained 10 µM of each primer, 200 µM of each deoxynucleoside triphosphate (Amersham Biosciences, Piscataway, NJ), and 2.5 U of Taq DNA polymerase (Gold, Applied Biosystems, Carlsbad, CA) or 1 µL of Taq DNA polymerase for exon 5 (Advantage 2 polymerase mix, Clontech Laboratories, Mountain View, CA) in the appropriate buffer.

PCR products were purified (Exosap-it, Affymetrix UK Ltd., High Wycombe, UK), and cycle sequenced by using BigDye terminator chemistry (ABI-PRISM BigDye Terminator v1.1 Cycle Sequencing Kits, Applied Biosystems). Sequences were analyzed on an automated fluorescence-based ABI Prism 3130 (Applied Biosystems).

Results

Molecular Data

From the 748 individuals systematically tested using molecular RHD analysis, a total of 459 individuals (61.3%) exhibited a D variant that could be named weak D according to the RhESUSBase classification. The different weak D types found in this study and their prevalence are reported in Table 1. The RHD allele characterized by the 602C>G, 667T>G, 744C>T, 957G>A, and 1025T>C polymorphisms in RHD exons 4, 5, and 7 was named RHD*weak D type 4.2.2 according to its first molecular description. This RHD allele may be dubbed DAR, an allele which shares the 602C>G, 667T>G, and 1025T>C nucleotide polymorphisms found in weak D type 4.2.2 but lacks the silent 744C>T and 957G>A polymorphisms. Weak D type 4.2.2 is currently listed as a “partial D” on the RhESUSBase; International Society of Blood Transfusion (ISBT) allele terminology describes this allele as carrying the 602C>G, 667T>G, 744C>T, and 1025T>C polymorphisms. We described seven novel variant RHD alleles (Table 1). The N1, N2, N3, N4, N5, N6, and N7 novel variant alleles were derived from a DCe (R’), DCE, DcE (R’), Dce (R’), DCE, DcE, and DCE haplotype, respectively.

Of the 748 individuals, 138 (18.5%) exhibited a partial D. As the focus of this study is weak D types, no further analysis or discussion of these samples is included here. Genomic DNA sequencing of all exons in 65 cases (8.7%) did not identify known variant RHD alleles. No exonic variants were found in most cases. cDNA analysis would be necessary to rule out other variants; these samples were not pursued further. An additional 86 samples (11.5%) were not pursued further; these included samples with two variant RHD alleles or incomplete or pending analysis.

Serologic Data

Anti-D was identified in 7 weak D type 1 individuals (5.1%), 6 weak D type 2 individuals (5.5%), 9 weak D type 4.0 individuals (19.1%), 14 weak D type 4.2.2 individuals (15.1%), 1 weak D type 11 individual (5.5%), 1 weak D type 15 individual (5.9%), and 1 weak D type 33 individual (Table 1). Weak D Type 4.0

All 47 individuals expressing a weak D type 4.0 exhibited a D+C–E–c+e+ phenotype. The reactivity of weak D type 4.0 RBCs with selected D MoAbs is detailed in Table 2.

When using the panel of anti-D MoAbs, the strength of the positive reaction obtained varied when using IgG MoAbs; RD7C2 gave a negative reaction (data not shown).

Anti-D was detected in 9 of the 47 individuals (19.1%) exhibiting a weak D type 4.0. Serologic data from five cases (C-1 to C-5) are reported in Table 3. Transfusion history and pregnancies related to these individuals are listed in Table 3. No data were reported for the four other cases as a result of recent transfusion. To sum up, anti-D was demonstrated to be an alloantibody in one case (C-1). In this case, the anti-D reactivity was not significantly reduced after autologous adsorptions. Autologous controls, DAT, and eluate were negative. In contrast, anti-D was demonstrated to be an autoantibody in two other cases (C-4 and C-5). In these cases, the titer and score were significantly reduced (at least 2 dilutions for the titer and 16 for the score) after autologous adsorptions. In C-4, the autologous controls and the DAT were positive, and anti-D was present in the eluate. In C-5, autologous controls and DAT were negative. Finally, no conclusion about the nature (alloantibody or autoantibody) of anti-D could be reached from the incomplete serologic data in the last two cases (C-2 and C-3). No alloantibody against other antigens of blood group systems was detected in C-1 to C-5.

Weak D Type 4.2.2

All of the 93 individuals expressing a weak D type 4.2.2 exhibited a D+C–E–c+e+ phenotype. The reactivity of weak D type 4.2.2 RBCs with selected D MoAbs is detailed in Table 2.

When using the panel of anti-D MoAbs, the strength of reaction obtained varied when using HIRO3, HIRO7, D7, 415-1E4, P3x249, P3x35, and HM16 IgG MoAbs (data not shown).
Anti-D was detected in 14 of the 93 individuals (15.1%) exhibiting a weak D type 4.2.2. Notably, it was found in combination with anti-Hr (RH18) in 8 of the 14 patients (C-12 to C-19). Serologic data related to anti-D investigation are reported in Table 4. Transfusion history and pregnancies related to these individuals are listed in Table 4. To sum up, anti-D was demonstrated to be an alloantibody in two cases (C-6 and C-7). After autologous adsorptions, the anti-D reactivity was not significantly reduced. Autologous controls, DAT, and eluate were negative. In the other 12 cases, no conclusion about the nature (allo- or autoantibody) of anti-D could be made from the incomplete serologic data.

**Weak D Type 11**

All 18 individuals expressing a weak D type 11 exhibited a D+C+E−c+c+ phenotype. Our results were concordant with previous studies reporting that the weak D type 11 was a weak form of D named DEL (D_{el}) when encoded by a DCe haplotype in the Caucasian population.

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**Table 1. Weak D type and antibody status in individuals exhibiting a weak D phenotype identified in this study**

<table>
<thead>
<tr>
<th>Trivial names*</th>
<th>Molecular changes carried by the corresponding RHD allele</th>
<th>N</th>
<th>% of the total weak Ds</th>
<th>Number of individuals with anti-D (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weak D type 1</td>
<td>809T&gt;G (RHD*01W.1)</td>
<td>138</td>
<td>30.1%</td>
<td>7 (6.1%)</td>
</tr>
<tr>
<td>Weak D type 2</td>
<td>1154G&gt;C (RHD*01W.2)</td>
<td>109</td>
<td>23.7%</td>
<td>6 (5.5%)</td>
</tr>
<tr>
<td>Weak D type 3</td>
<td>8C&gt;G (RHD*01W.3)</td>
<td>6</td>
<td>1.3%</td>
<td>0</td>
</tr>
<tr>
<td>Weak D type 4</td>
<td>602C&gt;G, 667T&gt;G, 819G&gt;A (RHD*weak partial 4.0)</td>
<td>47</td>
<td>10.2%</td>
<td>9 (19.1%)</td>
</tr>
<tr>
<td>Weak D type 4.2.2</td>
<td>602C&gt;G, 667T&gt;G, 744C&gt;T, 957G&gt;A, 1025T&gt;C (RHD*weak 4.2.2)</td>
<td>93</td>
<td>20.3%</td>
<td>14 (15.1%)</td>
</tr>
<tr>
<td>Weak D type 4.2.3</td>
<td>602C&gt;G, 667T&gt;G, 744C&gt;T, 1025T&gt;C</td>
<td>7</td>
<td>1.5%</td>
<td>0</td>
</tr>
<tr>
<td>Weak D type 5</td>
<td>446C&gt;A (RHD*01W.5)</td>
<td>1</td>
<td>NA</td>
<td>0</td>
</tr>
<tr>
<td>Weak D type 10</td>
<td>1177T&gt;C (RHD*01W.10)</td>
<td>2</td>
<td>NA</td>
<td>0</td>
</tr>
<tr>
<td>Weak D type 11</td>
<td>885G&gt;A (RHD*weak partial 11)</td>
<td>18</td>
<td>3.9%</td>
<td>1 (5.5%)</td>
</tr>
<tr>
<td>Weak D type 15</td>
<td>845G&gt;A (RHD*weak partial 15)</td>
<td>17</td>
<td>3.7%</td>
<td>1 (5.9%)</td>
</tr>
<tr>
<td>Weak D type 17</td>
<td>340C&gt;T (RHD*01W.17)</td>
<td>1</td>
<td>NA</td>
<td>0</td>
</tr>
<tr>
<td>Weak D type 18</td>
<td>19C&gt;T (RHD*01W.18)</td>
<td>4</td>
<td>NA</td>
<td>0</td>
</tr>
<tr>
<td>Weak D type 20</td>
<td>1250T&gt;C (RHD*01W.20)</td>
<td>1</td>
<td>NA</td>
<td>0</td>
</tr>
<tr>
<td>Weak D type 27</td>
<td>661C&gt;T (RHD*01W.27)</td>
<td>2</td>
<td>NA</td>
<td>0</td>
</tr>
<tr>
<td>Weak D type 33</td>
<td>520G&gt;A (RHD*01W.33)</td>
<td>1</td>
<td>NA</td>
<td>1</td>
</tr>
<tr>
<td>Weak D type 38</td>
<td>833G&gt;A (RHD*01W.38)</td>
<td>1</td>
<td>NA</td>
<td>0</td>
</tr>
<tr>
<td>Weak D type 56</td>
<td>65C&gt;A (RHD*01W.56)</td>
<td>3</td>
<td>NA</td>
<td>0</td>
</tr>
<tr>
<td>Weak D type 59</td>
<td>1148T&gt;C (RHD*01W.59)</td>
<td>1</td>
<td>NA</td>
<td>0</td>
</tr>
<tr>
<td>Novel weak D = N1</td>
<td>208C&gt;T, 818C&gt;T, 1195G&gt;A</td>
<td>1</td>
<td>NA</td>
<td>0</td>
</tr>
<tr>
<td>Novel weak D = N2</td>
<td>542T&gt;C</td>
<td>1</td>
<td>NA</td>
<td>0</td>
</tr>
<tr>
<td>Novel weak D = N3</td>
<td>730G&gt;C</td>
<td>1</td>
<td>NA</td>
<td>0</td>
</tr>
<tr>
<td>Novel weak D = N4</td>
<td>731C&gt;T</td>
<td>1</td>
<td>NA</td>
<td>0</td>
</tr>
<tr>
<td>Novel weak D = N5</td>
<td>751A&gt;C</td>
<td>1</td>
<td>NA</td>
<td>0</td>
</tr>
<tr>
<td>Novel weak D = N6</td>
<td>884T&gt;C</td>
<td>1</td>
<td>NA</td>
<td>0</td>
</tr>
<tr>
<td>Novel weak D = N7</td>
<td>1107A&gt;C</td>
<td>1</td>
<td>NA</td>
<td>0</td>
</tr>
</tbody>
</table>

**Table 2. Reaction strengths of samples with weak D type 4.0 and weak D type 4.2.2 in tests with MoAb anti-D**

<table>
<thead>
<tr>
<th>Strength</th>
<th>Weak D type 4.0 (N = 47)</th>
<th>Weak D type 4.2.2 (N = 93)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Monoclonal anti-D</td>
<td>Polyclonal anti-D</td>
</tr>
<tr>
<td>4+</td>
<td>25</td>
<td>4</td>
</tr>
<tr>
<td>3+</td>
<td>19</td>
<td>27</td>
</tr>
<tr>
<td>2+</td>
<td>1</td>
<td>14</td>
</tr>
<tr>
<td>1+</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Negative</td>
<td>2</td>
<td>1</td>
</tr>
</tbody>
</table>

* Trivial names assigned by RhesusBase.\(^{10}\)

The RHD allele was named RHD*weak D type 4.2.2 according to its first molecular description.\(^{12}\) This RHD allele may be dubbed DAR, the sequence of which shares the 602C>G, 667T>G, and 1025T>C polymorphisms found in RHD*weak D type 4.2.2 but lacks the silent 744C>T and 957G>A polymorphisms.\(^{20}\)

NA = not applicable.

