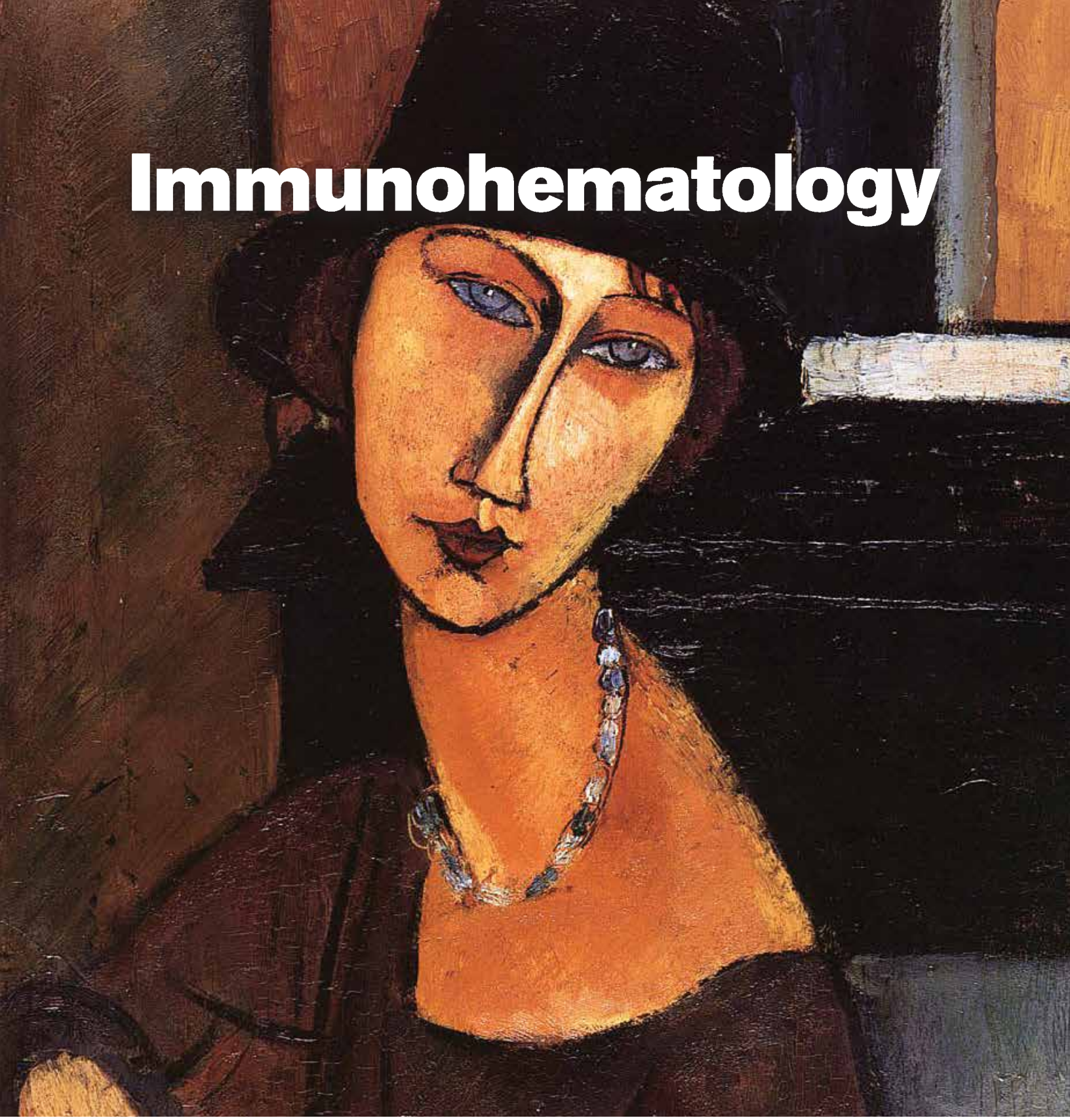

Immunohematology

VOLUME 28, NUMBER 2, 2012

American Red Cross

Immunohematology

Volume 28, Number 2, 2012

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Immunohematology is published quarterly (March, June, September, and December) by the American Red Cross, National Headquarters, Washington, DC 20006.

