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On Our Cover

Precisionism, with its clean lines and geometries and its unironic worship of the burgeoning industrial American landscape, was the first modernist art movement to originate in the United States. Charles Demuth was a key innovator in and contributor to the style. While many precisionists focused on the city, with its manifold technological novelties, health problems confined Demuth to his home in rural Lancaster, Pennsylvania, for much of his life. Nevertheless, as the grain elevators in My Egypt (1927) indicate, the bucolic landscape provided him with ample thematic subjects. Demuth’s decision to paint the enormous towers as seen from below enhances their monumental feel, while crisscrossing angles and rays—along with the title—coyly allude to the Great Pyramids. This issue of Immunohematology features reports by Goldman et al. and Schmidt et al. describing their experiences with automated testing platforms.

David Moolten, MD
Recipient red blood cell (RBC) phenotyping using serologic techniques, within 3 months of a transfusion, is considered unreliable. We conducted in vitro experiments to determine how long recipient RBC phenotyping results would be compromised after an allogeneic transfusion. In vitro models were created to mimic in vivo posttransfusion ratios of “transfused” RBCs with either a single or a double dose of an antigen and “autologous” RBCs negative for the corresponding antigen at 10-day intervals from day 0 to day 90 in hypothetical recipients with varying weights and hematoctrits (Hct) receiving varying numbers of RBC units. In general, a reliable recipient RBC phenotype was possible earlier after transfusion in larger recipients, those with higher Hct, and those transfused with fewer RBC units and if the transfused units had the antigen of interest in single, rather than double, dose. We believe that a reliable RBC phenotype, using routine serologic techniques, can often be obtained well before 3 months after transfusion. Similar studies with other donors, antigens, antisera, and methods and in actual patients will be useful. *Immunohematology* 2013;29:93–6.

**Key Words:** RBC, phenotyping, posttransfusion, in vitro model

Recipient red blood cell (RBC) phenotyping may be useful in determining what clinically significant alloantibodies a recipient could make by identifying the antigens that the recipient lacks. It has been considered difficult to do this after transfusion, but it is the regularly transfused patient in whom this information is most useful. Alternatives exist—e.g., genotyping—but this may not be possible everywhere. We used an experimental model to determine how soon after a hypothetical “transfusion” an unequivocal “recipient” RBC phenotype could be determined using conventional serologic methods.

**Materials and Methods**

In vitro mixtures of RBCs were created to mimic in vivo “posttransfusion” ratios of “autologous” to “transfused” RBCs at 10-day intervals from day 0 to day 90 after transfusion of varying numbers of RBC units in recipients of varying weights and with varying degrees of anemia.

In our model, C+, Jk(a+), or K+ RBC units were transfused to C−, Jk(a−), or K− recipients, respectively. RBC transfusions with a single or double dose of the antigen concerned were considered separately. The transfused and autologous RBCs used in the experiments came from four healthy blood donors or volunteers (blood groups: O, rr, K−k+, Jk[a−b+]; O, R1R1, K−k+, Jk[a+b+]; O, R1r, K+k−, Jk[a−b+]; O, R1r, K+k+, Jk[a+b+]) whose units were less than 1 week old.

Nominal male recipients weighing 50, 70, or 100 kg, with estimated blood volumes (EBV) appropriate to weight, with either of two levels of anemia (hematocrit [Hct] 25% or 15%) were considered. The EBV was taken as 70 mL/kg, i.e., the midpoint of the normal range in either sex (60–80 mL/kg). The volume of autologous RBCs in the recipient was calculated from the EBV and Hct. Recipients with Hct of 25 percent were considered to have received one, two, or three units of packed RBCs and those with Hct of 15 percent, two, three, or four units. Per the standard specifications for leukocyte-reduced RBC units in New Zealand, the average volume of RBC units was determined to be 300 mL, the average Hct was 60 percent, and posttransfusion RBC recovery (PTR) was 80 percent, resulting in an RBC volume per RBC unit transfused of 144 mL. It was assumed that transfused RBCs survived optimally. Thus, the loss of transfused RBCs per day was taken as 1/120 of the RBC volume transfused on day 0 (144 mL × number of RBC units transfused).

For instance, a 50-kg recipient with an EBV of 3500 mL (70 mL/kg) and Hct of 15 percent might have an RBC volume of approximately 525 mL. If such a recipient were to be transfused with three RBC units, the volume of transfused RBCs on day 0 would be 144 mL × 3 or 432 mL. Assuming that 1/120 of the transfused RBCs are destroyed each day, surviving transfused RBCs after 10 days would be 396 mL, after 20 days would be 363 mL, and so on. Similar calculations were done for all nominal recipients. Note that it was assumed that the autologous RBC volume stayed constant as a result of balanced production and destruction. Posttransfusion ratios of autologous to transfused RBCs at different times were calculated for all recipients, factoring in recipient weight, Hct, and days after transfusion as described previously. These are shown in Figure 1 expressed as calculated percentages of RBCs in the recipient that are likely to be transfused RBCs.
The actual volumes of the RBC suspensions—either a 3 percent or 0.8 percent suspension of the RBC mixture (Ortho Biovue and DiaMed-ID, respectively—see later section)—used varied, from the one with the highest autologous-to-transfused ratio (1750 µL:65.8 µL in the nominal 100-kg patient with an Hct of 25%, at day 90 after transfusion of one RBC unit) to the one with the lowest autologous-to-transfused ratio (576 µL:525 µL in the nominal 50-kg patient with an Hct of 15%, at day 0 after transfusion of four RBC units). In each case we started with the autologous-to-transfused RBC ratio most likely to show a clear negative reaction (a score of 0), i.e., a high ratio. Then, mixtures with successively lower autologous-to-transfused RBC ratios were tested until the point at which a clear negative reaction was no longer obtainable. The last ratio giving a clear negative reaction was taken as the day beyond which it was possible to ascertain the autologous, antigen-negative RBC phenotype. This was done separately for transfused RBCs with a single or double antigen dose.

RBC phenotyping was done using column agglutination methods (Ortho BioVue System, Ortho Clinical Diagnostics, Raritan, NJ) for C and K and (DiaMed-ID Micro Typing System, DiaMed AG, Morat, Switzerland) for Jkα. Commercially obtained antisera (anti-C and anti-Jkα, Lorne Laboratories, Reading, UK) were used. The anti-K was incorporated in the gel card (Ortho BioVue System, Ortho Clinical Diagnostics). Validated New Zealand Blood Service procedures were followed. Positive and negative controls were run in parallel with test samples.

**Results**

All four variables studied (recipient weight and Hct, the number of antigen-positive RBC units transfused, and the dose of the relevant antigens in the transfused units) affected which day after transfusion the autologous, antigen-negative RBC phenotype could be determined (Table 1).
Serologic determination of RBC phenotype in recently transfused patients is considered difficult and is generally not recommended within 3 months of a transfusion. Alternatives in this situation include using the resistance of RBCs from patients with sickle cell disease, relative to transfused RBCs, to hypotonic lysis; autologous reticulocyte phenotyping after initial separation in microhematocrit tubes or by using immunomagnetic or color flow cytometric methods; and genotyping using polymerase chain reaction and related methods. Many such methods are not generally available.

The 3-month period referred to previously does not take into account variables such as recipient weight and Hct, the number of transfused units, or the antigen dose in the transfused units—the effects of all of which were examined in this in vitro study. Patient weight determines, to an extent, the blood volume, which is the volume of dilution for transfused cellular components. If the volume of RBCs transfused is high relative to the patient’s volume of dilution and Hct, one might expect a relative delay in establishing an unequivocal patient RBC phenotype after transfusion. We might also expect similar delays in patients with chronic RBC synthesis failure or hemolysis as opposed to, say, otherwise well patients who are anemic after an acute blood loss in whom it might be expected that autologous RBC synthesis would be significantly increased after the acute event.

To our knowledge, an in vitro model of this sort has not previously been described in the literature. Our experiments suggest that, at least for the antigens we considered, a clear RBC phenotype can be obtained for some transfusion recipients well before 3 months after transfusion, using routine serologic methods, although this may not be possible in others, for instance, small-sized individuals with low Hct given relatively large RBC transfusions (Table 1). We expect that similar results will be obtained with other RBC antigens.

Both the Council of Europe and the US Food and Drug Administration guidelines require a PTR of at least 75 percent 24 hours after transfusion. We assumed a PTR of 80 percent, which is within the range that might be expected for transfused RBCs toward the end of shelf-life and we used this figure in our experiments to determine transfused-to-autologous RBC ratios at various time intervals. Of course,

<table>
<thead>
<tr>
<th>Patient weight (kg)</th>
<th>Hct (%)</th>
<th>Number of units transfused</th>
<th>Phenotype of transfused RBCs</th>
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<tr>
<td>50</td>
<td>15</td>
<td>4</td>
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Discussion

Recipient RBC phenotyping may be useful in at least three situations in recently transfused patients: first, to avoid further exposure to clinically significant RBC antigens in those likely to require RBC transfusions long-term (ideally such recipients should be phenotyped before the first transfusion); second, to corroborate alloantibody specificities; and third, to allow allodextrusion studies using phenotype-matched RBCs in patients with autoimmune hemolytic anemia to be more easily performed.