---

1 Trivial names assigned by RhesusBase.\(^{10}\)

2 The RHD allele was named RHD*weak D type 4.2.2 according to its first molecular description.\(^{12}\) This RHD allele may be dubbed DAR, the sequence of which shares the 602C>G, 667T>G, and 1025T>C polymorphisms found in RHD*weak D type 4.2.2 but lacks the silent 744C>T and 957G>A polymorphisms.\(^{20}\)
When using the panel of anti-D MoAbs, a very weak positive reaction was obtained with the HIRO3, D7, and P3x249 MoAbs only (data not shown).

Anti-D was detected in 1 of the 18 individuals (5.5%) exhibiting a weak D type 11. This patient produced an autoanti-D (Table 5, C-20). The anti-D reactivity (titer of 32, and score of 53 before autologous adsorptions) was not detected after autologous adsorptions, anti-D was present, without significant reduction exhibiting a weak D type 15 (Table 5, C-21). After autologous adsorptions, anti-D was present, without significant reduction of either the titer or the score (1 dilution for the titer, and 10 for the score). Autologous controls, DAT, and eluate were negative. No alloantibody against antigens of other blood group systems was detected.

**Weak D Type 15**

Among the 17 individuals expressing a weak D type 15, 9 exhibited the D+C–E+c+e+ phenotype described by Wagner et al. However, 8 individuals exhibited a D+C+E–c+e+ phenotype. When using monoclonal anti-D or polyclonal anti-D, a negative reaction was observed for all samples.

When using the panel of anti-D MoAbs, a weak positive reaction was obtained with HIRO3, HIRO7, and 415-1E4 IgG MoAbs (data not shown).

Alloanti-D was detected in 1 of the 17 individuals (5.9%) exhibiting a weak D type 15 (Table 5, C-21). After autologous adsorptions, anti-D was present, without significant reduction of either the titer or the score (1 dilution for the titer, and 10 for the score). Autologous controls, DAT, and eluate were negative. No alloantibody against antigens of other blood group systems was detected.

---

**Table 3. Serologic data of anti-D in weak D type 4.0 individuals**

<table>
<thead>
<tr>
<th>Case number/gender</th>
<th>Transfusion history</th>
<th>Pregnancy</th>
<th>Native RBCs</th>
<th>Papain-treated RBCs</th>
<th>Autoantibody controls</th>
<th>Before autologous adsorptions</th>
<th>After autologous adsorptions</th>
<th>Number of autologous adsorptions</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-1 Female</td>
<td>Yes</td>
<td>Unk</td>
<td>Negative</td>
<td>Positive</td>
<td>Negative</td>
<td>8/31</td>
<td>4/23</td>
<td>5</td>
</tr>
<tr>
<td>C-2 Female</td>
<td>Unk</td>
<td>Yes</td>
<td>Positive</td>
<td>Negative</td>
<td>Negative</td>
<td>128/55</td>
<td>NT</td>
<td>NA</td>
</tr>
<tr>
<td>C-3 Female</td>
<td>Yes</td>
<td>Unk</td>
<td>Negative</td>
<td>Positive</td>
<td>Negative</td>
<td>NT</td>
<td>NT</td>
<td>NA</td>
</tr>
<tr>
<td>C-4 Female</td>
<td>Yes</td>
<td>Unk</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>NT</td>
<td>16/44</td>
<td>1/5</td>
</tr>
<tr>
<td>C-5 Female</td>
<td>Yes</td>
<td>Unk</td>
<td>Positive</td>
<td>Positive</td>
<td>Negative</td>
<td>8/35</td>
<td>1/2</td>
<td>4</td>
</tr>
</tbody>
</table>

*Anti-D tested and titrated against papain-treated RBCs (gel-test). RBCs = red blood cells; DAT = direct antiglobulin test; Unk = unknown; NT = not tested; NA = not applicable; IgG = immunoglobulin G.

**Table 4. Serologic data of anti-D in weak D type 4.2.2 individuals**

<table>
<thead>
<tr>
<th>Case number/gender</th>
<th>Transfusion history</th>
<th>Pregnancy</th>
<th>Native RBCs</th>
<th>Papain-treated RBCs</th>
<th>Autoantibody controls</th>
<th>Before autologous adsorptions</th>
<th>After autologous adsorptions</th>
<th>Number of autologous adsorptions</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-6 Female</td>
<td>Unk</td>
<td>Yes</td>
<td>Negative</td>
<td>Positive</td>
<td>Negative</td>
<td>8/35</td>
<td>4/23</td>
<td>4</td>
</tr>
<tr>
<td>C-7 Female</td>
<td>No</td>
<td>Yes</td>
<td>Positive</td>
<td>Positive</td>
<td>Negative</td>
<td>16/40</td>
<td>8/25</td>
<td>3</td>
</tr>
<tr>
<td>C-8 Female</td>
<td>Unk</td>
<td>Yes</td>
<td>Positive</td>
<td>Positive</td>
<td>Negative</td>
<td>NT</td>
<td>NT</td>
<td>NA</td>
</tr>
<tr>
<td>C-9 Female</td>
<td>Unk</td>
<td>Yes</td>
<td>Positive</td>
<td>Positive</td>
<td>Negative</td>
<td>NT</td>
<td>NT</td>
<td>NA</td>
</tr>
<tr>
<td>C-10 Male</td>
<td>Yes</td>
<td>NA</td>
<td>Positive</td>
<td>Positive</td>
<td>Negative</td>
<td>NT</td>
<td>NT</td>
<td>NA</td>
</tr>
<tr>
<td>C-11 Male</td>
<td>Yes</td>
<td>NA</td>
<td>Negative</td>
<td>Positive</td>
<td>Positive</td>
<td>NT</td>
<td>NT</td>
<td>NA</td>
</tr>
<tr>
<td>C-12 Female</td>
<td>Unk</td>
<td>Yes</td>
<td>Negative</td>
<td>Positive</td>
<td>Negative</td>
<td>NT</td>
<td>NT</td>
<td>NA</td>
</tr>
<tr>
<td>C-13 Female</td>
<td>Unk</td>
<td>Yes</td>
<td>Negative</td>
<td>Positive</td>
<td>Negative</td>
<td>NT</td>
<td>NT</td>
<td>NA</td>
</tr>
<tr>
<td>C-14 Female</td>
<td>Yes</td>
<td>Yes</td>
<td>Negative</td>
<td>Positive</td>
<td>Negative</td>
<td>NT</td>
<td>NT</td>
<td>NA</td>
</tr>
<tr>
<td>C-15 Female</td>
<td>No</td>
<td>Yes</td>
<td>Negative</td>
<td>Positive</td>
<td>Negative</td>
<td>NT</td>
<td>NT</td>
<td>NA</td>
</tr>
<tr>
<td>C-16 Female</td>
<td>No</td>
<td>Yes</td>
<td>Negative</td>
<td>Positive</td>
<td>Negative</td>
<td>NT</td>
<td>NT</td>
<td>NA</td>
</tr>
<tr>
<td>C-17 Female</td>
<td>No</td>
<td>Yes</td>
<td>Negative</td>
<td>Positive</td>
<td>Negative</td>
<td>NT</td>
<td>NT</td>
<td>NA</td>
</tr>
<tr>
<td>C-18 Female</td>
<td>No</td>
<td>Yes</td>
<td>Positive</td>
<td>Positive</td>
<td>Negative</td>
<td>NT</td>
<td>NT</td>
<td>NA</td>
</tr>
<tr>
<td>C-19 Female</td>
<td>Yes</td>
<td>Negative</td>
<td>Negative</td>
<td>Positive</td>
<td>Negative</td>
<td>NT</td>
<td>NT</td>
<td>NA</td>
</tr>
</tbody>
</table>

*Anti-D tested and titrated against papain-treated RBCs (gel-test). RBCs = red blood cells; DAT = direct antiglobulin test; Unk = unknown; NT = not tested; NA = not applicable.
Table 5. Serologic data of anti-D in weak D type 11, 15, or 33 individuals

<table>
<thead>
<tr>
<th>Case number/gender</th>
<th>Transfusion history</th>
<th>Pregnancy</th>
<th>Weak D type</th>
<th>Autoantibody against native RBCs</th>
<th>Papain-treated RBCs</th>
<th>Direct elution</th>
<th>Before autologous adsorptions</th>
<th>After autologous adsorptions</th>
<th>Number of autologous adsorptions</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-20 Female</td>
<td>Yes</td>
<td>Unk</td>
<td>Weak D type 11</td>
<td>Negative</td>
<td>Positive</td>
<td>Negative</td>
<td>Negative</td>
<td>NT</td>
<td>32/53</td>
</tr>
<tr>
<td>C-21 Female</td>
<td>No</td>
<td>Yes</td>
<td>Weak D type 15</td>
<td>Negative</td>
<td>Positive</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>64/69</td>
</tr>
<tr>
<td>C-22 Female</td>
<td>Yes</td>
<td>Unk</td>
<td>Weak D type 33</td>
<td>Negative</td>
<td>Positive</td>
<td>Negative</td>
<td>Negative</td>
<td>Anti-D</td>
<td>4/20</td>
</tr>
</tbody>
</table>

*Anti-D tested and titrated against papain-treated RBCs (gel-test).
†Neg indicates titer = 0/score = 0.
RBCs = red blood cells; DAT = direct antiglobulin test; Unk = unknown; NT = not tested.

**WEAK D TYPE 33**

Only one D+C+E−c+e+ individual was found to express a weak D type 33. When using monoclonal anti-D or polyclonal anti-D, a negative reaction was observed when testing the blood sample from the weak D type 33 individual.