Immunohematology is indexed and included in *Index Medicus* and MEDLINE on the MEDLARS system. The contents are also cited in the EBASE/Excerpta Medica and Elsevier BIOBASE/Current Awareness in Biological Sciences (CABS) databases.

The subscription price is $40.00 (U.S.) and $50.00 (foreign) per year.

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ISSN 0894-203X

On Our Cover

One finds diverse influences—Cubism, Expressionism, Primitivism—in the sensual oeuvre of Amedeo Modigliani, an Italian of Sephardic Jewish origin whose work thrived in the artistic melting pot of early 20th century Paris. But above all else, Modigliani was a quintessential Bohemian, the magnificence of his craft contrasting with the squalor and erratic course of his life. The portrait on the cover, *Jeanne Hebuterne with Hat and Necklace,* is of the model and lover he would marry against her family's wishes, who leapt from a window to her death carrying their unborn child after he succumbed at age 35 to tuberculosis, drugs, and alcohol. The review of the Cartwright system in this issue includes a discussion of Anti-Yt^a, more commonly found in individuals of Jewish descent.

David Moolten, MD

Report

Low risk of hemolysis after transfusion of uncrossmatched red blood cells

L. Radkay, D.J. Triulzi, and M.H. Yazer

Transfusing uncrossmatched red blood cells (RBCs) can be a life-saving bridge until crossmatched RBCs are available. The risk of using uncrossmatched RBCs is that of hemolysis from unexpected clinically significant antibodies. This study sought to quantify the risk of hemolysis after the transfusion of uncrossmatched RBCs. The records of recipients of uncrossmatched RBCs over approximately 9 months were retrieved from the regional transfusion service. Basic immunohematologic data were recorded on all recipients including the number of uncrossmatched RBCs transfused. For recipients who had either previously identified clinically significant antibodies or those identified on the day of transfusion, clinical and biochemical data were evaluated to determine whether hemolysis had occurred after uncrossmatched RBC transfusion. There were 218 recipients of 1065 units of uncrossmatched RBCs. Most of the RBCs were administered in the emergency room (48%) followed by the operating room (24%) and intensive care unit (23%). Seven (3.2%) recipients had clinically significant antibodies that were active on the day of the transfusion, whereas in four patients a clinically significant antibody had been previously identified but was not active on the day of the transfusion. One patient with active antibodies who received three units of uncrossmatched RBCs for a gastrointestinal bleed demonstrated a reactive eluate several days later as well as positive biochemical hemolysis markers. Thus the overall rate of detectable hemolysis after uncrossmatched RBC transfusion was 1 of 218 (0.5%). The use of uncrossmatched RBCs is a relatively safe intervention, although close monitoring of recipients with clinically significant antibodies for evidence of hemolysis is recommended. ***Immunohematology* 2012;28:39–44.**

Key Words: RBC, hemolysis, uncrossmatched, antibody, transfusion

Under uncomplicated circumstances, crossmatched red blood cells (RBCs) can usually be available within approximately 60 minutes of the patient's blood sample arriving at the blood bank. However, there are circumstances under which this short delay in providing RBCs can be life threatening to the recipient. In these cases, uncrossmatched RBCs, which are RBC units whose compatibility with the recipient's plasma has not been serologically or electronically verified, can be provided almost immediately for use during an acute resuscitation while the blood bank performs a forward and reverse type and an antibody screen. As uncrossmatched RBCs are always group O, immediate hemolysis caused by naturally occurring anti-A and anti-B is avoided. The main risk of using uncrossmatched RBCs is the potential for hemolysis caused by unexpected non-ABO antibodies; thus the risk of hemolysis after receiving uncrossmatched RBCs is directly related to whether the recipient has either received a previous transfusion or been pregnant. In a situation in which uncrossmatched RBCs might be used, such as in trauma resuscitation in the emergency department, the patient's pregnancy and transfusion history are often unknown; however, the risk of immediate hemolysis caused by unexpected clinically significant antibodies should be low because the prevalence of these antibodies in the general population is quite low. In a study of almost 16,000 patients (corresponding to nearly 28,000 antibody screens) at a tertiary care hospital in Australia, only 1.9 percent of the recipients had a positive screen caused by a clinically significant antibody, of which the majority were directed toward antigens in the Rh or Kell systems.[1] When stratified by age, women generally had a higher incidence of alloimmunization than men, and patients with hematologic or oncologic diseases had higher rates of alloimmunization compared with patients in the emergency room or trauma patients. Similarly, Heddle and colleagues demonstrated that 96.5 percent of previously transfused patients at their hospital had a negative antibody screen,[2] whereas Stack et al. demonstrated a 2.4 percent alloimmunization rate among 18,750 transfused veterans.[3]