In general, in nominal recipients with an Hct of 25 percent, we were able to determine the autologous RBC phenotype relatively early after transfusion. This was especially true of larger recipients; of those transfused with fewer RBC units; and if the RBCs expressed a single, rather than a double, dose of the antigen concerned. In nominal recipients with an Hct of 15 percent weighing 50 or 70 kg, irrespective of the antigen, its dose, or the number of RBC units transfused, the autologous, antigen-negative phenotype could not be unequivocally determined even at day 90 after transfusion. This was also largely true for the 100-kg recipient with an Hct of 15 percent except that, in this case, a clear negative (autologous) phenotype could be established in the day 90 sample with two transfused RBC units if the C, Jk+, or K antigen was present in a single dose (Table 1).
higher recoveries (e.g., approximately 90%) may be obtained with fresher units,11 and this would tend to increase the proportion of transfused RBCs relative to autologous RBCs at various times after transfusion. However, Luten et al.11 also noted that PTR in transfused patients may be lower than in healthy volunteers, which would have the opposite effect, i.e., to decrease the proportion of transfused RBCs relative to autologous RBCs.

In most patients (again, with the exception of individuals with low EBV and low Hct given relatively large RBC transfusions), the proportion of antigen-positive, transfused RBCs is likely to be small relative to that of autologous RBCs. We considered only unequivocal negative results as indicative of the true autologous, antigen-negative RBC phenotype. However, in the context of RBC phenotyping in recently transfused patients, the relevant questions are, is there an antigen-negative RBC population, and, if so, could this be of recipient origin, taking into consideration the patient’s weight and Hct, the number of RBC units transfused, and the time after transfusion? Furthermore, mixed populations of RBCs are easily distinguishable with gel agglutination methods.

When considering patient RBC phenotyping after transfusion, these factors should be taken into account. Information on antigen doses in transfused units will usually be unavailable; however, in many instances and with many of the clinically significant RBC antigens, RBCs are more likely to have a single rather than double dose of the antigen. Studies with other antigens, test systems, and antisera, as well as in real patients, will be useful.

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Kumudini Gonsalkorale, MD, Post-graduate Trainee in Transfusion Medicine, New Zealand Blood Service, (currently, Consultant Transfusion Physician, Colombo South Teaching Hospital, Kalubowila, Dehiwela, Colombo, Sri Lanka); Charlotte Vanhecke, Registered Medical Laboratory Scientist, Blood Bank, New Zealand Blood Service, Christchurch Public Hospital; Krishna G. Badami, MD, FRCPATH (corresponding author), Transfusion Medicine Specialist, New Zealand Blood Service, 87, Riccarton Road, Christchurch, New Zealand.
Identifying D-positive donors using a second automated testing platform

M. Goldman, I. Resz, J. Cote, G. Ochoa, and N. Angus

Because of the variability of D expression, one method may be inadequate to correctly classify donors with variant RHD alleles. We evaluated the use of a solid-phase automated platform (ImmucorGamma Galileo) to confirm D− test results obtained on first-time donors on the Beckman Coulter PK7300 automated microplate test system. Samples with discordant results were analyzed by serologic tube methods, RHD genotyping using the BLOODchip platform (Progenika), and, if necessary, sequencing. We estimated the number of cases of alloimmunization in women younger than 50 years likely to be prevented by the addition of Galileo testing. From May 2011 to May 2012, 910,220 donor samples were tested; 15,441 were first-time donors with concordant D− results. Five donors tested D− on the PK7300 and weak D+ on the Galileo; one was found to be a false positive on further testing. On manual testing, the other four donors had positive indirect antiglobulin test results with one to three of the antisera used and were C+. On BLOODchip testing, two donors were classified as D+, and two were assigned a “no call.” D variants included weak D type 67, weak D type 9, and two novel variants. Approximately 10 percent of D− units are transfused to women younger than 50 years. Assuming an alloimmunization rate of 30 percent, use of the Galileo would prevent approximately one alloimmunization every 5 to 6 years in this patient group. We conclude that the yield of preventing alloimmunization in this population by adding a second automated serologic testing platform is very low. Immunohematology 2013; 29:97–100.

Key Words: weak D phenotype, RHD alleles

D is one of the most immunogenic blood group antigens, and may cause both hemolytic transfusion reactions and hemolytic disease of the fetus and newborn (HDFN). D typing can be problematic because the distinction between D+ and D− individuals is not always clear.2,3 Approximately 1 percent of white people carry variant RHD alleles, which may lead to reduced antigen expression.2,3 The more than 160 different RHD alleles that have been described may result in reduced antigen density (so-called weak D phenotypes) or qualitatively distinct D antigens missing some D epitopes (so-called partial D phenotypes). In addition to genetic variability, variable typing results may be caused by use of different typing reagents and methods. Monoclonal reagents generally give stronger reactivity than polyclonal reagents but are directed against a single epitope, and therefore may miss certain variants; therefore blends of more than one monoclonal reagent or monoclonal/polyclonal blends are currently used. Testing to detect D is performed by immediate spin (IS); so-called weak D testing to detect reduced D expression is performed by the indirect antiglobulin test (IAT).2

The classification of individuals as D+ or D− depends not only on laboratory results but also on the underlying reason for performing the typing. Standards from both the AABB and the Canadian Standards Association (CSA) require blood donors who appear to be D− to be tested by a method to detect weak D expression.4,5 Only donors with negative results are classified as D−. The rationale behind these standards is the concern that the red blood cells (RBCs) of some donors with variant antigen expression may carry enough D to alloimmunize D− recipients.

Canadian Blood Services (CBS) tests approximately 1,000,000 donor samples a year for ABO and D using an automated microplate system, the PK7300 (Beckman Coulter, Inc., Brea, CA). Two anti-D reagents were used to perform testing. First-time donors who appeared to be D− had manual tube testing done by both IS and IAT. More recently, the reagents used for automated testing on the PK7300 machine have been licensed to detect weak D expression. Because of concerns that one method may be inadequate to classify all donors with variant D alleles as D+, we performed an evaluation of the yield of identifying D+ donors by adding an automated solid-phase platform (Galileo, ImmucorGamma, Norcross, GA) to confirm typing of D− first-time donors. Samples with discordant results by the two automated methods were investigated further using manual serologic methods and genotyping. Because the ultimate goal of testing is not simply to identify variants but to prevent alloimmunization, particularly of girls and women of childbearing age, we also estimated the number of cases of alloimmunization likely to be prevented a year by testing using the solid-phase platform in addition to the PK7300.

Materials and Methods

Automated Testing

Blood samples in EDTA tubes were drawn from the diversion pouch of donor collection kits and sent to one of
two national donor testing laboratories. Routine ABO and D testing was performed on the Olympus PK7300 (Beckman Coulter) following manufacturer’s instructions and reagent package inserts. Samples were tested by the hemagglutination sedimentation method for D typing using the anti-D monoclonal blend reagent (IgM and IgG human monoclonal blend, composed of clones P3X61, P3X21223B10, P3X290, and P3X35) and the anti-D (PK1) monoclonal IgM reagent (clone P3X61; DIAGAST, Loos Cedex, France). Samples from first-time donors with a valid ABO group and D– result with both reagents were tested on the automated solid-phase platform (Galileo, ImmucorGamma), following manufacturer’s instructions and the reagent package inserts. Weak D testing was performed by the IAT using one of the Immucor monoclonal D reagents (anti-D Series 4 [IgG, IgM monoclonal blend, clones MS201 and MS26], ImmucorGamma).

**Manual Testing**

Donor samples testing weak D+ on the Galileo were investigated further using manual tube testing in our National Immunohematology Reference Laboratory (NIRL). Tube testing was done by both IS and IAT using three different anti-D reagents in accordance with the manufacturers’ package inserts: an IgM (D175-2) and IgG (D415 1E4) monoclonal blend (Novaclone, Dominion Biologicals Limited, Dartmouth, Nova Scotia, Canada); an IgM (GAMA401) and IgG (F8D8) monoclonal blend (Gamma-clone, ImmucorGamma); and a blend of monoclonal IgM (MAD2) and polyclonal human IgG anti-D (Bioclone, Ortho Clinical Diagnostics, Raritan, NJ).

**Genotyping and DNA Sequencing**

Samples with discordant D results were also sent for RHD genotyping to the Novartis/Progenika, Inc., reference laboratory (Medford, MA). Samples were analyzed by the Progenika BLOODchip Reference assay. BLOODchip Reference is a polymerase chain reaction– and hybridization-based genotyping test in oligonucleotide microarray format that interrogates 116 polymorphisms in genes encoding RBC antigens. For the RHD gene, it interrogates 72 polymorphisms that encode 23 D–, 20 weak D, 34 partial D, and 8 D0 variants. DNA sequencing was performed by the standard Sanger dideoxy method with specific primers that bind to intron regions flanking RHD exons 5, 6, and 7.

**Estimation of Alloimmunization**

The retention rate for first-time donors, mean frequency of whole blood donations per year per donor, and total number of RBCs issued per year were extracted from the CBS Progesa database, using our data warehouse. The percentage of RBC units transfused per year to women younger than 50 years was estimated using data from two sources: from the British Columbia (BC) Provincial Blood Coordinating Office (PBCO), which stores data on all transfusions in the province of BC, the data for a 2-year period (April 2010 to March 2012: 50,978 distinct recipients and 269,330 RBC units transfused); and from the McMaster University Hospital database of all transfusions in three large hospitals in the McMaster University Hospital system, data from a 3-year period in the city of Hamilton, Ontario (January 2009 to January 2012: 15,058 recipients and 76,702 RBC units transfused).

