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Subjects of the San rock paintings in the Drakensberg mountains of South Africa range from depictions of daily life to those of sacred events and of animals native to the region, such as the eland and reedbuck. Caves and overhangs are numerous in the mountains due to the ease with which the terrain’s abundant sandstone erodes. These caves shelter the paintings, some of which date back millennia. Tens of thousands of rock paintings have been documented in hundreds of locations. The artists made use of natural pigments such as red and yellow ocher, hematite, manganese oxide, and charcoal. Anthropological evidence suggests the San people may have been present in the region as far back as 100,000 years ago. As Reid et al. remind us in this issue of Immunohematology, anti-hrB (-RH31) and anti-HrB (-RH34) were discovered 40 years ago in the serum of a South African woman. Their report characterizes Rh in Bastiaan using molecular techniques.

David Moolten, MD
Blood group genotyping in a multitrauma patient: a case report

J. Curvers, V. Scharnhorst, M. de Haas, L. Warnier-Wandel, and D. van de Kerkhof

Currently DNA-based analysis of blood groups is mainly used to improve transfusion safety by reducing alloantibody formation in multiply transfused patients and by monitoring pregnancies at risk for hemolytic disease of the fetus and newborn. We present a case in which genotyping was performed after massive transfusion with unmatched group O, D– blood in a trauma setting. Our patient was genotyped as O1A1 and predicted to be D–, and we therefore transfused group A, D– red blood cell concentrates. This case demonstrates how the use of blood group genotyping in an acute setting can lead to a decrease in the unnecessary use of group O, D– blood products. *Immunohematology* 2012;28:85–7.

Key Words: genotyping, serology, massive transfusion, unmatched transfusion

Case Report

Our transfusion laboratory received an emergency request for six unmatched group O, D– red blood cell (RBC) concentrates for a patient. These units were to be transfused not inside the hospital but at the scene of a car accident (at a peripheral highway), where the patient was bleeding heavily while trapped in a car wreck. Again, another four unmatched RBC units were ordered together with five group AB, D– fresh-frozen plasma (FFP) units and one randomly selected unit of platelets obtained from five donors. All products were leukocyte reduced, with less than 10⁶/L white blood cells per unit. We had not received blood for crossmatching.

When released from the car wreck, the 62-year-old white female patient arrived at the hospital emergency department and was admitted to the operating room immediately. The patient suffered from complex high-impact trauma. Her car had slid underneath a truck, and the patient had been trapped for at least 1.5 hours. She had multiple fractures, in the nose, left wrist, left humerus, bilateral femur, and ramus superior and inferior, and she had a pneumothorax. Also, the patient exhibited various superficial wounds and a head injury that was closed using staples. Besides damage-control surgery, additional acute surgery was necessary to implant a stent because of dissection of the descending aorta. During this procedure the patient was in need of more blood products, and at that time the transfusion laboratory received the first sample for crossmatching. Serologically it was no longer possible to determine a blood group owing to the number of transfused RBC units. Even a mixed-field reaction was hardly visible (Fig. 1). Also, a reverse blood group typing was not possible as a result of the number of FFP units she had received. After the surgical procedure, the patient was stable and was transferred to the intensive care unit with a hemoglobin concentration of 8.8 g/dL (5.5 mmol/L). Three additional FFP units and two five-donor platelet concentrates were transfused because of low levels of fibrinogen (0.87 g/L) and platelets (76/nL), respectively. Altogether, our patient received 18 units of group O, D– leukocyte-reduced packed cells, 12 units of FFP (blood
group AB), and 6 units of leukocyte-reduced five-donor platelet concentrates (group O, D–) during her intensive care stay.

Unfortunately, we were unable to obtain information regarding the blood group of our patient (even after contacting the patient’s general practitioner and the local blood bank). Therefore, we chose to determine her blood group by means of DNA analysis to avoid further (unnecessary) use of group O, D– RBC products. We collected venous blood samples (anticoagulated in K₃-EDTA) and sent them to the laboratories of the Sanquin Blood Supply Foundation. ABO genotyping was performed using a sequence-specific primer polymerase chain reaction (SSP-PCR). RHD genotyping was performed using a multiplex reverse transcriptase (RT) PCR method. Our patient’s genotype was determined to be O1A1, and she was predicted to be D–.

Fortunately, the patient appeared to no longer need blood products. She stayed in the intensive care unit for another 20 days, during which time only one unit of RBCs was transfused (blood group A, D–). At that time the patient’s blood sample was serologically typed as group A, D– (without mixed-field reactions), and the screening for irregular antibodies was negative (using a three-cell screening panel, Ortho Clinical Diagnostics, Inc.). She was then moved to the surgery department for necrotomy of wounds and was transferred to a nursing home 2 months after the car accident.

Results

The chosen SSP-PCR is based on the presence of O1, O2, B, or A2 genes (the presence of glucosyltransferases on chromosome 9q34-2) in DNA isolated from peripheral blood samples. A PCR product is produced only when the specific chosen primer binds to and amplifies the sequence of interest. Eight mixtures of four allele-specific primer sets are used. The PCR products are transferred to an ethidium-bromide gel, and patterns are read and interpreted manually. Extrapolating results of eight different incubations (O1, O2, B, A2, non-O1, non-O2, non-B, and non-A2) led to the genotype. Therefore, this assay cannot be used for ABO variant analysis. In our case, blood group A1 was predicted as determined by exclusion of the other genotypes present. This phenotype has a frequency of 33 percent in the white population. The presence of an A₁ blood group was excluded, as was the presence of a B or Bombay (O₂) phenotype. The frequency of other type A (or B) blood groups is very rare in the white population, and would not have led to a mixed-field reaction of the patient’s RBCs with the anti-A serum (Fig. 1).

We were not able to confirm the blood group by reverse blood typing because of the amount of FFP received. However, after several days, serum anti-B was found but no anti-A; this is consistent with the ABO genotype we found.

The multiplex PCR for genotyping the Rh blood group system is based on single-nucleotide polymorphisms (SNPs) using six different primer sets that cover exons 3, 4, 5, 6, 7, and 9 of RHD and is able to identify most RHD variants. Based on the genotyping results, our patient was predicted to be D–, and for our white patient, the frequency of RHDψ or a variant D is very low. We did not confirm genotyping with buccal cells because the clinical consequences of a false-negative result are far less severe than those of a false-positive result. Serologically, on increasing volumes of RBCs to observe mixed-field reaction in our column technique, the D phenotype remained negative (Fig. 1). When blood group serology was repeated before transfusion 2 weeks after the accident, no mixed-field agglutination was observed, although transfused RBCs were likely to have been present. This was consistent with a D– phenotype and showed concordance between genotype and phenotype results. Again, using a three-cell screening panel, we found no irregular erythrocyte antibodies.

Of course, interpretation of genotyping results should be performed carefully because ethnic differences (e.g., presence of RHDψ, or the DAR variant) and silencing alleles (e.g., GATA box mutations) exist that could lead to discrepancies between genotype and phenotype. When the patient is of a certain ethnicity with a higher risk of ABO variants, sequencing may be an option, although costly and time-consuming.

Discussion

Recently, excellent reviews have been published regarding genotyping of the blood donor population and its possible drawbacks. However, in transfusion practice, genotyping is not commonly performed on patient samples. The Dutch national guideline currently states that a blood group can only be confirmed when it has been established by testing two different samples, irrespective of the method used. One method may be genotyping.