When using the panel of anti-D MoAbs, a positive reaction was obtained when using the P3BROU7 IgM MoAb and all IgG MoAbs except RD7C2.

This weak D type 33 patient produced an autoanti-D (Table 5, C-22). The anti-D reactivity was not detected after autologous adsorptions. Autologous controls were positive. Anti-D was present in the eluate despite a negative DAT. No alloantibody against antigens of other blood group systems was detected.

**Discussion**

From our experience, molecular RHD analysis appears to be the only reliable method to identify D variants. The D antigen expression of a given D variant appears variable when tested serologically. Our previous study clearly showed the variable reactivity with anti-D of RBCs expressing weak D type 1 and that of RBCs expressing weak D type 2. In the same way, the present study clearly showed that the reactivity of RBCs expressing weak D type 4.0 or weak D type 4.2.2 ranged from 4+ to 0, despite using a defined validated method with a CE-marked anti-D monoclonal or polyclonal reagent. These data reinforce our feeling that the identification of a D variant should never be based on serologic criteria owing to its variable expression. Consequently, our recommendation would be to perform molecular RHD analysis when D expression is weakened when compared with normal D expression, whatever the level of decreased reactivity, or if anti-D is produced by a D+ individual.

Molecular testing has allowed identification of more than 150 different D variants. Based on RHD sequence variation, the widely used classification found on RhesusBase has assigned names to D variants, weak D or partial D, referenced in the ISBT allele terminology. In our study, the most frequent weak D types were weak D type 1, type 2, type 4.0, type 4.2.2, type 11, and type 15. When available, it is useful to give RH haplotype information when describing a novel RHD allele. In accordance with data listed on RhesusBase, weak D type 1, type 2, type 4.0, and type 4.2.2 were found to be encoded by DCe, DcE, Dce, and Dce haplotypes respectively. Weak D type 11 was found to be encoded by a Dce haplotype, as expected when expression of this D variant corresponds to a “DEL phenotype.” Interestingly, our study first reports that weak D type 15 may be encoded either by the expected Dce haplotype or by a Dce haplotype. Whether the presence of C in cis affects the D antigen density requires further study. In our laboratory, the frequencies of weak D type 1, type 2, type 4.0, type 4.2.2, type 11, and type 15 were 30.1, 23.7, 10.2, 20.3, 3.9, and 3.7 percent, respectively, for the 459 weak D types identified using molecular methods. These particular frequencies are likely biased because of our recruitment strategy toward a population of African ancestry. Actually, weak D type 1, weak D type 2, and weak D type 3 have been reported to be the most prevalent D variants found in the white population. In contrast, weak D type 4.0 and weak D type 4.2.2 have been reported to be mostly found in the African population. Thus, our results are in accordance with the D variant frequencies expected when a mixed population is tested. Whether patients carrying certain molecular weak D types are prone to anti-D immunization has been questioned for many years and followed through a Rhesus immunization registry. After our recent study about anti-D immunization in weak D type 1 or type 2 individuals, we perform a complete serologic investigation whenever possible in individuals
expressing a weak D type other than type 1 or type 2. The serologic investigation includes autologous controls, DAT, elution, and titration of anti-D before and after adsorption of serum onto autologous RBCs. In our experience, titration of anti-D before and after adsorption of serum onto autologous RBCs is the most informative test, indicating the nature (allo- or auto-) of the antibody. In this study, anti-D was identified in 19.1 percent of weak D type 4.0 individuals, 15.1 percent of weak D type 4.2.2 individuals, 5.5 percent of weak D type 11 individuals, 5.9 percent of weak D type 15 individuals, and 1 weak D type 33 individual.

In weak D type 4.0 individuals, a complete serologic analysis was performed in three cases. Interestingly, the serologic data demonstrated that anti-D was an alloantibody in one case (C-1), but an autoantibody in the other two cases (C-4 and C-5). To our knowledge, only two cases of weak D type 4.0 individuals developing anti-D have been reported in the Rhesus immunization registry to date. Yet, the allo- or autoantibody nature of anti-D has not been determined. Based on a complete serologic investigation in C-1, we conclude that weak D type 4.0 individuals may produce alloanti-D. The presence of autoanti-D in weak D type 4.0 individuals may not weaken the case for our hypothesis because it may be attributable to an autoimmune reaction unrelated to the expression of this weak D type. However, other complete documented cases are required to definitively conclude this.

In weak D type 4.2.2 individuals, this report showed these anti-D to be alloantibodies. This conclusion is in accordance with the incomplete serologic data reported in previous studies. In the only weak D type 11 individual producing anti-D, the serologic investigation allowed us to conclude that it was an autoantibody. The discordance between our result and the one listed in the Rhesus immunization registry reporting an alloanti-D without serologic data reinforces our hypothesis that discussions about anti-D in weak D patients should systematically be based on the four different tests discussed previously to determine whether anti-D is an allo- or an autoantibody. In the only weak D type 15 individual producing anti-D, data obtained with a complete serologic investigation demonstrated that the anti-D was an alloantibody. This result was in accordance with the data reported by Wagner et al. Finally, in the only weak D type 33 individual producing anti-D, the latter was demonstrated to be an autoantibody.

The documentation of anti-D (auto- or alloantibody) is a major issue for weak D individuals. Consequences are recommendations provided for transfusion strategy and to pregnant women regarding the frequency of the weak D types. The first concern is that anti-D production may only be studied in individuals expressing a D variant whose frequency is high enough for it to be spotted. The second concern is that only complete serologic investigation should be taken into account. Therefore, the demonstration of only autoanti-D in individuals expressing a given weak D type should lead to transfusing patients expressing the same weak D type with D+ RBC units and not giving anti-D immunoprophylaxis to pregnant women. So our recommendation is to transfuse weak D type 1 and type 2 patients with D+ RBC units and not give anti-D immunoprophylaxis to pregnant women of these types. On the other hand, the demonstration of an alloanti-D immunization in an individual expressing a given weak D may lead to transfusing patients expressing the same weak D type with D− RBC units, and to proposing anti-D immunoprophylaxis to pregnant women of these types.

Finally, the lack of anti-D immunization is not a prerequisite for labeling a weak D type. However, the notion of anti-D immunization should be taken into account. The present study clearly confirms that the RBCs of individuals with some weak D types carry altered D antigens, as alloanti-D was shown to be produced by patients expressing a D variant associated with RHD variants encoding amino acid substitutions in the membrane-spanning or the cytoplasmic domain of the D protein, contrary to the concept that alloanti-D may be produced by patients expressing a D variant associated with RHD polymorphisms encoding amino acid substitutions in the extracellular loops of the D protein. These data point out that the discrimination between weak D types and partial D may be a “delicate affair” as the serologic definition, the predicted location of amino acid polymorphisms deduced from molecular sequences, or the notion of anti-D immunization may be flawed. Consequently, to make accurate clinical decisions in terms of transfusion policy and anti-D immunoprophylaxis, alloanti-D production in given weak D types should be the only criterion to consider.

Acknowledgments

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A review of the JR blood group system

L. Castilho and M.E. Reid

The JR blood group system (ISBT 032) consists of one antigen, Jr\(^a\), which is of high prevalence in all populations. The rare Jr(a–) phenotype has been found mostly in Japanese and other Asian populations, but also in people of northern European ancestry, in Bedouin Arabs, and in one Mexican. Anti-Jr\(^a\) has caused transfusion reactions and is involved in hemolytic disease of the fetus and newborn. The Jr\(^a\) antigen is located on ABCG2 transporter, a multipass membrane glycoprotein (also known as the breast cancer resistance protein, BCRP), which is encoded by the \(ABCG2\) gene on chromosome 4q22.1. The Jr(a–) phenotype mostly results from recessive inheritance of \(ABCG2\) null alleles caused by frameshift or nonsense changes.

Anti-Jr\(^a\) is stimulated by either transfusion or pregnancy. A review of the literature indicates that anti-Jr\(^a\) may be clinically significant because it has been implicated in cases of hemolytic disease of the fetus and newborn (HDFN)\(^{16,17,18,19}\) and hemolytic transfusion reactions (HTRs).\(^{22–28}\) Anti-Jr\(^a\) has caused significant transfusion reactions as well as severe HDFN in some cases,\(^{15–18}\) including two cases of fatal HDFN.\(^{17,18}\)

In 1990, the Jr\(^a\) antigen was placed in the 901 series of high-incidence antigens by the International Society of Blood Transfusion (ISBT) and assigned \#901005.\(^29\)

For many years, numerous laboratories using various methods have failed to characterize Jr\(^a\), and their attempts to immunoprecipitate and immunoblot the antigen using human anti-Jr\(^a\) were unsuccessful.

The rare Jr(a–) phenotype appeared to be inherited as an autosomal recessive trait as the children of Jr(a+) parents type Jr(a–), but the gene controlling the expression of Jr\(^a\) was not known until two recent studies\(^{30,31}\) that provided genetic and molecular evidence that null alleles in \(ABCG2\) define the Jr(a–) phenotype.

Jr Is Carried on ABCG2 Transporter and Encoded by ABCG2 Gene

Recently, in an attempt to identify the gene responsible for Jr\(^a\) expression, two groups using different approaches\(^{30,31}\) established the connection between the Jr(a–) blood group phenotype and ABCG2.

Zelinski and coworkers\(^30\) conducted a homozygosity-by-descent (HBD) mapping study to identify the chromosomal region containing the gene responsible for Jr\(^a\) expression. Genomic DNA was extracted from stored samples of six Jr(a–) individuals (four probands). The authors performed analysis of single nucleotide polymorphisms (SNPs), (GeneChip Human Mapping, 250K Nsp array, Affymetrix, Santa Clara, CA) and identified a homozygous region of 397,000 bp located at chromosome 4q22.1 that contained four validated genes: \(MEPE\), \(SPP1\), \(PKD2\), and \(ABCG2\), but only the product of \(ABCG2\) was known to be expressed on red blood cells (RBCs). DNA sequence analysis defined three nonsense \(ABCG2\) alleles (c.376C>T, c.706C>T, and c.736C>T) in the six study subjects.
Because HMR0921 monoclonal antibody (MoAb) reacted weakly with RBCs from humans, Saison and coworkers explored the existence of Jr in different mammalian species by analyzing their RBCs by flow cytometry using HMR0921. They observed that cat RBCs reacted much more strongly than human RBCs and decided to identify the antigen recognized by the HMR0921 MoAb on cat RBCs. Using a lysate from cat RBCs, they immunoprecipitated a single protein of around 70 kDa, identified by mass spectrometry as abc2, encoded by the cat ortholog (abcg2) of the human transporter gene ABCG2. They transfected K-562 cells with ABCG2 and observed strong expression of ABCG2 as well as Jr at the surface of the ABCG2-transfected K-562 cells. Using these cells to immunoprecipitate ABCG2 with the HMR0921, they verified expression of Jr on RBCs and concluded that Jr is carried on ABCG2. Further studies including Western blot analysis proved that ABCG2 was absent in the RBC membrane of Jr(a−) individuals. Sequencing of ABCG2 in a cohort of 18 unrelated Jr(a−) probands identified eight different mutations in ABCG2: five frameshift mutations (c.187_197delATATTATCGAA, c.542_543insA, c.791_792delTT, c.875_878dupACTT, c.1111_1112delAC) and three nonsense mutations (c.376C>T, c.706C>T, c.730C>T; Table 1). Interestingly, later the nonsense mutation c.706C>T was reported to be present in homozygous state in two subjects and the nonsense mutation c.376C>T was present in homozygous state in three subjects. The remaining individual was homozygous for the missense c.34G>A mutation and heterozygous for the nonsense mutation c.706C>T.