**Results**

During a 1-year period starting in May 2011, approximately 910,220 samples from whole blood donations were tested on the PK7300. Of these, 15,441 were first-time donors who tested D– on both the PK7300 and Galileo. Five donors tested D– on the PK7300 and weak D+ on the Galileo. One of these donors was found to be D– on manual testing and genotyping, and therefore is considered to be D–, with a falsely positive test result on the Galileo. Testing results on the Galileo for the other four donors, shown in Table 1, gave 2+ to 4+ results by the IAT. On manual tube testing, donor 1 reacted by IAT with all three antisera, whereas donors 2, 3, and 4 reacted by IAT with only one or two of the three antisera. Interestingly, all four donors were found to be C+ on extended Rh typing.

On genotyping using the BLOODchip, donors 1 and 3 were classified as D+, whereas donors 2 and 4 were assigned a “no

<table>
<thead>
<tr>
<th>Donor</th>
<th>Galileo</th>
<th>Manual tube</th>
<th>Bioclone</th>
<th>Genotyping</th>
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<td>3+</td>
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<tr>
<td>4</td>
<td>2+</td>
<td>0</td>
<td>0</td>
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</table>

Extended Rh typing: Ccee D+ Variant of exon 7, RHD*1018A

DNA sequencing: Ccee No call Variant of exons 5, 6, RHD*712A, 809G

DNA sequencing: Ccee D+ Weak D, type 67

DNA sequencing: Ccee No call Weak D, type 9
call. On DNA sequencing, donor 1 was found to have a novel variant of exon 7 RHD*1018A, similar to RHD*1018A, 1019T (weak D type 30). Donor 2 was found to have a novel variant of exons 5 and 6, RHD*712A, 809G. A similar listed variant, RHD*712C, 809G, was reported in a large French study in which direct sequencing was performed on 806 samples with ambiguous D phenotypes. Finally, donors 3 and 4 exhibited known weak D genotypes type 67 and type 9, respectively.

**Estimation of Alloimmunization of Female Recipients Younger Than 50 Years**

Table 2 summarizes the number of female recipients younger than 50 years that may have been alloimmunized, if the four donors with D-expressing variants had been classified as D− based on PK7300 testing. The return rate for new donors at CBS is approximately 50 percent, whereas the average donor donates two whole blood units annually (Tony Steed, CBS, Marketing and Recruitment, personal communication). Therefore, we would expect these four donors to contribute the initial four units plus two additional units during the year. In the province of BC, female recipients younger than 50 years represent 10.1 percent of patients being transfused and receive 9.3 percent of transfused RBC units (Tanya Petraszko, BCBlood Coordinating Office, personal communication). In Hamilton area hospitals, female recipients younger than 50 represent 10.6 percent of patients being transfused and receive 10.6 percent of units transfused (Nancy Heddle, McMaster University Transfusion Research Program, personal communication). Extrapolating these numbers nationally, women younger than 50 would receive approximately 10 percent of distributed D− units, or 10,306 D− units per year, of which 0.60 would be discordant. The exact alloimmunization potential of each of these D variants is unknown. A D+ unit, with the normal number of D sites, would be expected to cause alloimmunization of approximately 30 percent of recipients. The alloimmunization rate for these variant D units is likely to be less. However, using an alloimmunization rate of 30 percent, 0.18 female recipients younger than 50 could be alloimmunized annually. This rather simplistic analysis assumes that the percentage of D− units transfused to women younger than 50 is similar to the percentage of all units transfused to this group. Additionally, because a large percentage of D− units have had extended Rh typing and have the results printed on the unit label, these C+ units may not be selected for transfusion of this group.

**Discussion**

In this study, 4 of 15,441 (0.026%) samples with D− results on the PK7300 typed as weak D+ on the solid-phase Galileo platform. These donors also had positive results by the IAT in tube testing using one or more available reagents, although no reagent would have detected all four donors. Additionally, two of these donors have unreported D variants. These data illustrate that no one method will detect all the variants of the extremely polymorphic Rh system.

Our donor population is primarily white and of European origin. Therefore, we would expect approximately 0.4 to 0.8 percent of the donors to be weak D variants, predominantly type 1, 2, or 3, as described in the European literature. However, none of these variants was found in the four discordant donors. This suggests that the common D variants are being detected and classified as D+ by PK7300 testing, as has been shown to occur when using the PK7200 machine.8

Determination of the number of female recipients younger than 50 years who might be alloimmunized if Galileo weak D testing was stopped is difficult because the exact immunization rate associated with the four D variants detected is unknown. However, even assuming very conservatively that 30 percent of recipients of these units would be alloimmunized, this would result in one female recipient younger than 50 being alloimmunized every 5 to 6 years.

Interestingly, all four donors were C+. The presence of C is a well-known cause of reduced D expression for already weak D variants.1,9,10 Indeed, it has been proposed that individuals who are D− but C+ should undergo genotyping to detect D variants.9,10 Extensive phenotyping of D− units for C, c, E, e, and K is performed at CBS; the results are printed on the unit label once testing has been performed on two donations. Therefore, it would be possible to simply select rr (ccee) units for transfusion to women younger than 50 years because the majority of these would be true D− units. An advantage to
selecting rr units would be the avoidance of other very weak D variants that may be found by genotyping but are missed by the PK7300, the Galileo, or other serologic methods used for testing. Additionally, use of rr units would avoid C and G alloimmunization caused by transfusion in C− recipients. In some European countries, routine matching for C, c, and K is done for transfusion of women younger than 50. This approach would have the added advantage of avoiding alloimmunization to c in CC patients.

We conclude that the yield of identifying D+ donors by adding an automated solid-phase platform to confirm typing of D− first-time donors is extremely low. Matching for C and K would likely have a larger impact on reducing HDFN. Strategies for prevention of alloimmunization may change in the future, with the ongoing development of high throughput, inexpensive genotyping methods that could be used to routinely screen blood donors.

Acknowledgments

The authors would like to thank Dr. Tanya Petraszko, Jennifer Danielson, and Jimmy Chan from the BC Provincial Blood Coordinating Office and Nancy Heddle and Liu Yang from the McMaster Transfusion Research Program for providing data on the age and sex of transfusion recipients. We would also like to thank Dr. Irene Sadek, Balkar Gill, and the entire donor testing staff, as well as Jennifer Cuffari for secretarial assistance.

References

Our facility changed antibody screening methods from a gel microcolumn–based test (ID-Micro Typing System Gel Test; Ortho Clinical Diagnostics, Inc., Raritan, NJ) to an automated solid-phase test (Galileo/Capture-R Ready-Screen [I and II], Immucor, Inc., Norcross, GA). To determine whether detection rates for commonly encountered clinically significant red blood cell antibodies differed as a consequence of this change, preimplementation and postimplementation antibody identification records were retrospectively reviewed. A statistically significant difference in the percentage of positive screening tests during the gel microcolumn testing period (73,903 total screens, 1.56% confirmed positive) versus the solid-phase screening period (80,242 total screens, 1.81% confirmed positive; \( p < 0.0002 \)) was observed. The number of antibodies to K identified was significantly lower with solid phase than with gel (27% decrease; \( p = 0.004 \)). It is unknown whether there is a statistical difference in delayed or hemolytic transfusion reaction rates as this was not evaluated. *Immunohematology* 2013;29:101–04.

**Key Words:** gel microcolumn, solid phase, antibody screening, anti-K

When a transfusion service or laboratory implements new red blood cell (RBC) antibody detection methods, it is likely that differences in the sensitivity and specificity of these methods will be observed. The choice of the system must take into account its limitations as well as its advantages over the system currently in use (ease of use, reproducibility, cost effectiveness, turnaround time, etc.). Ultimately, a system should be chosen that most effectively and efficiently meets the needs of the patients and workflow of the laboratory. This has been the case at our institution as outlined later.

Our facility, a large academic tertiary-care facility with more than 2000 beds and 96 operating rooms serving 350,000 patients per year in various surgical and medical specialties, including a large solid-organ and hematopoietic progenitor cell transplant center. A blood donor center and a busy transfusion service are supported by the laboratory, and each year during the period of this study, more than 45,000 RBC units were transfused and approximately 49,000 antibody screens were performed.

In 1993, the polyethylene glycol (PEG) tube method for RBC antibody screening was implemented as the primary method for most patients requiring such testing at our institution. An early automated version of solid-phase technology (IBG Systems, Shoreham-by-Sea, England, later purchased by Immucor, Inc., Norcross, GA) was used for screening of blood donors and next-day surgical patients. When the early solid-phase system could no longer be maintained, we explored the options that were available to meet the automation needs of a high test-volume blood bank. At that time, automation options were limited.

In 2004, an automated instrument (Tecan MEGAFlex ID, Ortho Clinical Diagnostics, Inc., Raritan, NJ) was purchased for ABO determination and antibody screens for blood donors and next-day surgical patients. We decided to use the gel method (ID-Micro Typing System, Ortho Clinical Diagnostics Inc.) for our antibody screening because it best fit our automation needs at the time. At the same time, the manual gel microcolumn method replaced the PEG method for antibody screening of all other patients. (Both gel microcolumn methods will subsequently be identified as gel).

In 2007, the gel test and its supporting automation were replaced with the fully automated solid-phase testing platform (Capture-R Ready-Screen [I and II] on Galileo, Immucor, Inc.). This instrument performed antibody screening and ABO testing on patients and blood donors, while PEG tube was used as our backup method. The switch was made to accommodate a more automated workflow in our high test-volume institution.

Throughout the process, limitations of each automated system were considered. Each piece of automation is restricted in some manner, whether it is sensitivity, specificity, ease of use, reproducibility, cost of instrumentation, reagent costs, turnaround time, etc. This study was undertaken to allow comparisons of two methods and automated systems during a multiyear period at a single institution.