From 2001, leukocytes are depleted from all blood products within the Netherlands by means of filtration, resulting in the presence of less than 1 × 10⁶ white blood cells per unit. In case of massive transfusion of RBCs and platelet or plasma units, the load of donor white blood cells in the patient remains low (undetectable when compared with the patient’s white blood cell count, on average 5 × 10⁹/L), hence genotyping on DNA from the patient’s white blood cells is achievable. Our patient
had a white blood cell count of 4.5 leukocytes/nL at the time of DNA analysis. Of these, a maximum of 4 percent (0.16/nL: 16 blood products times 10^6 white blood cells) could potentially originate from the received RBC units. To circumvent these limitations or when white blood cell counts in peripheral blood are too low to perform genotyping, other material can be used, such as nails or buccal epithelial cells (with potential errors in bone marrow transplant patients).3,4,8 Therefore, leukocyte counting for the patient is essential before performing these analyses, and care should be taken in case of neutropenic or lymphopenic patients.

Ideally, all transfusions should be closely monitored, especially transfusions of unmatched RBC units, to detect acute transfusion reactions in case of transfusion of incompatible blood group(s). Unfortunately the clinical practice shows that unmatched units are transfused in emergency situations, in which close monitoring of transfusion can be difficult. Because transfused RBCs remain in circulation for several weeks and can cause mixed-field agglutination when antigen typing is performed, genotyping can determine the blood group of trauma patients who have received repeated unmatched RBC units.

Conclusions

As has previously been suggested, blood group genotyping can be helpful in acute situations, when serologic (hemagglutination with antisera) blood group typing is not possible or yields inconclusive results (i.e., mixed-field agglutination, discrepant results).6,8 The success of genotyping in cases of emergency depends on the speed of processing and the turnaround time. Improvements in these will eventually lead to fewer unmatched transfusions. The blood transfusion laboratory should, however, always be aware of the limitations of genotyping (vs. serologic determination of blood groups). In a trauma center, genotyping a patient’s sample to determine ABO and D might be a suitable alternative to transfusing group O, D− RBCs, thus reducing the unnecessary use of such blood products.

References

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Warm autoadsorption with enzyme-treated red blood cells

F. Tsimba-Chitsva, S. Bishop, and K. Kezeor

Patients demonstrating warm autoantibody specificity present serologic challenges for laboratory staff performing antibody identification in the blood bank. Autoantibody can be removed from plasma or serum by adsorption onto autologous red blood cells (RBCs) provided the patient has not been transfused in the previous 3 months. The adsorption process can be enhanced by enzyme pretreatment of autologous RBCs. Immunohematology 2012;28:88–90.

Key Words: warm autoadsorption, ficin, papain, autologous

Principle

Performing pretransfusion testing in the presence of warm autoantibodies optimally reactive at 37°C presents serologic challenges when attempting to determine the presence of underlying clinically significant alloantibodies.1 Warm-reactive autoantibodies may mask the presence of other antibodies because of their agglutination with all cells tested, including autologous red blood cells (RBCs). To determine the presence of clinically significant antibodies, the removal of autoantibody may be achieved by adsorption of patient plasma or serum with autologous RBCs.2–4 Allogeneic RBCs may be used for adsorption if the patient has been recently transfused or when the quantity of autologous RBCs is insufficient for autoadsorption.2

Indications

As circulating autologous RBCs are coated with autoantibody, antigen sites on the autologous cells may become blocked with immunoglobulin. To efficiently achieve autologous adsorption of warm-reactive autoantibodies, autoantibody must be dissociated from the RBC before the adsorption process.2 Autoantibody dissociation can be accomplished by several methods, including partial heat elution at 45°C, gentle heat elution at 56°C, a combination of 0.2 M dithiothreitol (DTT) and papain or ficin treatment, acid-EDTA treatment, ZZAP (a mixture of cysteine-activated proteolytic papain and DTT) treatment, and chloroquine diphosphate (CDP) treatment.5 (For specific procedures concerning these methods and reagents, see the applicable procedural reference documents or manufacturer’s package insert.) After dissociation of the autoantibody from the autologous RBCs, the antigen sites should be available for binding the autoantibody from the plasma or serum during the adsorption process.

To further enhance the adsorption process, autologous RBCs can be treated with proteolytic enzymes such as ficin or papain. This additional treatment enhances antibody uptake onto the autologous cells by cleaving some of the glycoprotein chains extending from the cell membrane, leading to improved accessibility of the antigens not removed by the dissociation method.4

Procedure

For papain or ficin (1%) treatment of RBCs, one volume of 1% cysteine-activated papain or 1% ficin is added to two volumes of packed RBCs. For example, if 2 mL of packed RBCs is treated, 1 mL of ficin or papain is added. The mixture

<table>
<thead>
<tr>
<th>Enzyme Treatment Method</th>
<th>Reagents</th>
<th>Supplies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Papain 1% cysteine-activated papain</td>
<td>• Isotonic saline • Patient autologous red blood cells</td>
<td>• 1 mL graduated pipettes • 37°C water bath • Large bore test tubes • Calibrated centrifuge</td>
</tr>
<tr>
<td>Ficin 1% ficin</td>
<td>•</td>
<td></td>
</tr>
</tbody>
</table>

Reagents/Supplies

Procedural Steps

<table>
<thead>
<tr>
<th>Treatment of Cells</th>
<th>• Mix enzyme with packed red cells • Incubate mixture • Wash mixture to remove enzyme • Centrifuge to pack the red cells • Remove supernatant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adsorption</td>
<td>• Mix enzyme-treated cells with patient plasma/serum • Incubate the mixture • Centrifuge the mixture • Remove plasma/serum</td>
</tr>
</tbody>
</table>
Warm autoadsorptions with enzyme-treated RBCs

is incubated at 37°C for a predetermined time to allow for the most effective enzyme treatment. If commercially prepared enzyme is used, the manufacturer’s insert is used to determine the incubation time. If enzyme is prepared in-house, qualification studies are performed to determine the appropriate treatment time.6

The enzyme-treated RBCs are washed with copious amounts of isotonic saline a minimum of three times, or until the supernatant saline is clear, to ensure complete removal of enzyme. The last wash is centrifuged a minimum of 5 minutes at 900 to 1000×g to completely pack the RBCs.7 This step is critical to achieve proper packing of RBCs and removal of maximum amounts of saline. To avoid plasma or serum dilution during adsorption, as much saline as possible is carefully removed after the final wash. This can be accomplished with suction using a small-bore pipette or filter paper.4

To perform adsorption, one volume of enzyme-treated RBCs is mixed with an equal volume of the patient’s plasma or serum. To enhance antibody uptake, the proportion of RBCs to plasma or serum can be increased. Adsorption is more effective if the area of contact between the RBCs and plasma or serum is large; use of a large-bore test tube (16 × 100 mm) increases the surface area and allows for optimum plasma or serum and RBC contact. The mixture is incubated at 37°C for 10 to 60 minutes.4 The mixture is then centrifuged and the plasma or serum is carefully removed. If the harvested adsorbed plasma or serum will be used for additional adsorptions, disturbing the packed RBCs to achieve maximum plasma removal is acceptable. If, however, the harvested adsorbed plasma or serum will be used for antibody detection, the packed RBCs should not be disturbed in an attempt to recover additional plasma.

The number of adsorptions required to exhaust autoantibody from the plasma or serum is proportionally related to the strength of its reactivity in plasma or serum; however, in some instances, additional adsorptions may be required to remove the antibody.7–9 If the original plasma or serum reactivity is 1+ with the indirect antiglobulin test (IAT), the first aliquot of adsorbed plasma or serum should be tested against group O reagent RBCs or direct antiglobulin test (DAT) negative autologous RBCs, if available. If reactivity persists, the adsorption procedure should be repeated, as needed, using a fresh volume of enzyme-treated RBCs. Once reactivity is abolished, the adsorbed plasma or serum is tested against the appropriate group O reagent panel RBCs to exclude underlying alloantibodies.