This study, the first to use an HBD gene mapping strategy to identify a gene for a blood group system, provided genetic proof that the Jr(a−) phenotype is defined by the ABCG2 null allele.

The reports of Zelinski et al. and Saison et al., published 42 years after the first examples of anti-Jr, provided the information that Jr is carried on the ABCG2 transporter and encoded by the ABCG2 gene. Based on these results, Jr, previously designated ISBT 901.005, was promoted from the 901 series of high-incidence antigens to a blood group system (JR or ISBT 032) by the ISBT Working Party on Red Cell Immunogenetics and Blood Group Terminology in July 2012 (www.isbtweb.org/, Working Party on Red Cell Immunogenetics, J.R. Storry, personal communication, 2012).

**ABCG2 Gene and ABCG2 Glycoprotein**

ABCG2 is located on chromosome 4q22.1. It is composed of 16 exons (15 coding exons) that span approximately 68.6 kb of gDNA. The gene encodes a 655–amino acid, 72.6-kDa ABCG2 protein. The amino acid sequence of the wild-type ABCG2 protein is shown in Figure 1.
ABCG2 (breast cancer resistance protein [BCRP], mitoxantrone resistance protein), a well-studied molecule with more than 2000 publications, is an abbreviation for ATP-binding cassette (ABC), subfamily G, member 2. ABC transporters form one of the largest protein families encoded in the human genome, and more than 48 human ABC protein genes have been identified.

ABCG2 is predicted to pass through the membrane six times and has a single N-linked glycan. The intracellular amino terminal harbors a nucleotide-binding domain with Walker A, Walker B, and ABC signature motifs. The functional molecule is likely a homodimer or homotetramer in the membrane. Figure 2 depicts the predicted structure of the ABCG2 protein in the membrane and shows the location of amino acid changes encoded by nonsense and missense alleles.

ABCG2 has high expression in the placenta and blood–brain barrier and has low expression in epithelial cells of small and large intestines, liver ducts, colon, and lobes of the breast, in endothelial cells of veins and capillaries, in brain microvessel endothelium, and in stem cells, lung, and the apical membrane of proximal tubules of the kidney.

**Functional Aspects of the ABCG2 Glycoprotein**

Numerous studies in Abcg2−/− mice have suggested roles for ABCG2 in a variety of physiologic processes. ABCG2, also named BCRP, is known to be an essential transporter in cell detoxification and has a wide variety of substrates. It is a high-capacity transporter of urate, it appears to have a role in folate and porphyrin homeostasis, it may protect normal cells from toxic agents or metabolites, it may play a role in removing xenobiotics from the brain, and it may be involved in brain-to-blood efflux. It transports a wide variety of drugs and does not require glutathione for transport of electroneutral amphipathic compounds. It also appears to play a major role in the multidrug resistance phenotype of several cancer cell lines and solid tumors, posing a problem in chemotherapy. The Gln126Stop and Gln141Lys variants of ABCG2 are associated with an increased risk for gout. In a recent paper, Tiribelli et al. demonstrated that the Gln141Lys variant is associated with poor outcome in patients with acute myeloid leukemia receiving idarubicin-based chemotherapy.

**Molecular Bases of Jr(a−) and Jr(a+W−) Phenotypes**

The Jr(a−) phenotype results from the recessive inheritance of ABCG2 null mutations caused by frameshift or nonsense changes, and the Jr(a+W−) phenotype results from missense nucleotide changes (Table 1, Fig. 2).

**Jrα Antigen**

Jrα is fully developed on cord blood cells. The antigen is resistant to the treatment of RBCs with papain, trypsin, chymotrypsin, pronase, neuraminidase, and 2-aminoethyliothiouronium bromide.

**Clinical Significance**

Anti-Jrα is generally IgG (some are IgG1; some are a mixture of IgG1 and IgG3) and reacts best by the antiglobulin test, especially when ficin- or papain-treated RBCs are used. IgM anti-Jrα was found in the plasma of two Jr(a−) brothers who had not been transfused, and it has been detected in

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**Fig. 1** Amino acid sequence of ABCG2 protein. Amino acid sequence taken from GenBank, accession NP_004818.2.

**Fig. 2** Predicted topology of ABCG2 protein with location of amino acid changes encoded by nonsense and missense alleles. The single-letter code is used for amino acids. RBC = red blood cell; S = disulfide bond; X = stop codon.
untransfused Jr(a–) women during their first pregnancy.\textsuperscript{12,13} Some anti-Jr\textsuperscript{a} may bind complement.\textsuperscript{43,57} A collaborative study of irregular erythrocyte antibodies in Japan showed that anti-Jr\textsuperscript{a} is more frequently determined in pregnant patients than in nonpregnant patients.\textsuperscript{58}

Anti-Jr\textsuperscript{a} may be stimulated by transfusion\textsuperscript{22} or by pregnancy.\textsuperscript{7,10} Rare cases of hemolysis associated with transfusion have been reported, mostly as delayed HTRs.\textsuperscript{7,22–26} One patient with anti-Jr\textsuperscript{a} in the plasma developed rigors after 150 mL of serologically incompatible blood had been transfused,\textsuperscript{24} and in another patient chromium-51 cell survival studies indicated reduced RBC survival.\textsuperscript{7} In contrast, another patient with anti-Jr\textsuperscript{a} was transfused with three units of Jr(a+) blood in an emergency situation,\textsuperscript{13} and no symptoms of either an immediate or a delayed HTR were seen, although the titer of anti-Jr\textsuperscript{a} increased from 32 to 2048 on the 20th day after transfusion and later fell to 64 (35 days after transfusion). Kwon et al.\textsuperscript{27} described two cases of anti-Jr\textsuperscript{a} in the setting of incompatible transfusions, one without consequences despite multiple transfusions of Jr(a+) blood and the other leading to an acute HTR. Ogasawara and Mazuda\textsuperscript{59} studied 20 plasma samples containing anti-Jr\textsuperscript{a} and reported that none mediated phagocytosis of RBCs (in vitro). Garratty et al.\textsuperscript{60} reported that only one of eight anti-Jr\textsuperscript{a} samples had a positive monocyte monolayer assay, suggesting clinical significance.

Anti-Jr\textsuperscript{a} has been responsible for a positive direct antiglobulin test on RBCs from newborns and, rarely, HDFN.\textsuperscript{4,9,12,13} A review of the literature indicates that anti-Jr\textsuperscript{a} has caused mild and even severe cases of HDFN with no evidence of hemolysis.\textsuperscript{11–21} There is one report of a fatality associated with anti-Jr\textsuperscript{a} alloimmunization.\textsuperscript{17} Collectively, these data suggest that Jr\textsuperscript{a}, like Kell and Gerbich,\textsuperscript{61,62} is expressed on erythroid progenitor cells and that in these cases of HDFN, anti-Jr\textsuperscript{a} causes suppression of erythropoiesis rather than hemolysis.

**Concluding Remarks**

To date, nearly 1300 synonymous and nonsynonymous SNPs in the gene sequence of \textit{ABCG2} have been described (http://www.ncbi.nlm.nih.gov/snp), and therefore, additional diversity within the JR blood group system is still expected.

Jr(a–) individuals provide a large cohort of “natural knockouts” for \textit{ABCG2} (\textit{ABCG2}–/–), opening the opportunity to study the exact role and function of \textit{ABCG2} in humans under normal physiology and in pathologic conditions such as cancer.

**References**


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Antigens in the SC blood group system are expressed by the human erythrocyte membrane-associated protein (ERMAP). Two molecular bases have been reported for the Scnull phenotype: SC*307del2 and SC*994C>T. We report our investigation of the molecular background of five Scnull individuals from the Pacific Islands and describe the successful transfusion of Sc3+ blood to a patient with anti-Sc3 in her plasma. SC (ERMAP) exons 2, 3, and 12 and their flanking intronic regions were analyzed. The SC*994C>T change introduces a restriction enzyme cleavage site for Tsp45I, and polymerase chain reaction (PCR) products from exon 12 were subjected to this PCR–restriction fragment length polymorphism (RFLP) assay. The five samples had the variant SC*994T/T. One sample, from a first cousin of one Marshallese proband, was heterozygous for SC*1514C/T (in the 3’ untranslated region); the other four samples were SC*1514C/C (consensus sequence). Samples from white donors (n = 100) and African American donors (n = 99) were tested using the Tsp45I PCR-RFLP assay; all gave a banding pattern that was consistent with the SC*994C/C consensus sequence. In all five samples, our analyses showed homozygosity for the nonsense nucleotide change SC*994C>T in an allele carrying the nucleotide associated with Sc1. Further investigation determined that one of the probands reported previously with the SC*994C>T change was from the Marshall Islands (which form part of the Micronesian Pacific Islands) and the other was from an unspecified location within the large collection of Pacific Islands. Taken together, the five known probands with the SC*994C>T silencing nucleotide change were from the Pacific Islands. *Immunohematology* 2013;29:69–72.