**Materials and Methods**

A retrospective review of RBC serologic records from November 1, 2005, to December 31, 2008, was performed.
For individual patients, data collection dated back to the first time a positive antibody screen result was documented using either a gel or a solid-phase method. Patients who had a positive antibody screen obtained via the PEG tube method and those who had an initial workup performed using an antibody identification panel were not included in the analysis. Blood donor data also were not included in this study. Subjects included in this analysis were not further selected by age or underlying diagnosis. Consent to undergo research investigation was verified for all study subjects, and the study was approved by the local institutional review board.

Data on antibody screen procedures performed using the gel methods (manual and automated) from November 2005 to April 2007 were collected. The automated and manual gel methods were performed using the manufacturer’s procedures and a two-cell screening set (0.8% Selectogen, Ortho Clinical Diagnostics). Reactions were graded and recorded using standard criteria.6,7

Data on solid-phase antibody screening from June 2007 to December 2008 were collected. Automated solid-phase testing was performed (Galileo, Capture-R Ready-Screen [I and II] Immucor, Inc.), which contains microtiter wells coated with screening cells. A two-cell screening technique was used. The manufacturer’s instructions for carrying out the assay and determining reaction grades were followed.8

The PEG tube method was used for antibody identification during both the gel and the solid-phase screening periods. Comparison of the identified antibodies was limited to common clinically significant specificities, to allow for statistical analysis. Antibodies identified singly as well as in conjunction with other specificities were included.

Poisson regression was used to assess the statistical difference in incidence (detection rate) of positive screens between the gel method and the Galileo method for overall positive screens, as well as for specific antibodies, with the dependent variable being the incidence of positive screens (overall, subsequently by antibody) and the independent variable being method of screening, with an offset being log-total number of screens by each method. All analyses were performed using a statistics program (SAS version 9.1.3, SAS Institute Inc., Cary, NC). Overall significance was defined as a probability value less than 0.05, with significance of specific antibodies less than 0.0045 to allow for multiple comparisons.

Results

Of 73,903 antibody screens performed with the gel method, 1153 positive screens (1.56%) were confirmed by manual PEG method. Using the Galileo method, 80,242 antibody screens were performed, and 1449 positive screens (1.81%) were confirmed by the manual PEG method. This difference was statistically significant (Poisson regression, p < 0.002). In Table 1, the specificities and total numbers of commonly encountered, clinically significant RBC alloantibodies identified with the two antibody screening methods are presented (anti-D was excluded from this study owing to the high incidence of passive antibody detected). A statistically significant decreased number of antibodies to K was identified with the switch from the gel method to the subsequent solid-phase method (190 vs. 150, 27% decrease in detection rate; p = 0.004). This is in contrast to the number of antibodies to Jka identified, which was higher with the

<table>
<thead>
<tr>
<th>Antibody specificity</th>
<th>Gel method</th>
<th>Solid-phase method</th>
<th>Comparison of total antibodies identified</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Single antibody</td>
<td>Multiple antibodies</td>
<td>Total identified</td>
</tr>
<tr>
<td>E</td>
<td>138</td>
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<td>225</td>
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<tr>
<td>c</td>
<td>17</td>
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<td>56</td>
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</tr>
<tr>
<td>Jkc</td>
<td>3</td>
<td>5</td>
<td>8</td>
</tr>
</tbody>
</table>

*Detection rates (incidence) were calculated to account for the variability in the total number of screens performed in each period: gel method = 73,903 screens, solid-phase method = 80,242 screens. For individual antibody comparisons, the statistical significance was defined as a probability value of ≤ 0.0045.
solid-phase method but did not reach statistical significance (35 vs. 61, 61% increase in detection rate; \( p = 0.03 \)). The relative numbers of antibodies to s, Fy\(^a\), and Jk\(^b\) detected with the solid-phase method compared with the gel method were 268 percent, 176 percent, and 73 percent higher, respectively. These differences, however, did not achieve statistical significance because of the overall low numbers of such antibodies.

**Discussion**

Overall, during the solid-phase screening period, a small but statistically significant increase in the number of antibodies detected relative to number of screens performed was identified (1.81% vs. 1.56%). Solid-phase screening was associated with a statistically significant decrease in the number of detected K antibodies. Although the numbers of other clinically significant antibodies such as anti-Jk\(^a\), anti-Jk\(^b\), and anti-Fy\(^b\) were increased with solid-phase testing as compared with gel screening, the small numbers overall led to a lack of a statistically significant change. Continued surveillance for such changes will be necessary to determine whether the change in frequency reaches clinical significance as the number of samples tested increases.

These differences may be attributable to the sensitivity and specificity of the assays or other clinical variables such as transfusion practices and patient populations, which may have changed during the study period. Such clinical differences may lead to significant changes in transfusions per patient and the subsequent number of antibodies formed. Differences in patient population or medical practice can be studied in a retrospective manner as has been done previously.\(^1\)\(^,\)\(^5\)\(^,\)\(^9\) These variables cannot be fully evaluated with the current data. A subsequent study is planned to more fully answer this question by looking at length of stay in the hospital as well as overall RBC transfusions during those periods. A study performing simultaneous screens with both methods would allow for some of these questions to be answered, but it is impractical at this time as the gel method is no longer performed at our institution.

Others have looked simultaneously at gel and solid-phase methods but have had varying results as to which method is more sensitive.\(^8\) In a comparison of multiple methods including three gel and two solid-phase methods, Weisbach et al.\(^10\) showed that one solid-phase method appeared superior to the gel methods in terms of specificity of antibody identification, although the other was inferior when tested on stored samples. In the study by Garozzo et al.,\(^11\) testing of clinical samples was performed simultaneously, as part of routine practice, using both solid-phase and gel methods, yielding similar results for screening and identification. Finally, in the most recent study of Haywood et al.,\(^12\) tube methods outperformed both solid-phase and gel methods on referral specimens for antibody identification at an immunohematology reference center. Our study extends these results. Importantly, all testing was performed as part of routine practice on fresh samples from a single institution, making it an accurate reflection of standard conditions for antibody screening. In addition, all data were analyzed in a statistically rigorous manner to determine whether significant differences in the overall methods and for specific antibody identification existed, which had not been done previously.

Inevitably, different methods will yield different sensitivities and specificities of antibody detection. In combination with other considerations such as cost, workflow, and time, laboratories must choose which methods are optimal for their patient populations and operations. Extended side-by-side comparisons of different methods would be optimal, but this is not always possible or practical. Alternatively, and perhaps regardless of such prospective comparison testing, it is advisable that laboratories continue to monitor antibody detection rates after new methods have been adopted. If changes are noted as occurred in our laboratory, further analysis of clinical parameters such as the incidence of delayed hemolytic or serologic transfusion reactions, transfusion practice, and hospital lengths of stay may make it possible to determine the clinical significance of such changes.

Shortly, we will be transitioning our antibody screening to the next-generation platform for solid-phase technology. We will continue to monitor the rates of antibodies identified and analyze them to determine whether statistically significant changes occur.

**Acknowledgments**

We would like to thank our colleagues in both the Reference Laboratory as well as those in the Transfusion Laboratory who unknowingly contributed to this paper by serving the needs of the Mayo Clinic’s patients. Without their efforts, there would not be a division of Transfusion Medicine fulfilling the institution’s transfusion needs. Thank you to Terri Coyle for providing the many details about the Galileo.
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Anne Schmidt, MT(ASCP), Division of Transfusion Medicine, Brenda J. Bendix, MLS(ASCP), SBB, Immunohematology Reference Laboratory Supervisor, Division of Transfusion Medicine, Eapen K. Jacob, MD, Assistant Professor and Director of the Immunohematology Reference Laboratory, Sandra C. Bryant, MS, Assistant Professor of Biostatistics, Division of Biomedical Statistics and Informatics, and James R. Stubbs, MD (corresponding author), Chairman of the Division of Transfusion Medicine, Department of Laboratory Medicine and Pathology, Mayo Clinic, 200 First St. SW, Rochester, MN 55905.

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Comparison of estimation of volume of fetomaternal hemorrhage using Kleihauer-Betke test and microcolumn gel method in D-negative nonisoimmunized mothers

K. Mittal, N. Marwaha, P. Kumar, S.C. Saha, and B. Thakral

In this study we assessed the efficacy of the microcolumn gel method in the detection and quantification of the volume of fetomaternal hemorrhage (FMH) in comparison with the Kleihauer-Betke test (KB) in nonisoimmunized D− mothers. We collected blood samples from 80 D− indirect antiglobulin test−negative mothers over a span of more than 1 year. FMH was determined by KB and microcolumn gel method, and the results were compared. FMH was recorded as less than 4 mL by KB if no fetal cells were seen after examining 25 fields using 10× objective. If fetal cells were seen, slides were examined further to quantify FMH. By microcolumn gel method, FMH was reported as less than 0.1 percent, 0.1 percent, 0.2 percent, and 0.4 percent or greater. None of the patients had FMH greater than 15 mL by KB. Sixty-two patients (77.5%) had FMH less than 4 mL by KB. In all these cases, FMH was less than or equal to 0.2 percent (approximately 4 mL) by microcolumn gel method. The mean volume of FMH in the remaining 18 (22.5%) cases by KB was 8.3 ± 1.7 mL. Fifteen (83.3%) of these 18 cases had FMH of at least 0.4 percent (approximately 8 mL) by gel technology. Microcolumn gel method is an effective screening test. Technologies like KB and flow cytometry are better options for detecting a large volume of FMH. Antepartum hemorrhage and cesarean delivery are risk factors for FMH. The recommended dose of Rh immune globulin (RhIG) prophylaxis for D− women delivering D+ infants is 300 μg within 72 hours of delivery without estimation of FMH. The dose is sufficient for 15 mL of fetal red cells or 30 mL of fetal whole blood volume.