Limitations

A recently transfused patient (within the last 3 months) is not a candidate for autologous adsorption. Circulating transfused RBCs may adsorb clinically significant plasma or serum alloantibodies.8 It has been demonstrated that very small amounts of antigen-positive RBC are capable of eliminating alloantibody reactivity from the plasma or serum.8,10

Large volumes of autologous RBCs from the patient are needed to perform autoadsorption. In the case of a low patient hematocrit, it may be difficult to obtain a sufficient volume of autologous cells for testing without exacerbating clinical symptoms of anemia.

Cell treatment may be unsuccessful in stripping autoantibody from the autologous, coated RBCs. As a result, adsorption may be less efficient in removing autoantibody.

Enzyme treatment of RBCs will destroy antigen sites on the RBCs. Enzymes remove sialic acid from the RBCs and will destroy some antigens (-M, -N, -S, -s, -Fya, -Fyb, -Ena, -Ge, -JMH, -Ch/Rg, -Inb). Autoantibodies specific for enzyme-sensitive antigens will not be removed by this method.4

Potential dilution of adsorbed plasma or serum must be considered a consequence of preparation of enzyme-treated cells. If the saline is not properly removed after the final wash, the residual saline will dilute the plasma or serum when adsorptions are performed. The more adsorptions performed, the greater the dilution factor. As a result, diluted antibody may become undetectable in the adsorbed plasma or serum.

Enzyme treatment of autologous RBCs can cause hemolysis. If the RBCs are hemolyzed by enzyme treatment, there may be an insufficient quantity of RBCs remaining to perform adsorptions.

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References


For information concerning the National Reference Laboratory for Blood Group Serology, including the American Rare Donor Program, contact Sandra Nance, by phone at (215) 451-4362, by fax at (215) 451-2538, or by e-mail at Sandra.Nance@redcross.org

For information concerning Immunohematology or the Immunohematology Methods and Procedures manual, contact us by e-mail at immuno@redcross.org
Implications of the Kidd blood group system in renal transplantation

A. Rourk and J.E. Squires

The association of the Kidd blood group system with hemolytic transfusion reactions and hemolytic disease of the newborn is well known. The Kidd antigens, which are localized to the HUT/UT-B urea transport protein, are found on red blood cells and the endothelial cells of the blood vessels of the medulla of the kidney. Recently it has been suggested that these antigens might play a role as minor histocompatibility antigens in renal transplantation. In the current case, the appearance of an anti-Jkb 10 years after renal transplantation associated with early renal allograft rejection further supports the potential importance of these antigens in renal transplantation and allograft rejection. Immunohematology 2012;28:91–4.

Key Words: Kidd blood group, renal transplantation, allograft rejection

The Kidd blood group antigens Jka and Jkb are expressed at position 280 of the membrane glycoprotein HUT11/UT-B. This protein is thought to function as a urea transporter that protects the red blood cell (RBC) from osmotic gradients, particularly as it passes through the kidney.1 The UT-B protein has also been localized to endothelial cells of the vasa recta of the medulla of the kidney where it facilitates the excretion of urea and concentration of the urine.2 More recently, the UT-B protein has been identified in a variety of other tissues such as testis3 and colon.4 Antibodies to Jka and Jkb have been associated with both hemolytic transfusion reactions (HTR) and hemolytic disease of the fetus and newborn (HDFN). More recently, they have also been associated with several cases of acute or delayed renal allograft rejection.5 We report a case in which the appearance of anti-Jkb is associated with early renal allograft rejection more than 10 years after the initial renal transplant surgery.

Case Report

At 14 months of age, an African American boy was treated for pneumonia caused by Streptococcus pneumoniae and associated hemolytic uremic syndrome (HUS). At that time, the patient received five group O+ RBC transfusions and one group O+ platelet transfusion; his antibody screen remained negative throughout the 8 days of his hospitalization. The patient was discharged from the hospital but, as a result of progressive renal disease, required peritoneal dialysis 48 months later. He did well on dialysis for 4 months but then underwent an uncomplicated cadaveric renal transplant procedure requiring no transfusions. For approximately 10 years, the patient did well and was maintained on a routine immunosuppressive regimen including tacrolimus, mycophenolate, and prednisone. Then, during a routine follow-up visit, he was found to have a slightly elevated creatinine (1.7 mg/dL compared with his usual baseline of 1.2 mg/dL). On further questioning, he admitted to oliguria and to “missing several doses” of his immunosuppressive medications. In preparation for a renal biopsy, a blood type and antibody screen was performed. His blood type was confirmed as O+, but for the first time his antibody screen was strongly positive (3+); anti-Jkb was identified. Kidney biopsy was consistent with acute allograft rejection, Banff classification type IA, plasma cell rich.6 The patient’s immunosuppressive regimen was reestablished while he was in the hospital, and now, 7 months later, his creatinine has returned to his usual baseline and his antibody screen is again negative.

Materials and Methods

Current blood type and antibody identification were performed by solid-phase red blood cell adherence assay using the Galileo immunohematology analyzer (Immucor Gamma, Norcross, GA).

Results

During the course of the patient’s treatment for HUS at the age of 14 months, he received 5 group O+ RBC transfusions and one group O+ platelet transfusion. His antibody screen at this time remained negative during the 8 days during which he was transfused. No further blood type or antibody screen was performed until the time of his renal transplant procedure 48 months later; his antibody screen at that time continued to be negative and he received no transfusions during or after this surgical procedure. The patient was routinely evaluated...
during the subsequent 10 years and apparently was doing well until, at the time of a routine follow-up visit, he was found to have an elevated creatinine level. When the patient admitted to oliguria and failing to adhere to his immunosuppressive regimen, a renal biopsy was scheduled. In preparation for this procedure, an antibody screen was performed and was found to be strongly positive (3+). The antibody was identified as anti-Jkb. Table 1 summarizes these results. The renal biopsy (Fig. 1) was considered diagnostic of acute allograft rejection (Banff type IA, plasma cell rich).

### Discussion

Antibodies to the Kidd blood group antigens, Jk<sup>a</sup> and Jk<sup>b</sup>, are well known to cause HTR and HDFN. However, recently a small number of reports have also suggested the importance of these antibodies in renal transplantation. The importance of these antibodies in renal transplantation presumably arises from the fact that the protein carrying the Kidd antigens, referred to as UT-B, is expressed on both the RBC membrane and the endothelial cells of the vasa recta of the medulla of the kidney.7

We report a case of renal allograft rejection occurring 10 years after transplantation. Transplant rejection in this case was heralded by a slight rise in serum creatinine and was probably related to the patient’s recent poor compliance with his immunosuppressive medications. Unexpectedly, anti-Jkb was identified when a routine blood type and antibody screen was performed in preparation for the renal biopsy. Because the patient had only been transfused with RBCs approximately 10 years before the appearance of anti-Jkb and had had a negative antibody screen during that period, it seems safe to assume that the transplanted kidney stimulated the development of this antibody. It is also important to note that the antibody appeared at a time when the patient admitted to poor compliance with his immunosuppressive medications. It is possible that anti-Jkb first appeared in this patient at the time of his transfusions at the age of 14 months and the reappearance of the antibody 10 years later is simply an anamnestic response to the exposure to the Jk<sup>b</sup> antigen presumably present on the transplanted kidney. Alternatively, it seems possible that the Kidd antigen on the transplanted kidney stimulated the de novo development of the antibody once the patient’s immunosuppressive regimen was interrupted. This observation is supported by several recent case reports.