**Key Words:** Scianna, blood groups, Micronesians, Pacific Islanders, blood group antigen, ERMAP

The Scianna (SC) blood group system (ISBT 013) consists of seven antigens: Sc1 (SC1), Sc2 (SC2), Sc3 (SC3), Rd (SC4), STAR (SC5), SCER (SC6), and SCAN (SC7). Sc1, a high-prevalence antigen, and Sc2, a low-prevalence antigen, are antithetical; Sc3, STAR, SCER, SCAN are high-prevalence antigens, and Rd is a low-prevalence antigen. These antigens are expressed by the human erythrocyte membrane-associated protein (ERMAP), which is a member of the immunoglobulin superfamily.1 The gene encoding this membrane protein, ERMAP, is located on chromosome 1p34.2, close to RH and RHCE.2,3

The SC−1,−2,−3 type represents the Scnull phenotype, and people with this phenotype are apparently healthy. Two molecular bases for this phenotype have been reported: a deletion of two nucleotides in exon 3 of SC (SC*307del2; ISBT provisional allele name SC*01N.01) in a Saudi Arabian, and a nucleotide change in exon 11 (now known to be exon 12) of SC (944C>T, Arg332Stop; ISBT provisional allele name SC*01N.02) in two probands, one with a concomitant 1514C>T change in the 3’-untranslated region of SC.1 The ethnicity for the latter two patients was not stated in that paper (3rd and 4th entries in Table 1).4 Since publication of the Flegel et al paper in 2005,5 it has been shown that ERMAP consists of 12, not 11, exons (reviewed by Brunker and Flegel6). Based on this knowledge, we believe the SC*944C>T nucleotide change occurs in exon 12. The reference sequence for the 12-exon ERMAP (SC) model in the human genome can be found at http://www.ncbi.nlm.nih.gov/gene/114625, and in the RefSeqGene it is found at NM_001017922.1. When we began the study described here, we used transcript NM_018538.3, which has 11 exons; fortunately, as the additional exon is upstream from the initiation sequence in both transcripts, the sequence for blood group antigen expression, whether the previous exon 11 or updated exon 12 is used, is the same. Throughout this manuscript, we use the 12-exon transcript model.

We report the molecular background of the Scnull phenotype in five individuals (three probands who were found because they had anti-Sc3 in their plasma) and for the first time report that the SC*994C>T nucleotide change is associated with Pacific Islanders. We also describe a case in which crossmatch-incompatible Sc3+ blood was successfully transfused to a patient with anti-Sc3 in her plasma.

**Case Report**

**Case 1**

Case 1 was a 24-year-old from the Marshall Islands with metastatic ovarian cancer. Despite considerable effort, we were unable to obtain a history regarding transfusion or pregnancy.
During routine testing her plasma was shown to contain an alloantibody to a high-prevalence antigen, strongly reactive in the indirect antiglobulin test (IAT), which was identified as anti-Sc3. Her red blood cells (RBCs) typed RH:1,2,–3,–4,5 (R_1 R_2); SC:–1,–2,–3, and typings for antigens in other blood group systems were unremarkable (and are not provided to protect the identity of the proband). Samples from two of her relatives (a first cousin and one for whom we could not obtain the relationship) were also tested.

Case 2
Case 2 was a 28-year-old from the Pacific Islands (despite considerable effort, a more specific location could not be ascertained), who was identified during her third pregnancy. She had no history of having been transfused, and there was no information about her prior pregnancies. Her plasma contained an alloantibody to a high-prevalence antigen, strongly reactive in the IAT, which was identified as anti-Sc3. Her RBCs typed RH:1,2,3,4,5 (R_1 R_2); SC:–1,–2,–3; typings for antigens in other blood group systems were unremarkable (and are not provided to protect the identity of the proband).

Case 3
Case 3 was a 51-year-old Marshallese female who had a diagnosis of type 2 diabetes mellitus and anemia secondary to chronic disease, renal failure, and recurrent urosepsis. The patient had received two leukocyte-reduced components approximately 3 months before being admitted to the hospital and had a history of three pregnancies, which required cesarean delivery. She was admitted to the hospital with fever and nausea, a hemoglobin level of 7.2 g/dL, and a hematocrit of 20.9 percent. Her plasma agglutinated all RBC samples in the IAT (titer 16, score 49) except her own. Her RBCs typed RH:1,2,–3,–4,5 (R_1 R_2); SC:–1,–2,–3, and her antibody was identified as anti-Sc3. Her RBCs were unremarkable for antigens in other blood group systems (and test results are not provided to protect the identity of the proband). Her hemoglobin dropped to 5.5 g/dL and she required transfusion. As no SC:–1,–2,–3 blood was available for transfusion, she was transfused with three units of phenotype-matched but crossmatch-incompatible leukocyte-reduced packed RBCs. The patient appeared to tolerate the transfusion well, and her hemoglobin increased to 11.1 g/dL, and her hematocrit, to 33.1 percent. On the fifth day after transfusion, she was discharged from the hospital with a hemoglobin of 10.2 g/dL and a hematocrit of 30.7 percent.

Materials and Methods

Samples and Hemagglutination Testing
Peripheral blood samples were freshly collected or recovered from liquid nitrogen storage after the institutional review board approved the protocol. Antigen testing, antibody detection, and antibody identification were performed in tubes using various enhancement media. RBC antigen typing for the Sc antigens was performed with in-house, unlicensed, single-donor-source polyclonal antibodies from our collections.

Polymerase Chain Reaction Sequence Analysis of Genomic DNA
Genomic DNA was isolated from whole blood (QIAamp DNA Blood Mini Kit, QIAGEN, Inc., Valencia, CA) from the three probands and from the two relatives of one of the Marshallese (Case 1). The regions of SC that included and flanked exons 2, 3, and 12 were amplified separately using the oligonucleotide primers listed in Table 1. The primers were synthesized (Life Technologies, Inc., Gaithersburg, MD). Five microliters of DNA per reaction was amplified by 5 U of Taq DNA polymerase (HotStarTaq, QIAGEN) in a 50-μL reaction mixture containing 1.5 mM MgCl_2 for exon 2 and 2.0 mM MgCl_2 for exons 3 and 12, 1× polymerase chain reaction (PCR) buffer, 0.2 mM deoxynucleoside triphosphates, and 100 ng of forward and reverse primers. Amplification was achieved over 35 cycles with a final extension time of 10 minutes. The PCR products were then treated (ExoSAP-IT, USB, Cleveland, OH) before sequencing (Sanger dideoxy sequencing, GENEWIZ, South Plainfield, NJ). The PCR products were sequenced in both directions.

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</table>
PCR–Restriction Fragment Length Polymorphism Analysis

Analysis of the nucleotide sequence of the variant revealed that the SC*994C>T nucleotide change induced a Tsp45I restriction enzyme cutting site, so the variant (SC*994T) could be distinguished from the consensus sequence (SC*994C) by a PCR–restriction fragment length polymorphism (RFLP) assay. PCR amplicons of 948 bp for exon 12 (see Table 1) were digested (Tsp45I restriction enzyme, New England Bio Labs, Ipswich, MA) and run on 8 percent polyacrylamide gels in a 1× Tris-borate-EDTA buffer, at a voltage of 200 V for 2 hours. The expected sizes for the consensus sequence and variant are listed in Figure 1. Samples from African Americans (n = 99) and from whites (n = 100) were amplified for this region of SC and analyzed.

Results

Hemagglutination

RBCs from each proband and the two Scnull family members typed SC:–1,–2,–3. Plasma of the three probands contained anti-Sc3, each reacting 3+ in the IAT, and were mutually compatible. The plasma of the compatible (Scnull) first cousin of the Case 1 proband had no detectable irregular antibody. Plasma from the other relative of Case 1 was not available for testing.

Sequence Analysis of Genomic DNA and PCR-RFLP Assay

Sequence analyses of the three probands and the two relatives demonstrated consensus sequence for exons 2 and 3, which is predicted to encode the SC1,–2 phenotype and eliminated the published 307del2 (provisional ISBT allele name SC*01N.01) as a cause of the Scnull phenotype in these people. However, all five samples were homozygous for the SC*994C>T change (provisional ISBT allele name SC*01N.02; GenBank accession number JF828030) in exon 12, which is predicted to encode Arg332Stop, and revealed the cause of their Scnull phenotype. The SC*994C>T change in exon 12 was confirmed using the Tsp45I restriction enzyme assay. Four samples were homozygous for SC*1514C/C (consensus), and one sample, the first cousin of Case 1, was heterozygous for SC*1514C/T. For a summary of these findings, see Table 2.

RFLP analyses of the PCR products using genomic DNA from the five samples confirmed the homozygous SC*994C>T change (Fig. 1). A cohort of samples from two unrelated ethnic groups, 100 random whites and 99 random African Americans, were screened using this assay; all demonstrated the expected digestion pattern for the consensus SC*994C/C.

Discussion

We describe three Scnull probands (five individuals in total) from the Pacific Islands who have a homozygous nonsense nucleotide change of SC*994C>T in exon 12 in an allele carrying the nucleotide associated with the Sc1 antigen. In the publication of Flegel and colleagues,4 two Scnull probands with the SC*994C>T change were reported to be living in California. Since that original description (in 2005), we were able to obtain the ethnicity of these probands: the one with SC*994T, 1514T was from an unspecified location within the large collection of Pacific Islands, and the one with SC*994T, 1514C was from a more precisely determined location, the Republic of...
of the Marshall Islands. Taking the cases together, of the five probands described with the SC*994C>T silencing nucleotide change, two were from an unspecified location within the Pacific Islands and three were definitely Marshallese. This provides strong evidence for an association between SC*994T and the Marshallese or Pacific Islander populations.

We found further evidence for an association between Pacific Islanders and Scnull in two of three reports. The first reported Scnull phenotype was in 1973 (then Sm– Bu(a–), now SC:−1,−2 or Sc1–Sc2–) in a proband from a Marshallese pedigree.7 The second Scnull proband was reported to be a Caucasian male.8 The third reported proband was a Melanesian girl from Papua New Guinea; her mother was also SC:−1,−2. In testing 29 additional samples, six more Scnull were found; four were among family members and two were unrelated villagers.9 To our knowledge, DNA-based testing was not performed on these cases.

Although four individuals reported in this manuscript were SC*1514C/C (consensus), the first cousin of the Case 1 proband was heterozygous SC*1514C/T in the 3′ untranslated region of exon 12 (rs111730740). This change in two probands (one in this report [heterozygote] and one in the original report [homozygote]7) shows there are two alleles with SC*994T, and it remains to be determined which is the founder allele. Interestingly, in the Single Nucleotide Polymorphism Database, the SC*1514C>T variant has been found in the heterozygous state (with the consensus SC*944C) in an individual from the Bushman population.

In the South Pacific, there are three cultural areas, namely Melanesia, Micronesia (which includes the Marshall Islands), and Polynesia. People from each of these three areas can be referred to as “Pacific Islanders.” In terms of blood groups, it is well known that Polynesians, who are Pacific Islanders, harbor the rare JK:−1,−2,−3 phenotype.10 In this paper, we reveal that a second rare null phenotype (SC:−1,−2,−3) is also found among Pacific Islanders. Thus, during the course of an antibody investigation in which an antibody to a high-prevalence antigen is suspected in an individual from the geographic area in the South Pacific, consider the possibility of either anti-Jk3 or anti-Sc3.

Anti-Sc3 has been reported to cause no or mild delayed transfusion reactions and mild hemolytic disease of the fetus and newborn.11,12 Case 3 in this paper documents an additional case in which the anti-Sc3 was apparently clinically insignificant in transfusion.