The Kleihauer-Betke test (KB) is among the earliest methods developed for FMH quantification and is still widely used. The method is sensitive but time-consuming and difficult to standardize, and it has poor reproducibility. The microcolumn gel method has been a major innovation in the field of immunohematology and has recently been introduced for the quantification of FMH; this method needs to be evaluated for its use in the clinical setting.

Materials and Methods

Eighty D−, indirect antiglobulin test (IAT)−negative women who delivered live babies in our institution from January 2008 to March 2009 constituted the study population. The study was approved by the institutional ethics committee, and informed consent was obtained from all patients. Previous obstetric history and detailed history of current pregnancy were noted. Peripheral venous blood samples were collected, two milliliters each in EDTA and plain vials, within 1 hour of delivery, before the administration of RhIG prophylaxis. Each sample was analyzed by both KB and microcolumn gel method, and the results were compared.

KB was performed using a commercial kit (Sigma Aldrich Chemie GmbH, Steinheim, Germany) in accordance with the manufacturer’s guidelines. Thin blood films were prepared.
on clean, dry slides. Control samples of cord blood were tested with each batch of slides stained. Slides were fixed in 80 percent ethanol. The slides were immersed in citrate phosphate buffer (pH 3.3) at 37.8°C for 5 minutes. The slides were rinsed in tap water and dried. They were placed in citrate phosphate buffer (pH 3.3). The slides were stained using acid hematoxylin for 3 minutes, rinsed in tap water, and counterstained with eosin for 3 minutes. The controls were first examined to ensure that the staining and the preparation were satisfactory. A minimum of 25 fields was examined using a 10× objective. Fetal cells containing hemoglobin F (HbF) were stained densely red and refractile, whereas the adult cells containing hemoglobin A (HbA) appeared pale and ghostlike as shown in Figure 1. If no fetal cells were seen, the FMH was reported as less than 4 mL of fetal red blood cells (RBCs). This critical value of 4 mL of RBCs was chosen because even a low dose of 100 μg of RhIG as administered in some countries can adequately neutralize up to 5 mL of FMH. If fetal cells were seen, slides were examined further to estimate the number of fetal cells present.

**Microcolumn Gel Method**

The test is based on consumption of antibody, indirectly measured by the use of D+ indicator RBCs. It uses anti-D, so that fetal D+ cells with available amounts of D are directly measured. The amount of residual anti-D is a function of the amount of D+ fetal RBCs present in the maternal sample that would have adsorbed the anti-D during incubation. More D+ fetal RBCs would be present in the maternal sample in case of a larger fetomaternal bleed. This would lead to more anti-D adsorbed by the D+ cells and a weaker gel reaction. The test was performed using commercial gel tubes (FMH kit, Diamed AG, Cressier sur Morat, Switzerland, which has since been replaced by Bio-Med ID FMH Screening Test). The test is currently not available in the United States. Two hundred fifty microliters each of washed packed RBCs of postdelivery maternal blood and of all standard cells with known percentages of D+ RBCs (≥ 0.4%, 0.2%, 0.1%, and < 0.1%) provided in the kit were pipetted into Eppendorf tubes. One hundred microliters of ID-Diluent 2 was added to each tube. Fifty microliters of ID-FMH anti-D was then added to each tube. Tubes were agitated for approximately 5 seconds on a vortex mixer and then for 60 minutes on a roller mixer. After incubation, tubes were centrifuged for 5 minutes at 800 g to harvest free anti-D in the supernatant. Fifty microliters of ID-FMH DiaCell II (D+ RBCs) was added to all microtubes of the ID-Card. Fifty microliters of the RBC-free supernatant containing unbound anti-D from each Eppendorf tube was then added to the appropriate microtubes of the ID-Card. The ID-Card was subsequently incubated and centrifuged. Results were read and recorded as follows, and are shown in Figure 2. The strength of reactivity in the microcolumn gel method corresponds to the amount of anti-D left in the supernatant. The strength of the cell reaction was compared with the reaction strengths of standard cells provided in the kit with known percentages of D+ RBCs in a mixture of D− RBCs. From this, the percentage of fetal D+ RBCs in the maternal sample was estimated and converted to volume of FMH with a formula, described later: (1) a compact button of RBCs or few agglutinates near the bottom of the microtube, indicating high consumption of the antibody equivalent to approximately 0.4 percent or more of D+ RBCs equivalent to at least 7.2 mL of FMH using the formula; (2) partial sedimentation of RBCs with agglutinated cells in the lower part of the microtube, indicating consumption of the antibody equivalent to approximately 0.2 percent of D+ RBCs equivalent to 3.6 mL of FMH using the formula; (3) agglutination distributed throughout the gel, indicating consumption of the antibody equivalent to approximately 0.1 percent of D+ RBCs equivalent to 1.8 mL of FMH.
Comparison of methods for fetomaternal bleeding in D-negative nonisoimmunized mothers

of FMH using the formula; and (4) agglutinated cells at the top of the gel; equivalent to insignificant consumption of the antibody, indicative of the presence of less than 0.1 percent D+ RBCs equivalent to 1.8 mL or less of FMH using the formula.

Calculation of Fetomaternal Hemorrhage

FMH was calculated using the formula as per the manufacturer’s instructions. Based on a maternal packed RBC volume of 1800 mL, the percentage of fetal RBCs can be assessed in milliliters using the following formula:

\[ X\% \times 1800/100 = y \text{ mL fetal packed RBCs} \]

The results of the tests using both methods were compared.

Results

The mean age of the patients was 25.3 ± 3.1 years (range 19–33 years), and the mean period of gestation was 37.9 ± 1.6 weeks (range 32–42 weeks). The mean gravida was 1.6 ± 0.8 (range 1–5), and included 50 primigravidae. The ABO blood group distribution of the female population showed 29 patients were group O (36.3%), 16 were group A (20%), 22 were group B (27.5%), and 13 (16.3%) were group AB. Sixty (75%) had spontaneous onset of labor, and 20 (25%) patients had cesarean delivery. Nine (11.25%) had antepartum hemorrhage, and 5 (6.3%) patients received antenatal RhIg prophylaxis.

There was no history of maternal trauma or antenatal obstetric procedures in any of the patients. There was no fetal demise, major congenital anomalies, significant anemia, or evidence of hydrops feticus in any of the fetuses.

The volume of FMH estimation was done using both KB and microcolumn gel method on 80 samples and is as shown in Table 1.

Table 1. Screening of patient samples for FMH by the KB and the microcolumn gel method

<table>
<thead>
<tr>
<th>FMH (mL)</th>
<th>Number of cases</th>
</tr>
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<tbody>
<tr>
<td>≤ 4 mL</td>
<td>KB 62 (77.5%)</td>
</tr>
<tr>
<td></td>
<td>Microcolumn gel method 65 (81.3%)*</td>
</tr>
<tr>
<td>&gt; 4–15 mL</td>
<td>KB 18 (22.5%)</td>
</tr>
<tr>
<td></td>
<td>Microcolumn gel method 15 (18.7%)</td>
</tr>
<tr>
<td>&gt; 15 mL</td>
<td>0</td>
</tr>
</tbody>
</table>

*Of 65 cases, 30 (37.5%) had FMH < 1.8 mL, 23 (28.8%) had FMH 1.8 mL, and 12 (15%) had FMH 3.6 mL.

FMH = fetomaternal hemorrhage; KB = Kleihauer-Betke test.

Volume of Fetomaternal Hemorrhage Using Kleihauer-Betke Test

Using KB, no fetal RBCs were seen in 62 patients (77.5%) after examining 25 fields, and hence the volume of FMH was recorded as less than 4 mL of fetal RBCs. In the remaining 18 patients (22.5%), the volume of FMH ranged between 5.6 mL and 10.4 mL, the mean ± 2 SD being 8.3 ± 1.7 mL. In none of the patients was the volume of FMH more than 15 mL of fetal RBCs, obviating the need of additional RhIG.

Volume of Fetomaternal Hemorrhage Using Microcolumn Gel Method

Using the microcolumn gel method, the volume of FMH was recorded as calculated using the formula. In 30 patients (37.5%), the volume of FMH was less than 1.8 mL, in 23 patients (28.8%) the volume of FMH was 1.8 mL, and the volume of FMH was 3.6 mL in 12 patients (15%). In the remaining 15 patients (18.7%), the volume of FMH was at least 7.2 mL of fetal RBCs.

Comparison of Volume of Fetomaternal Hemorrhage Estimation Using Both Methods

In 62 patients (77.5%) of 80 patients, the volume of FMH detected by KB was less than 4 mL, whereas a similar volume of FMH of 3.6 mL or less of FMH was detected in 65 (81.3%) patients by the microcolumn gel method. In 18 (22.5%) of the 80 patients, the volume of FMH ranged between 5.6 mL and 10.4 mL (mean ± 2 SD, 8.3 ± 1.7 mL). With the microcolumn gel method, a comparable volume of FMH was seen in 15 (18.7%) patients. The RBC equivalent antibody consumption was at least 7.2 mL of volume. In 3 patients (3.8%), there were discrepant results between the microcolumn gel method and KB, the former showing 1.8 mL of volume in each case as against KB, in which the FMH was 5.6, 5.9, and 6.4 mL as shown in Table 2. On the whole, however, both methods showed good correlation (Pearson correlation coefficient 0.892).
Table 2. Details of FMH volume in discrepant cases

<table>
<thead>
<tr>
<th>Cases</th>
<th>FMH by KB (mL)</th>
<th>FMH by microcolumn gel method (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.6</td>
<td>1.8</td>
</tr>
<tr>
<td>2</td>
<td>5.9</td>
<td>1.8</td>
</tr>
<tr>
<td>3</td>
<td>6.4</td>
<td>1.8</td>
</tr>
</tbody>
</table>

FMH = fetomaternal hemorrhage; KB = Kleihauer-Betke test.