In one of these, Hamilton et al.8 report the case of a 19-year-old woman who underwent a cadaveric kidney transplant procedure owing to a diagnosis of focal segmental glomerulosclerosis. The patient was noncompliant with her immunosuppressive medications, and approximately 24 months after her transplant she exhibited renal failure that ultimately necessitated dialysis and transplant nephrectomy. During her hospitalization she was found to have a positive antibody screen, and anti-Jkb was identified. Because this patient had no history of blood transfusion or pregnancy, the authors hypothesized that the antigen on the transplanted kidney

### Table 1. Summary of patient information

<table>
<thead>
<tr>
<th>Date</th>
<th>Purpose</th>
<th>Antibody screen</th>
<th>Antibody identification</th>
<th>Transfusion</th>
<th>Creatinine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nov. 1996</td>
<td>Treatment; HUS</td>
<td>Negative</td>
<td></td>
<td>5 RBC, 1 platelet</td>
<td>Not available</td>
</tr>
<tr>
<td>Dec. 2000</td>
<td>Clinic visit; follow-up</td>
<td>Negative</td>
<td></td>
<td>None</td>
<td>Not available</td>
</tr>
<tr>
<td>April 2001</td>
<td>Hospitalization; pre-renal transplant</td>
<td>Negative</td>
<td></td>
<td>None</td>
<td>Not available</td>
</tr>
<tr>
<td>Aug. 10, 2010</td>
<td>Clinic visit; follow-up</td>
<td>Not Done</td>
<td></td>
<td>None</td>
<td>1.7 mg/dL</td>
</tr>
<tr>
<td>Aug. 13, 2010</td>
<td>Hospitalization; treatment of acute rejection</td>
<td>Positive</td>
<td>Anti-Jkb&lt;sup&gt;b&lt;/sup&gt;</td>
<td>None</td>
<td>1.5 mg/dL</td>
</tr>
<tr>
<td>Aug. 15, 2010</td>
<td>Hospital discharge</td>
<td>Not Done</td>
<td></td>
<td>None</td>
<td>1.6 mg/dL</td>
</tr>
<tr>
<td>March 2011</td>
<td>Clinic visit; follow-up</td>
<td>Negative</td>
<td></td>
<td>None</td>
<td>1.3 mg/dL</td>
</tr>
</tbody>
</table>

HUS = hemolytic uremic syndrome; RBC = red blood cell.

---

**Fig. 1** Photomicrograph of kidney biopsy specimen demonstrates a prominent inflammatory infiltrate with prominent plasma cells consistent with Banff type 1 acute rejection, plasma cell rich.
kidney served as the immunizing antigen causing the development of the anti-Jk\(b\). In this case, a diagnosis of plasma cell-rich acute cellular rejection (PCAR) was made by renal biopsy. It has been suggested that in PCAR, which is a rather uncommon inflammatory manifestation of allograft rejection, patient-derived plasma cells infiltrate the renal allograft and are responsible for the production of antibody in situ.\(^9\) Hamilton et al.\(^8\) make the suggestion that the anti-Jk\(b\) was synthesized by the plasma cells identified in the kidney. Hamilton et al.\(^10\) described another 19-year-old woman who exhibited anti-Jk\(a\) 7 years after renal transplantation. Again, the patient was described as noncompliant with her immunosuppressive regimen. In this case, however, at the time of original renal transplantation the patient received two units of RBCs that were known to be Jk(a+).

In each of these three cases a cadaveric renal transplant was carried out on a patient with a negative antibody screen and 2 to 10 years later, in a setting of noncompliance with a routine immunosuppressive regimen, an antibody to the Kidd blood group system (anti-Jk\(a\) or anti-Jk\(b\)) appeared in conjunction with acute kidney transplant rejection. A comparison of these cases is found in Table 2.

### Table 2. Comparison of case reports of renal allograft rejection occurring after transplantation

<table>
<thead>
<tr>
<th>Case</th>
<th>Patient</th>
<th>Transfusion</th>
<th>Kidd antibody</th>
<th>Diagnosis: kidney rejection</th>
<th>Contributing factors to rejection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Current</td>
<td>Male</td>
<td>5 RBC; 1 platelet</td>
<td>Jk(b)</td>
<td>Acute rejection</td>
<td>Noncompliance with immunosuppressive medications</td>
</tr>
<tr>
<td>Hamilton, 2006</td>
<td>Female</td>
<td>None</td>
<td>Jk(b)</td>
<td>Acute rejection</td>
<td>Noncompliance with immunosuppressive medications</td>
</tr>
<tr>
<td>Hamilton, 2008</td>
<td>Female</td>
<td>2 RBC</td>
<td>Jk(a)</td>
<td>Acute rejection</td>
<td>Noncompliance with immunosuppressive medications</td>
</tr>
<tr>
<td>Holt, 2004</td>
<td>Female</td>
<td>Yes (total number not reported)</td>
<td>Jk(a)</td>
<td>Acute rejection</td>
<td>RBC transfusion reaction caused by anti-Jk(a)</td>
</tr>
</tbody>
</table>

The critical role that antibodies of the Kidd system may play in renal allograft rejection is further suggested by Holt et al.\(^11\) In this case a 29-year-old patient experienced a hyperacute renal allograft rejection presumably caused by a complement-fixing anti-Jk\(a\). In this case it was suspected that the patient had developed anti-Jk\(a\) as a result of a series of RBC transfusions that she had received approximately 18 years earlier. After her renal transplant procedure, she received three units of RBCs. Within 2 hours of this transfusion, the patient exhibited a fever and urine output slowed. Eventually the direct antiglobulin test was found to be positive, and an eluate demonstrated anti-Jk\(a\). The authors suggested that the transfusions shortly after her renal transplant stimulated an anamnestic anti-Jk\(a\), which was then responsible for both the transfusion reaction and ultimately failure of her graft.

Lerut et al.\(^5\) compared the matching versus mismatching status for Kidd and Duffy polymorphisms in 370 consecutive kidney transplants. With respect to the Kidd antigens, those transplants in which the donor and recipient were mismatched for Kidd antigens showed a greater degree of interstitial inflammation than those cases in which the donor and recipient were matched, thus suggesting the potential importance of these antigens in clinical transplantation.

Clearly, then, each of these case reports supports the suggestion that Kidd antigens do, in fact, act as minor histocompatibility antigens that are uncommon but potentially important causes of renal allograft rejection. Functionally, the explanation for this lies in the observation that the protein carrying the Kidd antigens derives from a single gene and is found on both the RBC and the endothelial cells of the vasa recta of the medulla of the kidney. The question that remains in the current case is whether the Kidd antigens on the transplanted kidney served as the primary stimulus for the development of the Jk\(a\) antibody (as suggested by the report of Hamilton et al.\(^8\)) or whether it served as the stimulus for an anamnestic antibody response of an anti-Jk\(b\) that originally arose because of the RBC transfusions 10 years earlier. In either event the important effect of the Kidd antigens on the kidney is apparent as the transplanted kidney must have either served as an immunogen in stimulating an anamnestic development of anti-Jk\(a\) or been the primary immunizing agent.

### Acknowledgment

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### References


Angela Rourk, MT (ASCP), Medical Technologist, and Jerry E. Squires, MD (corresponding author), Medical Director, Transfusion Service, Department of Pathology and Laboratory Medicine, Medical University of South Carolina, 171 Ashley Avenue, MSC 908, Charleston, SC 29425-9080.
EDTA glycine acid treatment of red blood cells

J. Kosanke

IgG dissociation is necessary when a sample is direct antiglobulin test (DAT) positive and antigen testing using blood grouping serum reactive by the antiglobulin test is performed. Exposure of IgG-coated red blood cells (RBCs) to a low pH of 3.0 with EDTA glycine acid successfully dissociates the IgG, rendering the RBCs DAT negative 82 to 85 percent of the time. The procedure takes one minute or less and leaves RBC antigens intact and able to be typed except for those antigens in the Kell blood group system and for the high-prevalence antigen Er*. Immunohematology 2012;28:95–6.