Acknowledgments

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References


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The Diego blood group system (DI) currently encompasses 22 antigens. Three of the antigens are of high prevalence and the other 19 are of low prevalence. The antigens of the Diego blood group system are carried on the erythroid band 3 protein anion exchanger 1 (AE1), the product of a single gene, SLC4A1 (solute carrier family 4, anion exchanger, member 1). AE1 is a member of a family of three anion exchangers or transporters expressed in a variety of tissues. This protein is involved in carbon dioxide transport from tissues to lungs. It is also found in the kidney, where it is involved in acid secretion. Antibodies to Diego system antigens with the exception of anti-Di\textsuperscript{a}, -Di\textsuperscript{b}, -Wr\textsuperscript{a}, -ELO and -DISK do not seem to be of clinical significance for transfusion or of importance in hemolytic disease of the fetus and newborn. *Immunohematology* 2013;29:73–81.

**Key Words:** Diego, AE1 protein, band 3, SLC4A1

**History**

Currently there are a total of 22 antigens in the Diego blood group system. Three of the antigens are of high prevalence, Di\textsuperscript{b}, Wr\textsuperscript{b}, and DISK. The other 19 antigens are of low prevalence. Within the 22 antigens there are only three sets of antithetical antigens: Di\textsuperscript{a}/Di\textsuperscript{b}, Wr\textsuperscript{a}/Wr\textsuperscript{b}, and Wu/DISK. None of the other 17 low-prevalence antigens has an antithetical high-prevalence antigen yet described. Figure 1 illustrates the molecule that carries the Diego system antigens.

The discoveries of these antigens exemplify the tenacity and curiosity of many immunohematologists and researchers. These individuals investigated many serum samples and in some cases came out with more questions (findings) than answers. On many occasions the new antibody discovered was present in sera that were under investigation for some other reason and contained antibody(ies) to other low-prevalence antigens. In other instances the antibody was discovered because it caused hemolytic disease of the fetus and newborn (HDFN) or an incompatible crossmatch in the presence of a negative antibody detection test.

The first antigen assigned to the Diego system, Di\textsuperscript{a}, was reported by Layrisse et al. in 1955. They reported an antibody to a low-prevalence antigen that caused a fatal HDFN.\textsuperscript{1}
Mutations of the band 3 gene have been implicated in Southeast Asian ovalocytosis (SAO), hereditary spherocytosis, congenital acanthocytosis and distal tubular acidosis. Band 3 SAO has the Memphis I variant and is not functional as anion transporter. Another variant is known as band 3 Memphis II. Spring et al. reported that the Di^a antigen is carried on the Memphis II variant of band 3.

It has been reported that naturally occurring antibodies to low-prevalence antigens in the Diego system are common in the plasma of patients with hyperactive immune systems, e.g., those with autoantibodies. This may be related to the exposure of the senescent cell antigen, which resides on protein residues in band 3.

**Band 3 and Glycophorin A**

Band 3 and glycophorin A (GPA) are the two most abundant integral proteins of the red blood cell (RBC) membrane (Figure 2). The extracellular domains of both proteins are highly polymorphic. Band 3 carries the antigens of the Diego blood group system, and GPA and glycophorin B (GPB) carry the antigens of the MNS system. There is evidence that band 3 and GPA associate in the RBC membrane, and the Wr^b antigen, although a product of the band 3 gene, requires a complex of GPA and band 3 for normal expression. The discovery of a novel GPA mutation (Ala84Pro) giving rise to aberrant Wr^b expression provided some of the initial information with regard to the site of interaction of the two proteins.

In contrast to GPA-related antigens (which are the result of events between two closely linked genes and different genetic mechanisms), the antigens on band 3 are exclusively caused by single-nucleotide polymorphisms (SNPs) in the band 3 gene, SLC4A1. All low-prevalence antigens in the DI system, with the exception of Bp^a, occur from extracellular amino acid substitutions. Some amino acid residues have been associated with more than one substitution, each giving rise to separate antigens, for example NFLD/BOW, Jn^a/KREP, and Hg^a/Mo^a. Table 1 lists the 22 phenotypes encoded by the alleles in the DI system and summarizes the alleles and amino acid substitutions.

**Effect of Enzymes on the Diego System Antigens**

The effect of enzymes on the Diego blood group antigens is directly related to their location. Band 3 has two α-chymotrypsin cleavage sites in the third extracellular loop, at Tyr553 and Tyr555. Therefore the antigens in the third loop are sensitive to α-chymotrypsin, and those in the fourth and

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**Fig. 2** Schematic diagram of the erythrocyte membrane (lateral view) showing major membrane-spanning proteins. Source: Kodippili GC, et al. Blood 2009;113:6237–45.
seventh loops are resistant. Data available for some of the DI system antigens indicate that they are resistant to the effects of dithiothreitol, sialidase, and acid. Refer to Table 2 for details on the effect of enzymes and other chemicals on the DI system antigens.

Antigens and Antibodies in the Diego System

Like most antigens, the low-prevalence antigens in the Diego system were discovered by first encountering the antibodies that define them. Most of the low-prevalence antigens in the Diego system, DI5 to DI21, are of very low prevalence. Some antigens have only been found in one family; therefore, information on their characteristics and clinical significance is very limited. It is important to mention that although the antigens are rare, the antibodies to them are very common. Table 3 summarizes the antibodies, their immunoglobulin types, and the limited information regarding their clinical significance.

**Di**<sup>a</sup> (DI1)

In 1955, Layrisse et al. reported an antibody to a low-prevalence antigen that caused a fatal HDFN. The Di<sup>a</sup> antigen is the result of a 2561C>T transition in exon 19, Pro854Leu, in the seventh ectoplasmic loop of band 3. The antibody was named Di<sup>a</sup> after the antibody producer, Mrs. Diego. Further family studies of the proposita and investigations using the maternal serum helped to unveil the interesting history of the Di<sup>a</sup> antigen and the genetic, anthropologic, and clinical relevance of the antigen.

The Di<sup>a</sup> antigen is characteristic of individuals of Mongoloid descent, and the DI<sup>a</sup>A alleles have never been found in individuals of unmixed European descent. The Di<sup>a</sup> antigen has been used as an anthropologic population migration marker. An example of this migration marker is the presence of the Di<sup>a</sup> antigen in Poles, which can be explained by the invasion of southern Poland by Tartars in the 13th to 17th centuries. Gene frequency in eastern Asians has been reported as 1 to 5 percent. In Korea and Tibet the frequency ranges from 7 to 8 percent. In some South American Indian tribes, the gene frequency can be as high as 40 percent; it is nonexistent in others. In Central American Indians, the frequency can also be high. In North American Indians, the frequency ranges from 2 to 12 percent in some tribes. The gene is absent in Eskimos.

Anti-Di<sup>a</sup> is most often RBC stimulated and mostly immunoglobulin (Ig) G1 and IgG3. A few examples of
agglutinins with anti-Di\textsuperscript{a} specificity have been reported in individuals with no known RBC exposure. A few examples of anti-Di\textsuperscript{a} have been shown to activate complement, and they have demonstrated the capability of causing in vitro hemolysis as well as causing severe immediate or delayed transfusion reactions.\textsuperscript{10} As previously mentioned, anti-Di\textsuperscript{a} has caused serious HDFN. It has been recommended that in populations at risk a Di(\(a^+\)) RBC be added to the routine antibody detection cells.

**Di\textsuperscript{b} (Di2)**

The Di\textsuperscript{b} antigen differs from Di\textsuperscript{a} by a single amino acid change, Leu854Pro, on band 3. The antigen is found in all populations. Di\textsuperscript{b} is weakly expressed in individuals with SAO, and it has also been reported as weakened in several Mexican American individuals.

**Anti-Di\textsuperscript{b}** was first reported by Thompson et al. in 1967.\textsuperscript{11} The antibody may cause mild cases of HDFN, yield a positive direct antiglobulin test (DAT), or exhibit no clinical manifestation. Anti-Di\textsuperscript{b} has caused moderate or delayed transfusion reactions. Anti-Di\textsuperscript{b} may show dosage in serologic testing. A few examples of autoanti-Di\textsuperscript{b} have been reported.\textsuperscript{12}

**Wr\textsuperscript{a} (Di3)**

The Wr\textsuperscript{a} antigen was first reported by Holman in 1953 and assigned to the DI system in 1995.\textsuperscript{13} Wr\textsuperscript{a} differs from the Wr\textsuperscript{b} antigen by a single nucleotide change, 1972G\(\rightarrow\)A, at exon 16, producing the amino acid change Glu658Lys.

Anti-Wr\textsuperscript{a} is a relatively common antibody and has a wide range of reactivity. The antibody can be an IgM agglutinin reacting at temperatures below 37\(^{\circ}\)C or an IgG agglutinin reacting only at the antiglobulin phase of testing (AGT). Anti-

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**Table 2. Effect of enzymes and other chemical treatments on the Diego system antigens**

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Ficin/ papain</th>
<th>Trypsin</th>
<th>α-chymotrypsin</th>
<th>Pronase</th>
<th>Sialidase</th>
<th>DTT (200 mM)</th>
<th>Acid</th>
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<tbody>
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<td>R</td>
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<td>S</td>
<td>PR</td>
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<td>S</td>
<td>R</td>
<td>R</td>
<td>PR</td>
<td>R</td>
<td>R</td>
</tr>
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<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
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<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
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<td>R</td>
<td>S</td>
<td>S</td>
<td>PR</td>
<td>R</td>
<td>PR</td>
</tr>
<tr>
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<td>V</td>
<td>PR</td>
<td>R</td>
<td>PR</td>
</tr>
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<td>PR</td>
</tr>
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<td>R</td>
<td>R</td>
<td>R</td>
<td>PR</td>
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</tr>
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<td>S</td>
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<td>PR</td>
<td>R</td>
<td>PS</td>
</tr>
<tr>
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<td>R</td>
<td>S</td>
<td>PS</td>
<td>PR</td>
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<td>PR</td>
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<td>R</td>
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<td>R</td>
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<td>S</td>
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<td>R</td>
<td>PR</td>
</tr>
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<td>PR</td>
</tr>
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<td>R</td>
<td>R</td>
<td>PR</td>
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<td>PR</td>
<td>PR</td>
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<td>PR</td>
<td>PR</td>
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<td>PR</td>
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<tr>
<td>Tr\textsuperscript{a}</td>
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<td>R</td>
<td>S</td>
<td>PS</td>
<td>PR</td>
<td>PR</td>
<td>PR</td>
</tr>
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<td>Vg\textsuperscript{a}</td>
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<td>R</td>
<td>S</td>
<td>S</td>
<td>PR</td>
<td>PR</td>
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</tr>
<tr>
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<td>R</td>
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<td>S</td>
<td>S</td>
<td>PR</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
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<td>R</td>
<td>S</td>
<td>PS</td>
<td>PS</td>
<td>PR</td>
<td>R</td>
</tr>
<tr>
<td>Wr\textsuperscript{a}</td>
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<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>Wr\textsuperscript{b}</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>Wu</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>PR</td>
<td>R</td>
<td>PR</td>
</tr>
</tbody>
</table>

**NOTE:** The effect of the listed chemicals on the DI antigens was collected from many of the references listed at the end of this review.