Cesarean delivery was associated significantly with an increased risk of FMH (p = 0.001) as 10 (50%) of 20 cases associated with cesarean delivery had a volume of FMH of greater than 4 mL. The volume of FMH ranged between 7.9 mL and 10.4 mL, with the mean ± 2 SD being 9.5 ± 0.8 mL after cesarean delivery. Seven (77.8%) of 9 patients with antepartum hemorrhage had a volume of FMH of greater than 4 mL (p < 0.001). The volume of FMH in cases associated with antepartum hemorrhage ranged between 7.9 mL and 10.4 mL, with the mean ± 2 SD being 9.5 ± 0.9 mL (Figures 3 and 4).

Discussion

In the present study, the microcolumn gel method was compared with KB for detection of FMH. None of the patients had large (> 15 mL) FMH that would require additional RhIg administration. Sixty-five patients had low-volume FMH of 4 mL or less by the microcolumn gel method. KB had FMH of less than 4 mL in 62 cases. This was in accordance with previous study results of Salama et al., Gómez-Arbonés et al., and Dass et al., which showed that the microcolumn gel method is a good screening test for estimation of FMH. FMH was greater than 10 mL in 3 cases by KB, whereas by the microcolumn gel method FMH was at least 7.2 mL. This was in accordance with a study conducted by Ben-Haroush et al., who found four episodes of FMH greater than 10 mL detected by flow cytometry and only one episode that was detected by gel agglutination method. Hence, for large-volume FMH, KB is required for FMH estimation, and the microcolumn gel method is not the test of choice. Cohen et al., Li et al., and Salim et al. were unable to confirm the mode of delivery as a risk factor for FMH. However, this conclusion was at variance with that of Ness et al., who reported that cesarean delivery was a risk factor for FMH. Our results indicate that the risk of FMH is significantly increased in cesarean delivery compared with vaginal delivery (p < 0.05); thus, the mode of delivery is a risk factor for FMH. In our study, none of the patients had manual removal of the placenta. Delivery maneuvers, episiotomy in labor, and gestational age were also explored as possible determinants of FMH but were found not to be significant. In a study conducted by Adeniji et al., antepartum complications of pregnancy (threatened abortion, pregnancy-induced hypertension, antepartum hemorrhage) were explored as possible determinants of FMH but were found not to be significant. In our study, antepartum hemorrhage was explored as a possible determinant of FMH and was found to be significant.

In India, all D– nonalloimmunized patients who deliver a D+ baby by either a normal delivery or a cesarean delivery receive a standard postnatal dose of 300 μg of RhIg within 72 hours of delivery with no requirement for a routine KB, which is sufficient for an FMH volume of approximately 15 mL of fetal RBCs. For a large majority of patients, this is an overdose. We need to analyze the relative cost-effectiveness of universal administration of 300 μg of anti-D immunoglobulin vs. quantitation of FMH with subsequent administration of titrated doses.
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Immunosuppressive protocols for transplantation and certain hematologic malignancies can prevent the primary immune response to the D blood group antigen

A. Seager and S.G. Sandler

A review of the published literature on Rh alloimmunization reveals that its incidence varies with the volume of infused D+ red blood cells (RBCs), the probable Rh genotype of the RBCs, and the immune competency of the D– recipient. Among the reports of Rh alloimmunization in different clinical circumstances, we identified five studies in which a combined total of 62 D– recipients of hematopoietic stem cell or solid-organ transplants were transfused with D+ RBCs and none (0%) formed anti-D. The observation that immunosuppressive protocols developed to prevent rejection of tissue and organ transplants also prevented alloimmunization to the D blood group antigen raises the possibility of practical applications in blood transfusion practice.


**Key Words:** Rh alloimmunization, anti-D, D+ RBCs, progenitor cell transplantation, solid-organ transplantation

The incidence of Rh alloimmunization, i.e., the formation of anti-D, when D+ red blood cells (RBCs) are transfused to D– recipients, varies with the volume of infused D+ RBCs, their Rh genotype, and the immune competency of the recipient.1 As an initial step in drafting a protocol to study transfusion-related Rh alloimmunization in patients, we conducted a review of published literature on Rh alloimmunization. The following report describes the result of our review and suggests how immunosuppressive protocols may benefit selected transfusion recipients who are at risk of alloimmunization to blood group antigens.

**Materials and Methods**

We conducted PubMed and OVID Medline searches of journal articles associated with the following key words: Rh alloimmunization, anti-D, and Rh-positive RBCs. The literature search included articles published during the years 1956 to 2013. We limited the search to journal articles published in the English language. We searched pertinent sections of standard textbooks in transfusion medicine for citations to additional original journal articles.1–4 We conducted a parallel search for journal articles on D-mismatched platelet transfusions and hematopoietic stem cell or solid-organ transplants, considering Rh alloimmunization in these patients to be a consequence of contaminating donor-derived D+ RBCs in the component. We summarized the results of our review in a figure that illustrates the incidence of Rh alloimmunization for various categories of immune-competent versus immune-compromised D– patients who had received transfusions of D+ RBCs (Figure 1).

**Fig. 1** Incidence of Rh alloimmunization in D– persons by recipients’ immune status and disease categories. The numbers in the graph refer to articles cited in References. *After a second (booster) infusion; †after one infusion.

**Results**

**Small Doses of Red Blood Cells Infused in Immune-Competent Recipients**

Several studies describe experimental Rh alloimmunization in immune-competent D– persons with D+ RBCs for purposes of basic research, as control subjects in studies of Rh immunoprophylaxis, or for immunizing volunteer donors...
for the collection of plasma containing anti-D for manufacture of Rh immune globulin (RhIG).2-9,29 The results of these studies indicate a general trend for an increasing incidence of Rh alloimmunization in relation to the volume of infused D+ RBCs and the Rh genotype (Table 1). Mollison et al.15 reviewed their studies, as well as those of others who infused small doses of D+ RBCs in immune-competent recipients. They concluded that approximately 20 percent of D– subjects, 84 percent receiving DcE/ce compared with 35 percent who received DcE/DcE or DcE/DcE RBCs. After a second infusion, the incidence of Rh alloimmunization nearly doubled (35% and 63%, respectively). At 1 year, 30 percent of subjects receiving DcE/ce RBCs formed anti-D compared with 84 percent receiving DcE/ce.1 Collectively, these studies demonstrate that for small-volume infusions in D– immune-competent recipients, DcE/DcE RBCs are more immunogenic than DcE/ce or DcE/DcE RBCs. This observation is supported by studies demonstrating a lower mean concentration of D on DcE/ce RBCs (240 pmol/mL) compared with DcE/DcE (473 pmol/mL).30

Table 1. Incidence of Rh alloimmunization in D– immune-competent recipients of small doses of D+ RBCs

<table>
<thead>
<tr>
<th>Dose of RBCs</th>
<th>No. subjects</th>
<th>Experimental conditions</th>
<th>Incidence of Rh alloimmunization</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0 mL</td>
<td>14</td>
<td>Tested for anti-D after 6 months</td>
<td>8%</td>
<td>5</td>
</tr>
<tr>
<td>1.0 mL/2 doses</td>
<td>31</td>
<td>DCe/ce RBCs/tested at 36.1 weeks</td>
<td>35%</td>
<td>6</td>
</tr>
<tr>
<td>1.0 mL/2 doses</td>
<td>12</td>
<td>DcE/DcE RBCs/tested at 6 months</td>
<td>42%</td>
<td>7</td>
</tr>
<tr>
<td>1.0 mL/2 or 3 doses</td>
<td>12</td>
<td>DcE/DcE RBCs/tested at 12 months</td>
<td>50%</td>
<td>8</td>
</tr>
<tr>
<td>0.5 mL</td>
<td>6</td>
<td>DcE/DcE RBCs/tested for anti-D at 210 days</td>
<td>83.3%</td>
<td>9</td>
</tr>
<tr>
<td>5.0 mL</td>
<td>6</td>
<td>DcE/DcE RBCs/tested for anti-D at 150 days</td>
<td>83.3%</td>
<td>9</td>
</tr>
</tbody>
</table>

RBCs = red blood cells.

Whole Units of Red Blood Cells in Immune-Competent Recipients

When larger volumes of D+ RBCs (500-mL units) were transfused to immune-competent recipients, the incidence of Rh alloimmunization was significantly higher, obscuring the effect of the Rh genotype on the incidence of Rh alloimmunization.11,30

Immune-Competent D– Volunteers

In a study of Rh prophylaxis, Pollack et al.10 reported an incidence of 81.8 percent (18 of 22) Rh alloimmunization 5 months after transfusion of one 500-mL unit of DcE/ce RBCs in D– volunteers. Urbaniak and Robertson11 reported their experience in a program infusing D+ RBCs to D– men for manufacture of RhIG. They infused 200 mL of frozen-thawed RBCs (DcE/DcE, DcE/DcE, or DcE/DcE) followed by serial boosters of 5 mL of RBCs from the same donor. The incidence of Rh alloimmunization was 87 percent (24 of 28) after the initial 200-mL infusion and 93 percent (26 of 28) after the booster infusions. The Rh genotype of the immunizing RBCs did not appear to be an important factor in determining the incidence of Rh alloimmunization. DcE/DcE RBCs were as effective as DcE/DcE. All D– recipients of DcE/DcE RBCs formed anti-D.