Key Words: EGA, EDTA glycine acid, IgG dissociation, direct antiglobulin test, DAT

Principle

The chemical bonds in antibody-antigen reactions are weak noncovalent bonds. The reactions between an antibody and antigen are often described as a lock and key fit and are held together by van der Waals, electrostatic, and hydrophobic forces. These forces are easily dissociated by extremes in pH. EDTA glycine acid (GA) treatment uses an acidic pH of 1.5 to dissociate IgG from red blood cells (RBCs).

The effectiveness of EDTA-GA was first described in an abstract by Louie, Jiang, and Zaroulis in 1986.1 Fifty samples were treated using an EDTA-GA method after in vitro sensitization with alloantibodies. After one treatment, 48 samples had a negative direct antiglobulin test (DAT) and the remaining two had a negative DAT after a second treatment. When 30 in vivo strongly sensitized samples were treated with EDTA-GA and compared with treatment with chloroquine diphosphate (CDP), the results indicated the EDTA-GA method was more effective than CDP. All 30 samples had negative DATs after EDTA-GA treatment, and 26 had negative DATs with CDP treatment. EDTA-GA has the added benefit of only requiring a 1-minute incubation instead of a minimum of 30 minutes with CDP. Additional testing of 100 consecutive samples had IgG dissociated from the patient’s RBCs 85 percent of the time. Another abstract, in 1989, reported the findings with 45 patient samples with DAT reactivity ranging from 1+ to 4+ with a success rate of 82 percent dissociation of IgG.2

Reagents/Supplies

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Supplies</th>
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<tr>
<td>Saline</td>
<td>Centrifuge</td>
</tr>
<tr>
<td>Reagents for EDTA-GA solution</td>
<td>Test tubes</td>
</tr>
<tr>
<td>TRIS buffer</td>
<td>Pipettes</td>
</tr>
<tr>
<td>Reagents for DAT</td>
<td>Agglutination viewer</td>
</tr>
</tbody>
</table>

GA = glycine acid; DAT = direct antiglobulin test.

Procedural Steps

<table>
<thead>
<tr>
<th>Process</th>
<th>Mix EDTA-GA with packed red blood cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Add buffering solution</td>
</tr>
<tr>
<td></td>
<td>Wash cells</td>
</tr>
<tr>
<td></td>
<td>Perform DAT on treated cells</td>
</tr>
</tbody>
</table>

GA = glycine acid; DAT = direct antiglobulin test.

Indications

EDTA-GA is used to dissociate IgG from a patient’s autologous RBCs to prepare them for antigen testing with blood grouping reagents. An autologous RBC sample would be defined minimally as a sample collected more than 3 months from the last transfusion. If a patient has been transfused within this 3-month period, a cell separation to recover autologous cells should be performed before the EDTA-GA treatment of the RBCs.

RBCs sensitized with IgG invalidate test results with blood grouping reagents that require an indirect antiglobulin test (IAT). Some monoclonal blood grouping reagents that do not require an IAT still may be considered invalid unless one of the antigens of the tested allelic pair is nonreactive. IgG dissociation by EDTA-GA treatment leaves cells intact to perform antigen typing. If the autologous cells have a negative DAT after treatment, a valid antigen typing can be obtained for common antigens except for K. EDTA-GA is known to denature all antigens in the Kell blood group system and the high-prevalence antigen Er*.3
**Materials**

Because an acidic pH in normal saline would cause hemolysis of RBCs, it is necessary to prepare the low-pH reagent in an EDTA glycine solution to maintain osmolarity of the RBCs (Table 1). A high-pH buffer is needed to bring the pH to 7.0 to 7.5 after the RBCs are exposed to EDTA-GA. Normal saline is used to wash the cells before and after treatment.

The reagents for preparing EDTA-GA–treated cells may be prepared from stock chemicals or purchased commercially as a kit with three individual reagent vials. Two of the reagents are used to prepare the EDTA-GA solution for addition to the cells, and the third reagent is used to neutralize the pH before washing the treated cells.

**Procedure**

A volume of packed RBCs is exposed to EDTA-GA for 1 to 2 minutes at room temperature (Table 2).

- Although washing the cells to prepare a cell suspension may have no impact on the effectiveness of the EDTA-GA, standard laboratory practices often start with RBCs washed at least one time in normal saline, if indeed not three times.
- The ratio of RBCs, EDTA, and GA reported by Louie, Jiang, and Zaroulis is 1:1:4. Although the incubation time was up to 2 minutes, an incubation of 1 minute or less is an effective time for the low pH to dissociate IgG from the cells.
- As a reagent, EDTA-GA typically is used to disrupt the binding interaction of antibody and antigen and also in immunoaffinity purification of monoclonal and polyclonal antibodies.

A few drops of buffering solution are added.

- Although EDTA-GA can disrupt the bonds without denaturing either the antibody or the antigen, prolonged exposure to an acidic environment may damage some proteins; an adjustment to neutral pH should be performed quickly.

The cells are washed three to four times, and a DAT is performed on the treated cells to determine effectiveness of the treatment.

**Limitations**

EDTA-GA is effective at dissociating IgG, but it does not remove complement components from RBCs. Anti-IgG rather than polyspecific anti-human globulin reagent should be used when testing EDTA-GA–treated cells.

If antigen typing the patient’s EDTA-GA–treated RBCs and the effect of acid is unknown, RBCs known to be antigen-positive should be EDTA-GA treated and tested in parallel with the patient’s cells to determine the effect of the acid.

EDTA-GA is not successful 100 percent of the time. If treatment of RBCs from a patient who has not been transfused in the previous 3 months is unsuccessful, recovering the patient’s reticulocytes and treating them with EDTA-GA may yield successful results.4

Some patients’ RBCs may turn brown when incubated a full minute with the EDTA-GA solution. Preparing a new suspension and incubating for a shorter amount of time may prevent browning and still be effective at dissociating IgG.

**Quality Control**

An inert control such as 6 percent albumin should be tested in parallel with blood grouping reagents to detect weak reactivity that may occur with EDTA-GA–treated DAT-negative cells. If the 6 percent albumin control is positive, antigen typing results are invalid.

Whenever implementing a new procedure, verification studies should be performed as required by 42 CFR 493.1253.

**References**


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Molecular background of RH in Bastiaan, the RH:–31,–34 index case, and two novel RHD alleles


Anti-hr\(^a\) (–RH31) and anti-Hr\(^a\) (–RH34) were found nearly 40 years ago in the serum of a South African woman. The anti-hr\(^a\) was revealed after adsorption with Dce/Dce red blood cells (RBCs). Numerous anti-hr\(^a\), in the absence of anti-Hr\(^a\), have since been identified. We obtained a sample of blood from this index case (Bastiaan) and report the molecular basis of her D+ C– E–e+/-, V–VS+Hr+h+r–HrA– phenotype as well as results of testing her RBCs using currently available reagents. We tested a cohort of African Americans to estimate the frequency of the RHCE*48C, 733G, 1006T allele, and in addition found two novel RHD alleles. Hemagglutination tests and DNA analyses were performed by standard methods. Analyses revealed homozygosity for RHCE*48C, 733G, 1006T in Bastiaan. RBCs from Bastiaan were strongly agglutinated by three commercial anti-e reagents. Testing RBCs from people homozygous for RHCE*48C, 733G, 1006T allele, and in addition found two novel RHD alleles. Moreover, these antibodies often exist concomitantly, they can also exist separately in unabsorbed plasma from different patients.