Several previous studies have evaluated patient outcomes after receipt of uncrossmatched RBCs.[4–11] In general, the risk of hemolysis was either low or absent, but it was not always clear whether the recipients in these studies had active antibodies on the day that they received their uncrossmatched RBC transfusion, thereby putting them at risk of hemolysis. A detailed study of 265 uncrossmatched RBC transfusion episodes by Goodell and colleagues found that 6.4 percent of these incidents were complicated by the presence of a clinically significant antibody.[4] However, only one of seven of the recipients with a clinically significant antibody who received at least one incompatible uncrossmatched RBC unit actually had a hemolytic reaction. Thus, we sought to determine the incidence of hemolysis after the administration of uncrossmatched RBCs throughout our hospital system.

Materials and Methods

The records of patients who were at least 16 years of age and received at least one uncrossmatched RBC unit over an approximately 9-month period were retrieved from the electronic records of a regional transfusion service. This transfusion service covers 16 hospitals in southwestern Pennsylvania including several level 1 trauma centers, active solid organ and stem cell transplantation services, and a variety of intensive care units. From the transfusion service's electronic files, basic serologic information on each recipient was recorded; this included the previous detection of alloantibodies, whether antibodies were detected on the day of receipt of the uncrossmatched RBCs, the detection of new antibodies after receipt of uncrossmatched RBCs, and the number of uncrossmatched RBC units transfused. Reports of transfusion reactions temporally associated with the uncrossmatched RBC transfusions were also recorded. Clinically significant antibodies were defined as those capable of causing hemolysis or shortening the lifespan of the transfused RBCs, and for which antigen-negative, crossmatch-compatible RBC units should be transfused.[12] Basic demographic data from the clinical records of the patients who received uncrossmatched RBCs were noted. For patients who had either known historical or active clinically significant antibodies on the day that they received uncrossmatched RBCs, biochemical variables including bilirubin, haptoglobin, lactate dehydrogenase, reticulocyte counts, and hemoglobin levels as well as the results of direct antiglobulin tests (DATs) and eluates were also analyzed, if available, to determine whether immune-mediated hemolysis had occurred at any point after the uncrossmatched RBC transfusion. The patient's clinical chart was also reviewed to determine whether there was a clinical suspicion of hemolysis. These laboratory and clinical variables were also analyzed in those recipients who subsequently produced antibodies after receipt of uncrossmatched RBCs. The physician and nursing notes from around the time of the uncrossmatched transfusions were also examined to determine whether there was a clinical suspicion of hemolysis.

Antibody detection was performed using a manual saline tube technique (Immucor, Norcross, GA) or automated techniques including column agglutination (Ortho ProVue; Ortho Clinical Diagnostics, Rochester, NY) and solid-phase (Galileo; Immucor) methodologies according to the manufacturer's specifications. The polyspecific and monospecific DATs (Ortho, Raritan, NJ) and eluates (Gamma Elu-kit II; Immucor) were performed using commercially available reagents and kits. As per our reference laboratory protocols, an eluate would have been performed on a specimen that demonstrated a newly positive DAT or one that had increased in strength from a previous test in a patient with a recent transfusion history, or if specifically ordered by a physician. Most of the uncrossmatched RBCs would not have been leukoreduced and would be stored in AS-3 or AS-5 solutions. The decision to use uncrossmatched RBCs was made by the patient's clinical team, and uncrossmatched RBCs were available from the blood bank and from remote monitored refrigerators in the emergency room and on selected wards. The prescribing physician was required to have signed and returned to the blood bank an authorization form for the use of uncrossmatched RBCs.

Descriptive statistics were used for continuous variables using the software package in Microsoft Excel 2010. Results are presented as mean ± standard deviation. This protocol was approved by the University of Pittsburgh's Total Quality Council.