D– Patients Not Receiving Immunosuppressive Therapy

Yazer and Triulzi33 reported a 22 percent (22 of 98) incidence of Rh alloimmunization in D– nononcology patients who were transfused with D+ RBCs and followed for a mean of 182 days. Frohn et al.14 reported Rh alloimmunization in 30.4 percent (16 of 78) of D– recipients of D+ RBCs. The patients in this study had various medical and surgical diagnoses, but hematologic disorders requiring treatment with immunosuppressive regimens were excluded. Gonzalez-Porras et al.12 reported a 21.4 percent (34 of 159) incidence of Rh alloimmunization in a general hospital population after a prospective median follow-up of 41 days.

Immune-Compromised Patients

Expectedly, the incidence of alloimmunization to blood group antigens was decreased when D+ RBCs were transfused to D– patients whose immune function is compromised by disease or immunosuppressive therapy. Ting et al.31 reported the formation of antibodies in 8.7 percent (13 of 150) transfusion recipients after bone marrow transplantation. They suggested that the blood group antibodies could be the result of transfusion of lymphocytes with the donor’s bone marrow, the ability of the grafted immune system to produce alloantibodies, or the viability of the residual host’s immune system despite chemotherapy and irradiation.31 After eliminating antibodies formed by three recipients whose transfused RBCs did not express the corresponding antigens, and excluding other antibodies that were transient and attributed to residual host lymphocytes, the incidence of alloimmunization was only 2 percent (3 of 150).31 Abou-Elella et al.32 also reported a relatively low incidence of alloimmunization to blood group antigens after bone marrow transplantation, namely, 2 percent.
or 0.1 percent per unit of transfused RBCs to 193 bone marrow transplant recipients.

We identified five reports of Rh alloimmunization in D– transplantation patients who had been treated with immunosuppressive protocols and transfused with D+ RBCs.\textsuperscript{15–19} Typically, the more intensive immunosuppressive protocols were for non–ABO-incompatible hematopoietic progenitor cell transplants, followed by non–ABO-incompatible solid-organ transplants, ABO-compatible hematopoietic cell transplants, and ABO-compatible solid-organ transplants, as follows:

Hematopoietic Stem Cell Transplants. Mijovic\textsuperscript{15} reported that none of nine (0%) D– recipients of nonmyeloablative hematopoietic stem cell transplants from D+ donors developed anti-D, although they had also received transfusions (7 to 499 mL) of D+ RBCs. There were six unrelated bone marrow donors and three sibling peripheral blood progenitor cell donors. The nonmyeloablative conditioning regimens were fludarabine, alemtuzumab (Campath, Bayer Healthcare Pharmaceuticals, Montville, NJ), and busulfan (FBC or FB16C) or bis-chloroethylnitrosourea (BCNU), etoposide, alemtuzumab (Campath, Genzyme Corporation, Cambridge, MA), melphalan, and cytosine arabinoside (BEAM-C). Cid et al.\textsuperscript{16} reported that none of 15 (0%) D– bone marrow transplant recipients developed anti-D, although all had received allogeneic hematopoietic stem cell transplants and transfusions of platelets from D+ donors. The patients had been treated with myeloablative (cyclophosphamide, plus total body irradiation of fludarabine) or nonmyeloablative (fludarabine and busulfan or melphalan) conditioning.

Solid-Organ Transplants. Ramsey et al.\textsuperscript{17} reported that none of 16 (0%) D– liver, heart, and heart-lung transplant recipients developed anti-D when tested 2.5 to 51 months after transfusions of D+ RBCs (3–153 units, median 10 units). The immunosuppressive regimen consisted of cyclosporine, corticosteroids, adjunctive rabbit antilymphocyte globulin, and in some cases OKT3 monoclonal antibody. Casameuva et al.\textsuperscript{18} reported no anti-D in 17 (0%) D– liver transplant patients receiving D– orthotopic liver transplants who were transfused with D+ units of red cells. The conditioning regimen was cyclosporine A, prednisone, azathioprine, and adjunctive rabbit antilymphocyte globulin or monoclonal OKT3 antibody. Yaun et al.\textsuperscript{19} also reported 0 percent (0 of 15) Rh alloimmunization in D– liver transplant recipients who received D– orthotopic liver transplants, but were transfused with D+ RBCs. In all of these reports, the investigators proposed that immunosuppression was the major contributing factor for the absence of anti-D in the D– recipients.\textsuperscript{15–19}

In summary, all 62 D– recipients (100%) in these five reports of immunosuppression for hematopoietic stem cell or solid-organ transplants received transfusions of D+ RBCs without forming detectable anti-D.\textsuperscript{15–19}

Immune-Compromised Patients With AIDS

Boctor et al.\textsuperscript{20} reported absence (0%) of Rh alloimmunization in eight patients with AIDS who were transfused with D+ RBCs. This study underscores the importance of T cells to the humoral (B cell) immune response.

D-Mismatched Platelet Transfusions

Although D is not expressed on platelet membranes in persons who inherit RHD,\textsuperscript{23} the methods for preparing whole blood (WB)-derived (random donor) platelet concentrates result in sufficient volumes of contaminating RBCs to potentially cause Rh alloimmunization. The volume of RBCs in one unit of WB-derived platelet concentrates varies from trace to 0.5 mL.\textsuperscript{34} The volume of RBCs in one unit of apheresis platelets, which does not appear to be adequate to stimulate an immune response, is 0.0002 to 0.007 mL.\textsuperscript{34} Goldfinger and McGinniss\textsuperscript{24} observed a 7.8 percent (8 of 102) incidence of Rh alloimmunization when D– oncology patients were transfused with platelet-rich plasma or pooled platelet concentrates from D+ donors. All but one of the recipients had received immunosuppressive treatment for malignant disease. Lichtiger et al.\textsuperscript{21} reported that none of 30 (0%) D– oncology patients formed anti-D after transfusions with platelets from D+ donors. Baldwin et al.\textsuperscript{25} reported an 18 percent (9 of 49) incidence of Rh alloimmunization in D– patients with leukemia and multiple myeloma, including two who received a bone marrow transplant and had received platelets or granulocyte transfusions from D+ donors. Cid et al.\textsuperscript{22} conducted a prospective study and reported that none of 22 (0%) D– adult patients with hematologic diseases who were transfused with platelets from D+ donors developed anti-D. The recipients had been treated with various immunosuppressive regimens, including autologous bone marrow transplantation. Molnar et al.\textsuperscript{23} observed no Rh alloimmunization in 42 (0%) D– pediatric oncology patients (7 hematopoietic cell transplant recipients and 35 chemotherapy only) who were transfused with leukocyte-reduced apheresis platelets from D+ donors. Asfour et al.\textsuperscript{24} reported a 4 percent (3 of 78) incidence of Rh alloimmunization when D– hematopoietic cell transplant recipients received a mix of WB-derived and apheresis platelets from D+ donors. Cid et al.\textsuperscript{27} reported Rh alloimmunization in 3.8 percent (12 of 315) D– patients who had received transfusions of platelet concentrates from D+ donors. In this
study, 4.8 percent of immunosuppressed and 1.9 percent of immune-competent recipients formed anti-D, but the difference in the incidence of Rh alloimmunization was not statistically significant. Similarly, Atoyebi et al. observed no Rh alloimmunization when 24 (0%) D− oncology patients were transfused with a mix of WB-derived and apheresis platelets, whereas 13.5 percent (8 of 59) patients with nonhematologic diseases developed anti-D after transfusion with similar platelet components from D+ donors.

**Discussion**

Two significant observations are apparent from this review. First, when small volumes of D+ RBCs (0.5–1.0 mL) are infused in D− immune-competent volunteers, there is a relatively low incidence of Rh alloimmunization. Given the incomplete and variable immune response to small doses of D+ RBCs, it is possible to distinguish the relatively greater immunogenicity of DcE/ce versus DcE/ce RBCs and of DcE/DcE versus DcE/DcE RBCs. The incidence of Rh alloimmunization increases generally with the volume of infused D+ RBCs, peaking at approximately 85 percent. There is no known explanation for the failure of approximately 15 percent of apparently immune-competent D− recipients, who are conventionally designated as nonresponders of D+ RBCs, to form anti-D.

Second, and most importantly, the results of this review indicate that recently developed immunosuppressive protocols for hematopoietic cell or solid-organ transplantation are capable of preventing the primary humoral immune response when D+ RBCs are transfused to D− recipients. This effect has been observed for transfusions of D+ WB and for WB-derived platelet concentrates from D+ donors to D− transplant recipients. The effect is not unexpected as the agents for transplant recipients are selected because of their capability of suppressing hosts’ immune responses to antigens expressed on donors’ organs and tissues. Newer nonmyeloablative protocols for bone marrow transplantation in sickle cell disease should offer not only improved clinical outcomes but also concomitant prevention of alloimmunization to D and other blood group antigens with less toxicity.