**Table 3. Immunoglobulin type and clinical significance of Diego system antibodies**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Immunoglobulin class</th>
<th>HDFN</th>
<th>HTR</th>
</tr>
</thead>
<tbody>
<tr>
<td>BOW</td>
<td>IgG, some IgM</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>Bp\textsuperscript{a}</td>
<td>IgM</td>
<td>No</td>
<td>NI</td>
</tr>
<tr>
<td>Di\textsuperscript{a}</td>
<td>IgG</td>
<td>Mild–severe</td>
<td>None–severe</td>
</tr>
<tr>
<td>Di\textsuperscript{b}</td>
<td>IgG</td>
<td>Mild</td>
<td>No–moderate</td>
</tr>
<tr>
<td>DISK</td>
<td>IgM, IgG</td>
<td>Possible</td>
<td>Possible</td>
</tr>
<tr>
<td>ELO</td>
<td>IgG</td>
<td>Mild–severe</td>
<td>NI</td>
</tr>
<tr>
<td>Fr\textsuperscript{a}</td>
<td>IgG, IgM</td>
<td>Pos DAT only</td>
<td>NI</td>
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<tr>
<td>Hg\textsuperscript{a}</td>
<td>IgM, IgG</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>Jn\textsuperscript{a}</td>
<td>IgM</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>KREP</td>
<td>IgM</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>Mo\textsuperscript{a}</td>
<td>IgM, IgG</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>NFLD</td>
<td>IgM, IgG</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>Rb\textsuperscript{a}</td>
<td>IgM</td>
<td>NI</td>
<td>NI</td>
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<td>IgM, IgG</td>
<td>NI</td>
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<td>IgM, IgG</td>
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<td>IgM, IgG</td>
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<td>NI</td>
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<td>IgM</td>
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<td>NI</td>
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<td>WARR</td>
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<td>Mild</td>
<td>NI</td>
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<td>IgM</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>Wr\textsuperscript{a}</td>
<td>IgM, IgG</td>
<td>Mild–severe</td>
<td>None, severe, immediate, delayed</td>
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<tr>
<td>Wr\textsuperscript{b}</td>
<td>IgM, IgG</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>Wu</td>
<td>IgM, few IgG</td>
<td>NI</td>
<td>NI</td>
</tr>
</tbody>
</table>

HDFN = hemolytic disease of the fetus and newborn; HTR = hemolytic transfusion reaction; DAT = direct antiglobulin test; Ig = immunoglobulin; NI = no information.

**NOTE:** The HDFN and HTR information presented was collected from many of the references listed at the end of this review.
Wr² has caused severe HDFN and hemolytic transfusion reactions (HTRs). It has been speculated that there may be some examples of anti-Wr² that are benign, as routine antibody detection reagent RBCs do not contain the Wr² antigen and HTR caused by anti-Wr² has not been reported since the widespread use of immediate spin (IS) and electronic compatibility testing.¹⁴

**Wr² (DI4)**

The Wr² antigen was first described in 1971 by Adams et al., but not assigned to the DI system until 1995.¹⁵ The Wr² antigen expression is dependent on the presence of GPA and band 3.

Wr² is absent from En(a–) erythrocytes, which lack GPA. However, GPA from En(a+), Wr(b–) RBCs has an amino acid sequence identical to that of GPA from En(a+), Wr(b+) erythrocytes. Wr² requires the interaction of GPA with erythrocyte integral membrane protein, band 3.⁴,⁷,¹⁶

The Wr(b–) phenotype with normal GPA may be explained by the change from a negatively to a positively charged residue at position 658 of band 3. Erythrocytes that are Hill-positive and Dantu-positive carry a hybrid sialoglycoprotein containing part of the GPA and GPB amino acid sequence and are also Wr(b–).¹⁷

Owing to the rarity of the Wr(b–) phenotype, data on the clinical significance of anti-Wr² are scanty. One alloanti-Wr², in a Wr(a–b–), GPA-variant individual, was evaluated by chemiluminescence assay, and the findings suggested the antibody was likely to cause destruction of incompatible RBCs.¹⁸ Many examples of autoanti-Wr² have been reported. Some examples of the autoantibody have been clinically significant and others benign.

**Wd² (DI5)**

The Wd² antigen was reported in two large nuclear Hutterite families. In 1981, Lewis and Kaita first reported the antibody defining the Wd² antigen.¹⁹ Wd² is destroyed by α-chymotrypsin.²⁰ Wd² is the result of a single point mutation that causes the amino acid change Val557Met.

Anti-Wd² can be found in sera containing other low-prevalence antigens. The antibody often reacts as a saline agglutinin. Only one IgG example has been reported. No cases of HTR or HDFN have been reported.

**Rb² (DI6)**

The Rb² antigen was described by Contreras in 1978.²¹ Rb² is destroyed by α-chymotrypsin.²² Rb² is the result of a single point mutation that causes the amino acid change Pro548Leu.

The antibody can be found in sera containing antibodies to other low-prevalence antigens and often reacts as a saline agglutinin. Only one IgG example of anti-Rb² has been reported. No cases of HTR or HDFN have been reported.

**WARR (DI7)**

Coghlan et al. reported a case in 1991 of mild HDFN caused by an antibody to a low-prevalence antigen that they named WARR.²² The antigen is very rare and has been found in two related kindreds. The antigen was detected in the oldest member of the kindred, an absentee Shawnee. It is believed that the antigen may be unique to Native American individuals. Issitt and Anstee reported that even though the antigen is rare, the antibody is not.²² WARR is the result of a single point mutation that causes the amino acid change Thr552Ile.

**ELO (DI8)**

The low-prevalence RBC antigen ELO was detected in 1979 (unpublished observations cited by Tippett). The findings were published by Coghlan et al. in 1993.²⁴ ELO was assigned to the DI system in 1998, when Zelinski et al. published their findings that established the association between ELO and the gene controlling Diego blood group polymorphisms.²⁵ The ELO antigen (Arg432Trp) is carried in the first ectoplasmic loop of band 3.

HDFNs caused by anti-ELO have been reported. In experiments with enzyme-treated erythrocytes, Jarolim et al. reported that in the two anti-ELO samples they studied, one reacted with α-chymotrypsin and pronase-treated RBCs, which was an unexpected finding.²⁶

**Wu (DI9)**

The Wu antigen was also called Hov and Haakestad by different investigators. The antigen was reported by three different individuals and given three different names. In 1972, an abstract describing Moe¹¹ was published that mentioned a new low-prevalence antigen called Haakestad. Then in 1973, Szaloky et al.²¹ described an antibody they named anti-Hov, and in 1976, Kornstad described the Wu antigen.²³ In 1987, Kornstad published data that showed that Haakestad and Hov were the same antigen, and in 1992, Moulds et al.²¹ showed that Hov and Wu were the same antigen. Wu was selected as the official name. This antigen is well developed in two related kindreds. The antigen was detected in the oldest member, an absentee Shawnee. It is believed that the antigen may be unique to Native American individuals. Issitt and Anstee reported that even though the antigen is rare, the antibody is not.²² WARR is the result of a single point mutation that causes the amino acid change Thr552Ile.
later. Adsorption studies demonstrated that RBCs positive for either antigen will remove all three antibodies. This is likely to happen as these antigens are the result of amino acid substitutions at the same position, and cross-reactivity surely occurs. The Wu antigen is antithetical to the DISK antigen (DI22).

**Bp** (DI10)

In 1964, Cleghorn (unpublished observations 1962–1967) found a serum that contained multiple antibodies to multiple known low-prevalence antigens and also contained a new specificity that he named anti-Bp. The Bp antigen is associated with the amino acid substitution Asn569Lys in the transmembrane domain close to the third extracellular loop. This area is sensitive to trypsin, α-chymotrypsin, pronase, and papain.

**Mo** (DI11)

Kornstad and Brocteur, in 1972, described the first example of anti-Mo in a serum being used to screen random donors for the low-prevalence antigen Jn. Mo is the result of a mutation at the fourth ectoplasmic loop that changed the amino acid at position 656 (Arg656His). Another mutation at the same amino acid position gives rise to the Hg antigen (see next section). Mo antigens are not destroyed by trypsin, α-chymotrypsin, ficin, or pronase.

**Hg** (DI12)

Anti-Hg was reported by Rowe and Hammond in a serum that contained a warm reactive autoantibody along with anti-Pt, -Wd, and -BOW. The fourth ectoplasmic loop carries the Hg (Hughes) antigen and is associated with the amino acid residue substitution Arg656Cys. Hg is not destroyed by trypsin, α-chymotrypsin, ficin, or pronase.

**Vg** (DI13)

Young, in 1981, reported the presence of anti-Vg in one serum that also contained anti-Wu. Anti-Vg is not uncommon in sera containing multiple antibodies to low-prevalence antigens. Vg (Van Vugt), Tyr555His, is carried in the third ectoplasmic loop of band 3 and is destroyed by α-chymotrypsin and pronase.

**Sw** (DI14)

Anti-Sw was described by Cleghorn in 1959. In 1987, Contreras et al. reported heterogeneity among anti-Sw and reported the existence of a second antigen related to Sw. It was found that some Sw(a+) RBCs reacted with all examples of anti-Sw, but others only reacted with some of the Sw antisera. The difference was shown to be qualitative, not quantitative. The RBCs that reacted with all anti-Sw were said to contain two antigens, Sw and SW1. The RBCs that only reacted with some of the anti-Sw were said to be Sw(a+) but negative for SW1. Family studies confirmed the theory that the difference between the two antigens is a qualitative one. In addition, a serologic relationship between Sw, SW1, and the low-prevalence antigen Fr has also been reported.

Two mutations in exon 16 have been associated with the Sw antigen, 1937G>A, which changes Arg646Gln, and 1936C>T, which causes the Arg646Trp change.

**BOW** (DI15)

Anti-BOW was first described by Chaves et al. in 1988. The antibody caused an unexplained incompatible crossmatch. BOW is the result of a single point mutation that causes the amino acid change Pro561Ser, and it is carried in the third ectoplasmic loop of band 3. The antigen is destroyed by pronase and α-chymotrypsin. The BOW antigen is antithetical to the NFLD antigen.