Before reporting Bastiaan, Shapiro reported a similar case, in which RBCs from a Bantu woman named Shabalala (after whom the antigen was named) had an Hr– hrS– (RH:–31,–34) phenotype. Her plasma contained anti-Hr with an underlying anti-hr\(^a\) that remained after absorption with R,R\(_2\) RBCs. Issitt recorded that “when e+hrS– and e+hr\(^a\)– RBCs first became available for use in serological tests, some anti-e-like specificities were called anti-hr\(^a\) or anti-hr\(^b\) because of their failure to react with hr\(^a\)– and hr\(^b\)– RBCs, even when tested with only one example of the antibody. Those sera were then used to classify, not necessarily accurately, other RBCs with e variants. Thus, the ‘new’ examples of anti-hr\(^a\) and anti-hr\(^b\) were not the same specificity as the originals. As these inaccurately classified antibodies and RBC phenotypes were used to ‘resolve’ further cases, the situation grew to where now it is difficult, serologically, to accurately define e variants. While RBC samples and sera in the collections of immunohematologists are labeled in terms of hr\(^a\) and hr\(^b\), they would be more accurately described as being RBCs or sera from people with e variants. Terms anti-hr\(^a\)-like and anti-hr\(^b\)-like should be abandoned.”4 Issitt concluded that “at the serological level the situation is remarkably complex and is unlikely soon (or easily) to be resolved. . . . It is clear that some of the apparent heterogeneity of anti-hr\(^a\) and anti-hr\(^b\) relates directly to the labels that have been applied to various similar but not identical specificity antibodies.”4 Added to this situation and complexity is the fact that the e antigen can have altered expression not only in inherited variants (alloanti-e-like)
but also on RBCs from patients with warm autoantibodies. Furthermore, e+ people with alloanti-e are frequently D+ with alloanti-D.8,9

As of August 2011, there were 291 RHD and 135 RHCE alleles listed in two databases.10,11 The numerous alleles occur because of the high degree of homology between RHD and RHCE, their close proximity at chromosome 1p36.11, and their opposite orientation.12 The variant alleles result from single and multiple inserts of RHD into RHCE or vice versa, novel nucleotide changes, deletion of nucleotide(s), or insertion of nucleotide(s).

With knowledge of the DNA sequence of alleles encoding e variants, we now have tools to more accurately predict an RBC phenotype and therefore determine the specificity of an antibody. The e/E polymorphism is fundamentally defined by a single amino acid change (Ala226Pro) on the RhCE protein13; however, amino acid changes at other positions on the Rhce protein affect expression of e. Noizat-Pirenne et al.6 reported the molecular bases of several e variants, including two cases of anti-RH34 in patients with the rS phenotype. Pham et al.14 showed that in addition to the existence of the rS haplotype with exons 1 to 3 of the RHD hybrid allele derived from RHD*DIIIa (rS type 1),15 the haplotype also exists with exon 1 to 3 being derived from RHD (rS type 2). These workers performed DNA and hemagglutination testing to show that RBCs from people with either of these rS phenotypes lacked hrS and HrB. Among the samples tested, they found four that contained anti-hrS plus anti-HrB, two with anti-hrS, and two with anti-HrB.5

The hrS− phenotype is often, but not invariably, VS+, which led to the speculation that hrS and VS are not, in the classical sense, antithetical.16–18 Four RHCE alleles with 48G>C and 733C>G changes (ISBT provisional name RHCE*01.20.02 [RHCE*ceVS.02], RHCE*01.20.03 [RHCE*ceVS.03], RHCE*01.20.04 [RHCE*ceVS.04], and RHCE*01.20.08 [RHCE*ceVS.08]) encode the VS+ and hrS− phenotype, three alleles (ISBT name RHCE*01.20.01 [RHCE*ceVS.01], RHCE*01.20.06 [RHCE*ceCF], and RHCE*01.20.07 [RHCE*ceJAL]) encode altered VS and altered hrS, and one allele (ISBT name RHCE*01.20.05 [RHCE*ceVS.05]) encodes VS, but the hrS status is unknown (www.isbt-web.org).19,20 The RHCE*ceMO (ISBT name RHCE*01.07.01) encodes a partial e antigen and the VS− hrS− hrR− phenotype.18 Thus, it is now known that hrS and VS are not, in the classical sense, antithetical.

The understanding of the underlying molecular bases of numerous e variants is beginning to provide a tool, based on DNA analyses, to more precisely match blood to a patient. To aid the antibody identification process, determining the molecular basis of the serologically defined index cases is valuable. Unfortunately, despite considerable effort, we have been unable to obtain a blood (or DNA) sample from Shabalala, the index Hr− hrS− case. However, we did succeed in obtaining a sample from Bastiaan, the index hrS− HrR− case. We have established that her DIIfa, C+E−c+e+/−, V− VS+, hrS+, hrR−, HrR− phenotype is owing to the homozygous presence of RHCE*ce 48C, 733G, 1006T (ceS) and heterozygosity for RHD*DIIIa 150C/DIIIa-CE(4-7)-D. We performed hemagglutination tests on RBCs from Bastiaan and others with the same RHCE genotype, using currently available anti-e reagents. We also tested samples from a cohort of people of African descent to estimate the frequency of the RHCE allele and the RHD alleles associated with them, and these analyses revealed two novel RHD alleles: RHD*DI86T (accession number JN635688) and RHD*DIIla 150C (accession number JN635689). No ISBT allele name was obtained because these novel RHD alleles do not, apparently, exhibit a phenotype.

Materials and Methods

Whole blood samples were freshly collected as part of antibody identification or recovered from storage in liquid nitrogen. (The names of donors are codes and not related to the donor’s actual name; they are used for the benefit of immunohematologists who have blood samples from these donors in their collections). Hemagglutination tests were performed by standard methods using reagents from our libraries of reagents and from Bio-Rad (Dreieich, Germany), Immucor (Norcross, GA), and Ortho Clinical Diagnostics (Raritan, NJ) according to the manufacturer’s instructions.

Genomic DNA and RNA Extraction, Amplification, Cloning, Sequencing, and Restriction Fragment Length Polymorphism

Genomic DNA was extracted by standard methods from peripheral white blood cells with a commercial kit (QIAsymphony; Qiagen Inc., Valencia, CA). DNA was subjected to polymerase chain reaction (PCR) amplification of specific exons of RHD or RHCE with HotStarTaq DNA Master Mix (Qiagen, Inc.) according to the manufacturer’s instructions, using primers and conditions as described previously.20

RNA was isolated from reticulocytes in peripheral blood samples by standard methods (TriZol, and PureLink RNA Mini kit; Invitrogen, Carlsbad, CA). Reverse transcription (RT) was carried out with gene-specific RHD and RHCE primers and Superscript III according to the manufacturer’s instructions (Superscript III first-strand synthesis SuperMix;
Molecular background of RHCE in Bastiaan

Invitrogen). PCR amplification of RHD and RHCE cDNA was carried out with a kit (HotStarTaq DNA Master Mix, Qiagen, Inc.) according to the manufacturer’s instructions, with primers to amplify exons 1–4 and exons 5–10 in RHD and RHCE, as described previously.21

PCR and RT-PCR products were checked for purity on agarose gels, cleaned (ExoSAP-IT Affymetrix, Santa Clara, CA) according to the manufacturer’s instructions, and directly sequenced through contract service provided by Genewiz Inc. (South Plainfield, NJ). Cloning and sequencing of clones of RHD-specific cDNA or genomic DNA were also carried out by Genewiz Inc.

DNA sequence results were aligned to reference alleles NM_020485 for RHCE and NM_016124 for RHD, and predicted protein sequence comparisons were performed (Sequencher v4.9, GeneCodes, Ann Arbor, MI).

MscI digestion of RHD exon 2 products from Bastiaan and a donor, Alson, was conducted at 37°C for 2 hours and run on a 2 percent agarose gel with a 100-bp DNA ladder. RHD zygosity analyses were performed to detect the presence or absence of the hybrid Rhesus box in Bastiaan and Alson as described previously.22 No hybrid box was detected in either sample, indicating that both samples carry two copies of RHD. However, it should be noted that this assay can give a false-negative result in people of African origin because of the altered nucleotide sequence of their hybrid Rhesus boxes.23–25

DNA Array Testing

DNA arrays containing multiple probes either for RHD or for RHCE were tested and analyzed, and interpretations were determined (BioArray Solutions, Warren, NJ) using the manufacturer’s BeadChip technology.