Given the chronic shortage of D− RBCs in transfusion services worldwide, these observations have potential practical application for transfusion practice. The safe transfusion of D− transplant recipients with D+ RBCs without Rh alloimmunization raises the possibility that similar immunosuppressive protocols may allow transfusion of D+ RBCs in other categories of D− patients in times of critical shortage when, otherwise, they would be highly likely to become alloimmunized to D. Extending the implications of these findings to the subject of alloimmunization to other (non-D) blood group antigens, the possibility arises that a relatively short course of pretransfusion or posttransfusion immunosuppression could prevent the formation of other blood group alloantibodies in different patient populations, for example, sickle cell disease. If prevention of the primary humoral immune response to blood group antigens can be achieved with clinically tolerable doses of immunosuppressive agents—without suppressing erythropoiesis in sickle cell patients—the potential applications are considerable. Recognizing that RhIG prevents Rh alloimmunization in postpartum women when administered as late as 72 hours after exposure to D+ RBCs, it is not unreasonable to encourage investigation of postexposure immunosuppression after D− persons have been transfused with D+ RBCs. It is conceivable that the observed side effect of suppression of Rh alloimmunization in D− transplant recipients will open opportunities for prophylactic protocols for future patients whose inherited blood groups place them at risk of complications, alloimmunization, and a lifelong requirement for uncommon and rare RBCs.

**References**


Adair Seager, MD, and S. Gerald Sandler, MD, FCAP, FACP (corresponding author), Department of Pathology and Laboratory Medicine, MedStar-Georgetown University Hospital, 3800 Reservoir Road NW, Washington, DC 20007.
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<td>Marie P. Holub</td>
<td>602-996-2396</td>
<td><a href="mailto:mholub@bloodsystems.org">mholub@bloodsystems.org</a></td>
<td><a href="http://www.bloodsystemslaboratories.org">www.bloodsystemslaboratories.org</a></td>
</tr>
<tr>
<td>Walter Reed Army Medical Center</td>
<td>William Turcan</td>
<td>301-295-8605</td>
<td><a href="mailto:William.Turcan@med.navy.mil">William.Turcan@med.navy.mil</a></td>
<td><a href="http://www.militaryblood.dod.mil/Fellow/default.aspx">www.militaryblood.dod.mil/Fellow/default.aspx</a></td>
</tr>
<tr>
<td>American Red Cross, Southern California Region</td>
<td>Catherine Hernandez</td>
<td>909-858-7496</td>
<td><a href="mailto:Catherine.Hernandez@redcross.org">Catherine.Hernandez@redcross.org</a></td>
<td><a href="http://www.redcrossblood.org/local/communityeducation">www.redcrossblood.org/local/communityeducation</a></td>
</tr>
<tr>
<td>ARC-Central OH Region</td>
<td>Joanne Kosanke</td>
<td>614-253-2740 ext. 2270</td>
<td><a href="mailto:Joanne.Kosanke@redcross.org">Joanne.Kosanke@redcross.org</a></td>
<td>none</td>
</tr>
<tr>
<td>Blood Center of Wisconsin</td>
<td>Phyllis Kirchner</td>
<td>414-937-6271</td>
<td><a href="mailto:Phyllis.Kirchner@bcw.edu">Phyllis.Kirchner@bcw.edu</a></td>
<td><a href="http://www.bcw.edu">www.bcw.edu</a></td>
</tr>
<tr>
<td>Community Blood Center/CTS Dayton, Ohio</td>
<td>Nancy Lohr</td>
<td>937-461-3293</td>
<td><a href="mailto:nlhorn@cbcts.org">nlhorn@cbcts.org</a></td>
<td><a href="http://www.cbcts.org/education/sbb.htm">www.cbcts.org/education/sbb.htm</a></td>
</tr>
<tr>
<td>Gulf Coast Regional Blood Center</td>
<td>Clare Wong</td>
<td>713-791-6201</td>
<td><a href="mailto:cwong@giveblood.org">cwong@giveblood.org</a></td>
<td><a href="http://www.giveblood.org/services/education/sbb-distance-program">www.giveblood.org/services/education/sbb-distance-program</a></td>
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<tr>
<td>Havworth Blood Center, University of Cincinnati Medical Center</td>
<td>Pamela Inglish</td>
<td>513-558-1275</td>
<td><a href="mailto:Inglishpf@ucmail.uc.edu">Inglishpf@ucmail.uc.edu</a></td>
<td><a href="http://www.ucmed.uc.edu">www.ucmed.uc.edu</a></td>
</tr>
<tr>
<td>Indiana Blood Center</td>
<td>Jayanna Stayton</td>
<td>317-916-5196</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/sbb.cfm">http://pathology.jhu.edu/department/divisions/transfusion/sbb.cfm</a></td>
</tr>
<tr>
<td>Medical Center of Louisiana</td>
<td>Karen Kirkley</td>
<td>504-963-3854</td>
<td><a href="mailto:kkirkley@lsuhsc.edu">kkirkley@lsuhsc.edu</a></td>
<td><a href="http://www.lsuhsc.edu/webresources/index.html">www.lsuhsc.edu/webresources/index.html</a></td>
</tr>
<tr>
<td>NIH Clinical Center Blood Bank</td>
<td>Karen Byrne</td>
<td>301-496-8335</td>
<td><a href="mailto:kbyrne@mail.ccc.nih.gov">kbyrne@mail.ccc.nih.gov</a></td>
<td><a href="http://www.ccc.nih.gov/otm">www.ccc.nih.gov/otm</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.rush.edu/cls">www.rush.edu/cls</a></td>
</tr>
<tr>
<td>Transfusion Medicine Center at Florida Blood Services</td>
<td>Murphie Dwyer</td>
<td>727-568-5433 ext. 1154</td>
<td><a href="mailto:mdwyer@flblood.org">mdwyer@flblood.org</a></td>
<td><a href="http://www.flblood.org">www.flblood.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:lmyers@bloodtissue.org">lmyers@bloodtissue.org</a></td>
<td><a href="http://www.bloodtissue.org">www.bloodtissue.org</a></td>
</tr>
<tr>
<td>University of Texas Medical Branch at Galveston</td>
<td>Janet Vincent</td>
<td>409-772-3055</td>
<td><a href="mailto:jvincent@utmb.edu">jvincent@utmb.edu</a></td>
<td><a href="http://www.utmb.edu/sbb">www.utmb.edu/sbb</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-proessions/programs/certificate-programs/medical-laboratory-sciences/index.html">www.utsouthwestern.edu/education/school-of-health-proessions/programs/certificate-programs/medical-laboratory-sciences/index.html</a></td>
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</table>

Revised May 2012
**Instructions for Authors**

**I. GENERAL INSTRUCTIONS**

Before submitting a manuscript, consult current issues of *Immunohematology* for style. Number the pages consecutively, beginning with the title page.

**II. SCIENTIFIC ARTICLE, REVIEW, OR CASE REPORT WITH LITERATURE REVIEW**

A. Each component of the manuscript must start on a new page in the following order:
1. Title page
2. Abstract
3. Text
4. Acknowledgments
5. References
6. Author information
7. Tables
8. Figures

B. Preparation of manuscript

1. **Title page**
   a. Full title of manuscript with only first letter of first word capitalized (bold title)
   b. Initials and last name of each author (no degrees; all CAPS), e.g., M.T. JONES, J.H. BROWN, AND S.R. SMITH
   c. Running title of ≤40 characters, including spaces
   d. Three to ten key words

2. **Abstract**
   a. One paragraph, no longer than 300 words
   b. Purpose, methods, findings, and conclusion of study

3. **Key words**
   a. List under abstract

4. **Text (serial pages):** Most manuscripts can usually, but not necessarily, be divided into sections (as described below). Survey results and review papers may need individualized sections
   a. **Introduction** — Purpose and rationale for study, including pertinent background references
   b. **Case Report (if indicated by study)** — Clinical and/or hematologic data and background serology/molecular
   c. **Materials and Methods** — Selection and number of subjects, samples, items, etc. studied and description of appropriate controls, procedures, methods, equipment, reagents, etc. Equipment and reagents should be identified in parentheses by model or lot and manufacturer’s name, city, and state. Do not use patient’s names or hospital numbers.
   d. **Results** — Presentation of concise and sequential results, referring to pertinent tables and/or figures, if applicable
   e. **Discussion** — Implication and limitations of the study, links to other studies; if appropriate, link conclusions to purpose of study as stated in introduction

5. **Acknowledgments:** Acknowledge those who have made substantial contributions to the study, including secretarial assistance; list any grants.

6. **References**
   a. In text, use superscript, Arabic numbers.
   b. Number references consecutively in the order they occur in the text.

7. **Tables**
   a. Head each with a brief title; capitalize the first letter of first word (e.g., Table 1. Results of….) use no punctuation at the end of the title.

8. **Figures**
   a. Figures can be submitted either by e-mail or as photographs (5 x 7: glossy).
   b. Place caption for a figure on a separate page (e.g. Fig. 1 Results of…), ending with a period. If figure is submitted as a glossy, place first author’s name and figure number on back of each glossy submitted.
   c. When plotting points on a figure, use the following symbols if possible: ● ▲ △ □ ■

9. **Author information**
   a. List first name, middle initial, last name, highest degree, position held, institution and department, and complete address (including ZIP code) for all authors. List country when applicable. Provide e-mail addresses of all authors.

**III. EDUCATIONAL FORUM**

A. All submitted manuscripts should be approximately 2000 to 2500 words with pertinent references. Submissions may include:
1. An immunohematologic case that illustrates a sound investigative approach with clinical correlation, reflecting appropriate collaboration to sharpen problem solving skills
2. Annoted 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)
   - 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 RHBDIVA2)

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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