Results

During the approximately 9-month period, there were 218 recipients of at least one unit of uncrossmatched RBCs. The mean age of these recipients was 54 ± 21 years, and 65 percent were male. Overall, 1065 uncrossmatched RBC units were transfused to these 218 recipients, which represents an average of 4.9 ± 4.9 uncrossmatched RBC units per recipient. Most of the uncrossmatched RBCs were administered in the emergency room (48%), followed by the operating room (24%), and the intensive care unit (23%). The remaining units were administered on medical floors, in labor and delivery suites, in the interventional radiology department, and to one patient who was receiving extracorporeal membrane oxygenation. Transfusion reactions were reported in two recipients of uncrossmatched RBCs who did not have known historical or active antibodies, and as expected, neither reaction was hemolytic in nature; one patient had hypotension in the operating room, the etiology of which was believed to be related to the patient's underlying hypovolemia from dialysis and ongoing bleeding. The signs and symptoms of the other reaction were not specific in nature, and it was reported in a patient who received the uncrossmatched RBCs during her unsuccessful resuscitation for hemorrhage.

Figure 1 presents the immunohematologic outcomes after receipt of the uncrossmatched RBCs. Of the 218 recipients of uncrossmatched RBCs, seven had active antibodies on the day of transfusion, and four others had historical