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<th>Name</th>
<th>C</th>
<th>e</th>
<th>hrª</th>
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<th>V</th>
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<th>RHHD*</th>
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<th>MS 17</th>
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</table>

BAS = BioArray Solutions; LDT = laboratory developed test; NT = not tested; NYBC = New York Blood Center; SCD = sickle cell disease.

ªRed blood cells tested with plasma containing both anti-V and anti-VS.

²Based on a large family pedigree, Bastiaan’s RHD genotype has to be Dilla/Dilla-CE(4-7)-D.

³Negative reaction when the control reacted 1+W.
(EuroBio, France) was also nonreactive. The epitope detected by MS21 is the only one that is unaffected by the nucleotide changes encoding the r\(^S\) phenotype.

RBCs from Bastiaan typed D+ DAK+, as would be expected with the presence of the DIIIa phenotype.\(^{26}\)

**Molecular Analyses of RHCE**

Analyses of RHCE gDNA from the Bastiaan sample, by both PCR amplification and sequencing, and by the RHCE DNA array, showed homozygosity for RHCE\(^*\)ce 48C, 733G, 1006T (ce\(^S\), ISBT allele name RHCE\(^*\)ceVS.03 or RHCE\(^*\)01.20.03; Table 2). This allele encodes a V–VS+hr\(^S–\)HrB– phenotype, and our findings are consistent with the RBC typings on Bastiaan, her second child, and his father, as reported by Shapiro et al. (Table 3).\(^1\)

In analyzing the sequences of RHD and RHCE alleles in 605 samples from random African Americans (donors and patients with sickle cell disease), we found 43 samples and 44 alleles (one sample had a homozygous change) exhibiting RHCE\(^*\)ce 48C, 733G, 1006T, which corresponds to an allele frequency of 0.036. Of these, 31 were assumed to be in cis to RHD\(^*\)DIIIa-CE(4-7)-D, 12 in cis to RHD\(^*\)DIIIa, and one in cis to RHD. The latter haplotype is rare, if not novel.

**Molecular Analyses of RHD**

Sequence analyses of RHD gDNA from the Bastiaan sample showed RHD\(^*\)DIIIa (ISBT allele name RHD\(^*\)03.01) and RHD\(^*\)DIIIa 150C, a novel allele reported here (Table 2). Testing gDNA on the RHD DNA array showed RHD\(^*\)DIIIa/RHD\(^*\)DIIIa, which is consistent given that the array does not probe for the nucleotide at position 150. Our DNA result is inconsistent with the RBC typings on Bastiaan, her second child, and his father that were reported by Shapiro et al. (Table 3).\(^1\) As the father was D–C–, the child must have inherited the D–C+ phenotype from his mother. As Bastiaan typed D+ but Rh\(^D–\) (i.e., nonreactive with an anti-D from a person with the DIIIa phenotype) and C+hr\(^S–\) (Table 3), one can predict that her RHD alleles are RHD\(^*\)DIIIa 150C and RHD\(^*\)DIIIa-CE(4-7)-D, and her RH haplotypes are RHD\(^*\)DIIIa 150C/RHCE\(^*\)ce 48C, 733G, 1006T (which encodes a D+ [DIIIa] C–V–VS+hr\(^S–\)–HrB– phenotype) and RHD\(^*\)DIIIa-CE(4-7)-D/RHCE\(^*\)ce 48C, 733G, 1006T (previously known as r\(^S\); now as r\(^S\) type 1, which encodes a D–C+V–VS+hr\(^S–\)–HrB– phenotype).\(^{27}\)

As several attempts to extract cDNA from the Bastiaan sample failed, we were limited to analyzing gDNA to determine the alleles present in this sample. As stated, gDNA revealed an unexpected RHD\(^*\)DIIIa/RHD\(^*\)DIIIa result and no detection of a hybrid allele. However, we have previously noticed the inability to detect an RHD\(^*\)DIIIa-CE(4-7)-D allele in trans to an RHD\(^*\)DIIIa allele using genomic DNA. In contrast, analyses of cDNA clearly show the hybrid allele. In earlier investigations, three samples that gave a cDNA sequencing result of RHD\(^*\)DIIIa/RHD\(^*\)DIIIa-CE(4-7)-D were followed up with gDNA analyses, which gave a sequencing result of RHD\(^*\)DIIIa/RHD\(^*\)DIIIa. The RHD DNA BeadChip (which uses gDNA) gave the same RHD\(^*\)DIIIa/RHD\(^*\)DIIIa result. This discrepancy between gDNA and cDNA has also been demonstrated by others.\(^{28}\)

After an upgrade by BioArray Solutions of their BeadChip interpretative software, RHD\(^*\)DIIIa and hybrid samples were more accurately detected on the RHD BeadChip and the interpretation was in concordance with cDNA results: the DIIIa-CE(4-7)-D hybrid was detected when paired with normal D (n = 15), DIIIa-CE(4-7)-D (n = 10), DAU-0 (n = 5), DAU-3 (n = 2), DOL (n = 1), and pseudo D (n = 1), but still not when paired with DIIIa (n = 3). Three samples that were typed by cDNA as RHD\(^*\)DIIIa-CE(4-7)-D/RHD\(^*\)DIIIa were still called RHD\(^*\)DIIIa/RHD\(^*\)DIIIa by RHD Bead Chip using the new software and by sequencing of gDNA (Table 4). Therefore, without access to cDNA for Bastiaan, it is not possible to verify our interpretation that, based on both gDNA and hemagglutination results, Bastiaan is actually RHD\(^*\)DIIIa-CE(4-7)-D/RHD\(^*\)DIIIa 150C.
Table 4. RHD*DIIIa and RHD*DIIia-CE-D hybrid samples tested by both cDNA sequencing and by gDNA analysis.

<table>
<thead>
<tr>
<th>RHD* alleles</th>
<th>No. of samples tested by cDNA and gDNA</th>
<th>Concordance</th>
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<tr>
<td>DIIla/D</td>
<td>13</td>
<td>13 concordant</td>
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<tr>
<td>DIIla/DIIla</td>
<td>12</td>
<td>12 concordant</td>
</tr>
<tr>
<td>DIIla/other D allele</td>
<td>4</td>
<td>4 concordant</td>
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<tr>
<td>DIIla-CE(4-7)-D/D</td>
<td>15</td>
<td>15 concordant</td>
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<tr>
<td>DIIla-CE(4-7)-D/DIIla-CE(4-7)-D</td>
<td>10</td>
<td>10 concordant</td>
</tr>
<tr>
<td>DIIla-CE(4-7)-D/other D allele</td>
<td>8</td>
<td>8 concordant</td>
</tr>
<tr>
<td>DIIla-CE(4-7)-D/DIIla</td>
<td>3</td>
<td>3 not concordant</td>
</tr>
</tbody>
</table>

Discussion

We observed that RHD*DIIIa from Bastiaan had a novel nucleotide change: RHD*DIIIa 150C. This RHD 150T>C change is a silent nucleotide change from RHCE (Val[GTT]50Val[GTC]). Of the 605 samples from random African Americans (blood donors or patients with sickle cell disease screened for another study), none had a RHD*150T>C change even though 38 carried an RHD*DIIla (186G>T, 410C>T, 455A>C, 602C>G, 667T>G, 819G>A) and 29 carried an RHD*DIIla-CE-D hybrid allele (186G>T, 410C>T, 455A>C, RHCE exons 4–7). Of the 118 samples from patients or donors of African ancestry who were selected because they were known to have diverse Rh phenotypes (based on typing discrepancies, the presence of alloantibodies in antigen-positive people, or the presence of a low-prevalence antigen marker), 25 carried an RHD*DIIIa of which one (Alson) had an RHD*150T>C change, and 19 carried an RHD*DIIla-CE-D hybrid allele. To confirm the presence of the 150T>C change found in sequencing genomic DNA from Bastiaan and RHD-specific cDNA from Alson, the genomic RHD exon 2 was amplified (as described previously) from both samples and cloned. Further verification was obtained by digesting the genomic RHD exon 2 products with MscI, which loses a cut site in the presence of 150C. Bastiaan showed a heterozygous banding pattern for 150T/C, and Alson showed a homozygous (or hemizygous) banding pattern for 150C/C (Table 2).