**NFLD** (DI16)

Lewis et al., in 1984, described a new low-prevalence antigen, NFLD, in a white family of French extraction. In 1988, Okubo et al. described the presence of the NFLD antigen in the Japanese population. It is interesting to mention that tests with NFLD+ RBCs in Japan detected 67 examples of anti-NFLD, and most of these examples were single antibodies.

NFLD is associated with the amino acid residue substitution Pro561Ala as the result of the single nucleotide change 1681C>G. As expression of the BOW antigen is also the result of a substitution at amino acid residue 561 (Pro561Ser), NFLD and BOW can be considered to be antithetical antigens. Expression of NFLD is also associated with a second mutation, 1287A>T, which causes the amino acid change, Glu429Asp. This suggests that the NFLD epitope may be formed through an association or interaction between the first (residue 429) and third (residue 561) extracellular loops of band 3.

**Jn** (DI17)

Kornstad et al. reported a new antibody, anti-Jn, in a serum containing anti-Wr and anti-Bp. They presented studies of three unrelated individuals with Jn(a+) RBCs. Two of them presented with nucleotide substitutions of Pro556Ser. The third individual showed a change that predicted Pro556Ala and was later shown to define the KREP antigen (see next section). These changes were recognized at the serologic level.
before the polymorphisms were established by Poole et al. in 1997.43

KREP (DI18)

Found in 1997 during investigation of the second Jn(a+) proband, KREP was named after the antigen-positive donor and assigned to the Diego blood group system in 1998. This antigen is the result of an SNP at 1696C>G, which causes the amino acid change Pro556Ala. As expression of both Jn(a) and KREP antigens is the result of substitutions of Pro556, they can be considered antithetical antigens.43

Tr(a) (DI19)

Cleghorn found anti-Tr(a) in 12 of 18 sera that also contained anti-Wr(a).33 This antigen has been called Lanthois in some publications. Tr(a) is the result of a single point mutation that causes the amino acid change Lys551Asn.

Fr(a) (DI20)

The Fr(a) antigen has been reported in three Mennonite kindreds in Manitoba, Canada. A point mutation in exon 13 of SLC4A1 accounts for a Glu480Lys substitution in band 3, which controls Fr(a) antigen expression.44 Based on these results Fr(a) was assigned to the Diego blood group system. Fr(a) seems to be related to Sw(a). Both Sw(a+) SW1 and Sw(a+) SW2−1 will adsorb anti-Sw(a) and anti-Fr(a), but Fr(a+) RBCs will adsorb anti-Fr(a) but not anti-Sw(a).45

SW1 (DI21)

SW1 was documented in 1987, after being revealed by heterogeneity among sera containing anti-Sw(a). It was assigned to the Diego blood group system in 2000.46 The SW1 antigen is linked to a mutation in exon 16 (1936C>T), which causes the Arg646Trp amino acid change. Refer to the Sw(a) review in a previous section.

DISK (DI22)

A high-prevalence antigen antithetical to DI9 (Wu) has been identified.47 The new antigen, DI22 (DISK), was characterized by an apparently naturally occurring, strongly agglutinating antibody reactive at both 18°C and 37°C and by the indirect antiglobulin test. DISK was shown to be sensitive to α-chymotrypsin treatment, but resistant to other commonly used proteases. Targeted sequence analysis of SLC4A1 exon 14 revealed homozygosity in the proband and heterozygosity in a sample from her brother for the mutation 1694G>C that encodes Gly565Ala. The RBCs of her brother reacted more weakly with her antibody, suggesting that the anti-DISK exhibits dosage in DI:9, 22 individuals.

Poole et al. suggest that anti-DISK may be of clinical significance based on its serologic characteristics even though it could not be confirmed at the time.47

The Diego Null Phenotype

In 1983, Biro et al.48 described a healthy Mexican blood donor initially thought to be Di(a−b−), but further testing showed the donor’s RBCs were Di(a−b+). The donor’s RBCs were tested with eight samples of anti-Di(b) and failed to react with two of them, but it was shown that the donor’s RBCs will adsorb and elute the anti-Di(b) present in the two antisera that were macroscopically nonreactive. Other reports have mentioned the weakened reactivity of the Di(b) antigens of some individuals.48−50

In 2000, Ribeiro and colleagues reported the case of a child with severe hereditary spherocytosis caused by the absence of band 3.51 Defects in SLC4A1 are the cause of spherocytosis type 4. Spherocytosis is a hematologic disorder characterized by numerous abnormally shaped erythrocytes, which are spheroidal and may cause chronic hemolytic anemia. The absence of band 3 suggests that the patient represents the Di(a−b−) phenotype.

Contrary to initial belief, homozygosity for mutations in SLC4A1 (lack of band 3) is not incompatible with life. That the lack of band 3 is not incompatible with life has been demonstrated in experimental evidence using engineered knockout mice and naturally occurring knockouts in Japanese black cattle, and it also has been reported in humans.3,52,53

In the article, Ribeiro et al.51 indicated that the child probably survived because the family history was known, including the underlying mutation that caused band 3 and protein 4.2 to be absent. At the time, the prognosis of the child was uncertain; she was transfusion dependent and also sustained by oral doses of bicarbonate to counteract the renal acidosis that resulted from the absence of band 3 in her kidneys.

Summary

For 40 years (1955–1995), Di(a) and Di(b) were the Diego system. In 1995, Wr(a) and Wr(b) were assigned to the system. Since then, a combination of serologic knowledge with discoveries from biochemical and molecular research has allowed another 18 antigens to be assigned to the system; all but one are of low prevalence.
There is scanty or no data on the clinical significance of most of the antibodies in the Diego system. Anti-Di, -Wr, -ELO, -BOW, -Sw, -Fr, and -SW1 have been associated with HDFN, and anti-Wr has also been associated with HTR. Antibodies against the high-prevalence antigen Di have been associated with mild HTR.

The antibodies directed against low-prevalence antigens in the Diego system are produced with no obvious immunizing stimulus. Antibodies to all low-prevalence antigens are almost invariably found in antisera that contain multiple antibodies to low-prevalence antigens. In contrast, anti-Di is usually found as a single specificity and only occasionally does it occur in plasma containing multiple antibodies to low-prevalence antigens.

Mutations in the SLC4A1 gene that encodes the Di system antigens can result in various defects, some of which are critical to the organism, such as hereditary spherocytosis or renal tubule acidosis. Other mutations seem to be benign, producing changes that result in new antigens.

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<tr>
<td>NIH Clinical Center Blood Bank</td>
<td>Karen Byrne</td>
<td>301-496-8335</td>
<td><a href="mailto:kbyrne@mail.cc.nih.gov">kbyrne@mail.cc.nih.gov</a></td>
<td><a href="http://www.cc.nih.gov/dtm">www.cc.nih.gov/dtm</a></td>
</tr>
<tr>
<td>Rush University</td>
<td>Yolanda Sanchez</td>
<td>312-942-2402</td>
<td><a href="mailto:Yolanda_Sanchez@rush.edu">Yolanda_Sanchez@rush.edu</a></td>
<td><a href="http://www.rushu.rush.edu/csl">www.rushu.rush.edu/csl</a></td>
</tr>
<tr>
<td>Transfusion Medicine Center at Florida Blood Services</td>
<td>Marjorie Duty</td>
<td>727-568-5433 ext. 1514</td>
<td><a href="mailto:mdoby@tfblblood.org">mdoby@tfblblood.org</a></td>
<td><a href="http://www.tfblblood.org">www.tfblblood.org</a></td>
</tr>
<tr>
<td>Univ. of Texas Health Science Center at San Antonio</td>
<td>Linda Myers</td>
<td>210-731-5526</td>
<td><a href="mailto:lmiers@bloodtissue.org">lmiers@bloodtissue.org</a></td>
<td><a href="http://www.sbbfosa.org">www.sbbfosa.org</a></td>
</tr>
<tr>
<td>University of Texas Medical Branch at Galveston</td>
<td>Janet Vincent</td>
<td>400-772-3055</td>
<td><a href="mailto:jvincent@utmb.edu">jvincent@utmb.edu</a></td>
<td><a href="http://www.utmb.edu/abb">www.utmb.edu/abb</a></td>
</tr>
<tr>
<td>University of Texas SW Medical Center</td>
<td>Lesley Lee</td>
<td>214-649-1785</td>
<td><a href="mailto:lesley.lee@utsouthwestern.edu">lesley.lee@utsouthwestern.edu</a></td>
<td><a href="http://www.utsouthwestern.edu/education/school-of-health-professions/programs/certificate-programs/medical-laboratory-sciences/index.html">www.utsouthwestern.edu/education/school-of-health-professions/programs/certificate-programs/medical-laboratory-sciences/index.html</a></td>
</tr>
</tbody>
</table>

Why become an SBB?

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

How does one become an SBB?

CAAHEP-accredited SBB Technology program or grandfather the exam based on ASCP education and experience criteria.

Fact: In recent years, a greater percentage of individuals who graduate from CAAHEP-accredited programs pass the SBB exam compared to individuals who grandfather the exam. The BEST route for obtaining an SBB certification is to attend a CAAHEP-accredited Specialist in Blood Bank Technology Program.

Which approach are you more compatible with?

Contact the following programs for more information:

Additional information can be found by visiting the following Web sites: www.ascp.org, www.caahep.org, and www.aabb.org
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
   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.

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.)
   3. Key words
   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 — Clinical and/or hematologic data
      c. Materials and Methods — Selection and number of subjects, samples, items, etc. studied and description of appropriate controls, procedures, methods, equipment, reagents, etc.
      d. Results — Presentation of concise and sequential results
      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...)
   8. Figures
      a. Figures can be submitted either by e-mail or as photographs (5 × 7 inch 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:
         s s n n .
   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)

Send all manuscripts by e-mail to immuno@redcross.org
A. For describing an allele which has not been described in a peer-reviewed publication and for which an allele name or provisional allele name has been assigned by the ISBT Working Party on Blood Group Allele Terminology (http://www.isbtweb.org/working-parties/red-cell-immunogenetics-and-blood-group-terminology/blood-group-terminology/blood-group-allele-terminology/)

B. Preparation

1. Title: Allele Name (Allele Detail)
   ex. RHCE*01.01 (RHCE*ce48C)

2. Author Names (initials and last name of each (no degrees, ALL CAPS)

C. Text

1. Case Report
   i. Clinical and immunohematologic data
   ii. Race/ethnicity and country of origin of proband, if known

2. Materials and Methods
   Description of appropriate controls, procedures, methods, equipment, reagents, etc. Equipment and reagents should be identified in parentheses by model or lot and manufacturer’s name, city, and state. Do not use patient names or hospital numbers.

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

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

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

D. Acknowledgments

E. References

F. Author Information

List first name, middle initial, last name, highest degree, position held, institution and department, and complete address (including ZIP code) for all authors. List country when applicable.
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