In analyzing samples for the presence of RHD*DIIIa (186G>T, 410C>T, 455A>C, 602C>G, 667T>G, 819G>A), we discovered a new RHD allele that contained only one nucleotide change (186G>T, Leu62Phe). Of the 605 samples from the random African American donors and patients with sickle cell disease, 470 carried at least one wild-type D allele, of which none had the lone 186G>T change. However, of the 118 selected patient or donor samples with known diverse Rh phenotypes, 60 had a wild-type D allele, and of these, two (Dennis and Sybil) had the 186G>T change. Both samples also carried a Weak D Type 4.2.2 allele (602C>G, 667T>G, 744C>T, 957G>A, 1025T>C). All changes were discovered by the sequencing of RHD-specific cDNA. To determine which allele carried the 186T>G change, the RHD exons 1–4 cDNA product from Dennis was cloned. Clones with 186G (in exon 2) had 602C>G (in exon 4), whereas clones with 186G>T had 602C, confirming that the 186G>T was carried by RHD and not by RHD*Weak D Type 4.2.2.

Testing the sample from Bastiaan, the original proband with anti-hrα and anti-Hrβ, has shown that her V–VS+hrα–Hrβ– phenotype is encoded by RHCE*ce 48C, 733G, 1006T regardless of whether it is in trans to RHD*DIIla or to RHD*DIIla-CE(4-7)-D. As the effect of the Rh complex constituents in the RBC membrane on the expression of antigens is not known, it should rather be stated that Bastiaan's genotype (RHD*DIIla-RHCE*ceS and RHD*DIIla-CE(4-7)-D-RHCE*ceS) is the gold standard for the hrα–Hrβ– phenotype. The RHCE*ce 48G>C, 733C>G, 1006G>T allele is frequently in cis to three different RHD alleles: RHD*DIIla-CE(4-7)-D, RHD*D-CE(4-7)-D, and RHD*DIIla (previously DIII type 5), which explains why many patients with this haplotype are stimulated to produce alloanti-D as well as alloanti-e, or alloanti-hrα. Such cases can be complex to resolve.

As the antibodies in Bastiaan's plasma caused HDFN in her second child, Shapiro et al. tested and reported phenotyping results not only on Bastiaan's husband and affected child but also on 98 other family members in three generations. The pedigree allowed the Rh phenotype of Bastiaan to be determined. Although the phenotype denotations were in the Wiener terminology system, which is no longer familiar, the RBC typings given by Shapiro et al. were sufficient to allow us to interpret the Rh phenotypes using current terminology (Table 2). That the father was D–C– made the child's phenotype particularly informative. The child's D–C+ phenotype most likely was a consequence of the r'S haplotype (D–C+V–VS+) inherited from Bastiaan. Clearly, Bastiaan has the DIIla 150C-ceS/RHD*DIIla-CE(4-7)-D haplotype.
We report here the first examples of alleles that had either a single 186G>T change (Leu62Phe) in RHD or a single 150T>C change in RHD*DIIIa. RHD alleles known to harbor a 186G>T change (silent) include ceS-type 1, DIIIa (DIII type 5), DIII type 4, and DIVa (DIVa.2). RHD alleles known to harbor a 150T>C silent nucleotide change include RHD*DIIIb, RHD*DIII type 7, RHD*DKK, and other hybrid alleles with exon 2 from RHCE.

In conclusion, the gold standard RHCE allele that is associated with the hr B– HrB– phenotype is r′S; the genotype is RHD*DIIIa-RHCE*ceS/RHD*DIIIa-CE(4-7)-D-RHCE*ceS. Phenotypes that lack hrB and HrB are Rhnull, D– –, Dc–, DCW–, r′S type 1, r′S type 2, and DIIIa-ceS.

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Letter from the Editor

Serologic Method Reviews—A New Feature

The editors of *Immunohematology* are excited to announce a new review series, Serologic Method Reviews. Authors have been recruited from the immunohematology reference laboratory community to draft reviews of specific serologic methods. (See table for a sampling of methods.) Each review will address aspects of the method such as its principle, indications, specific reagents and supplies, high-level procedural steps in a table format, detailed procedural information, limitations, and references. We will be publishing these reviews individually in successive issues of *Immunohematology*, and we expect to compile them and publish them as a collection. We anticipate that the individual method reviews will be a resource for incumbent and new laboratory staff and that a copy of the collected methods will be a must-have for transfusion medicine facilities, libraries, and personal bookshelves.

Sandra Nance
Editor-in-Chief
*Immunohematology*

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<td><a href="http://www.giveblood.org/services/education/sbb-distance-program">www.giveblood.org/services/education/sbb-distance-program</a></td>
</tr>
<tr>
<td>Hoxworth Blood Center, University of Cincinnati Medical Center</td>
<td>Pamela Inglish</td>
<td>513-558-1275</td>
<td><a href="mailto:inglishp@ucmail.uc.edu">inglishp@ucmail.uc.edu</a></td>
<td><a href="http://www.grad.uc.edu">www.grad.uc.edu</a></td>
</tr>
<tr>
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<td>Jayanna Stayton</td>
<td>317-916-5186</td>
<td><a href="mailto:jstayton@indianablood.org">jstayton@indianablood.org</a></td>
<td><a href="http://www.indianablood.org">www.indianablood.org</a></td>
</tr>
<tr>
<td>Johns Hopkins Hospital</td>
<td>Lorraine N. Blagg</td>
<td>410-502-9584</td>
<td><a href="mailto:lblagg1@jhmi.edu">lblagg1@jhmi.edu</a></td>
<td><a href="http://pathology.jhu.edu/department/divisions/transfusion/abb.cfm">http://pathology.jhu.edu/department/divisions/transfusion/abb.cfm</a></td>
</tr>
<tr>
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<td><a href="mailto:kkirkley@louisiana.edu">kkirkley@louisiana.edu</a></td>
<td><a href="http://www.mcm.edu/webresources/index.html">www.mcm.edu/webresources/index.html</a></td>
</tr>
<tr>
<td>NIH Clinical Center Blood Bank</td>
<td>Karen Byrne</td>
<td>301-486-8335</td>
<td><a href="mailto:kbyrne@email.cc.nih.gov">kbyrne@email.cc.nih.gov</a></td>
<td><a href="http://www.cc.nih.gov/dtm">www.cc.nih.gov/dtm</a></td>
</tr>
<tr>
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<td>Yalanda Sanchez</td>
<td>312-942-2402</td>
<td><a href="mailto:yalanda_Sanchez@rush.edu">yalanda_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:mduety@fsutblood.org">mduety@fsutblood.org</a></td>
<td><a href="http://www.fsutblood.org">www.fsutblood.org</a></td>
</tr>
<tr>
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<td>Linda Myers</td>
<td>210-731-5526</td>
<td><a href="mailto:lmyers@bloodnitsissue.org">lmyers@bloodnitsissue.org</a></td>
<td><a href="http://www.sbbofsa.org">www.sbbofsa.org</a></td>
</tr>
<tr>
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<td>Janet Vincent</td>
<td>400-772-3055</td>
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<td><a href="http://www.utmb.edu/abb">www.utmb.edu/abb</a></td>
</tr>
<tr>
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<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>
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