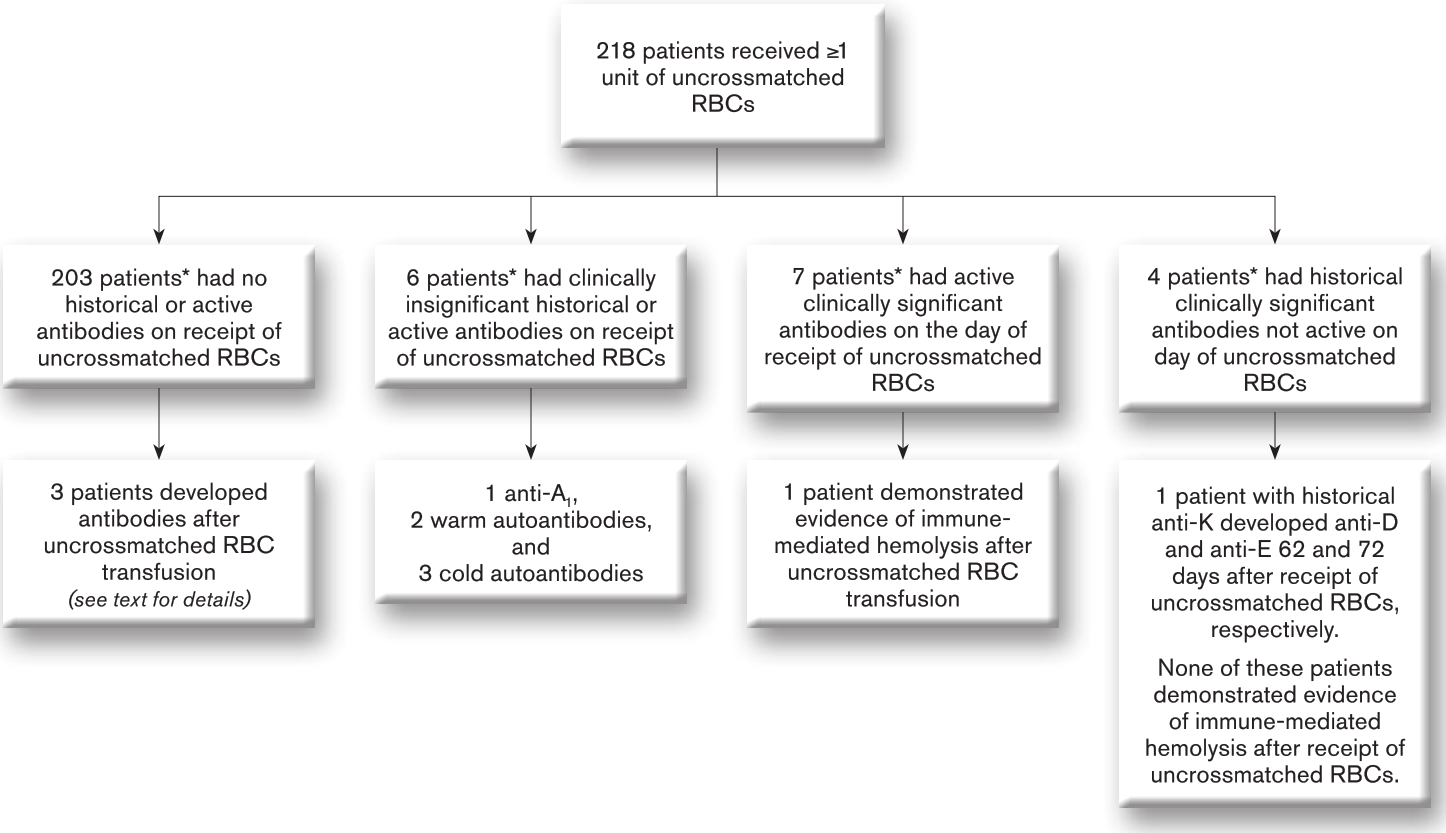

Fig. 1 Flow diagram demonstrating the immunohematologic consequences of receiving uncrossmatched RBCs. *The numbers do not add to 218 because 2 patients are counted in multiple categories: both patients had active clinically significant antibodies along with an insignificant antibody on the day of receipt of the uncrossmatched RBCs.

clinically significant antibodies that were not active on the day of the transfusion. Table 1 presents the demographic and immunohematologic details of the 11 patients who had historical or active clinically significant antibodies when they received their uncrossmatched RBC units. In one of four recipients with historical clinically significant antibodies no further antibody screens were performed after receipt of the uncrossmatched RBCs; in another recipient only K– selected antibody screening cells were used for antibody detection, so it was not possible to determine whether the historical anti-K had reappeared. In the remaining two patients, their historical anti-Jk^a and -K had not reappeared in antibody screens performed 14 and 100 days, respectively, after receipt of uncrossmatched RBCs.

Overall, only one recipient demonstrated biochemical or clinical evidence of immune-mediated hemolysis after receipt of uncrossmatched RBCs. The patient (Patient 4 in Table 1) was a 72-year-old man who received three units of uncrossmatched RBCs as a result of gastrointestinal bleeding. The antibody screen performed on a pretransfusion sample revealed anti-K, -Fy^a, -E, and -C^w, which had been detected previously. On this sample the anti-IgG DAT was 2+ with a nonreactive eluate. Because of the patient's condition, uncrossmatched RBCs had to be issued before the pretransfusion evaluation could be completed. A sample drawn 3 days later revealed a 2+ anti-IgG DAT with a weak positive anti-C3d, and on this sample the eluate revealed both anti-E and -Fy^a (the eluate was performed because of the clinical suspicion of hemolysis). The patient's hemoglobin before the uncrossmatched RBC transfusion was 7.2 g/dL (normal, 12.3–15.5 g/dL) and increased to 10.8 g/dL on the following day. His hemoglobin gradually decreased over the next 2 days to a nadir of 8.2 g/dL without further evidence of bleeding. The patient's creatinine was 1.9 mg/dL (normal, 0.5–1.17 mg/dL) before the uncrossmatched RBC transfusion and then increased to 2.4 mg/dL the day after receipt of uncrossmatched RBCs, at which time hemodialysis was instituted. The total bilirubin was only measured 6 days after the transfusion and was 16.1 mg/dL (normal, 0.1–1.2 mg/dL). The other biochemical markers of hemolysis such as reticulocyte count, lactate dehydrogenase, haptoglobin, or urine

Table 1. Demographic and immunohematologic characteristics of the patients with clinically significant historical or active antibodies at the time of the uncrossmatched RBC transfusions in this study

Patient	Age	Sex	Location of uncrossmatched RBC transfusion	Number of uncrossmatched RBCs	Transfusion reaction reported to blood bank	Previously identified clinically significant antibodies	New antibodies identified on the day of receipt of uncrossmatched RBCs	Previous RBC transfusions*	Evidence of immune-mediated hemolysis after uncrossmatched RBC transfusion
1	74	F	ICU	6	N	**D, K**		Y	N
2	67	F	ICU	2	N	S, Kp^a		Y	N
3	53	M	OR	6	N	Jk^a		Y	N
4	72	M	ICU	3	N	**K, Fy^a, E, C^w**		Y	Y
5	28	F	OR	8	N	**K, Fy^a**		Y	N
6	59	F	ED	2	N	K		Y	N
7	81	M	ED	2	N	**E**		Y	N
8	29	F	OR	5	N		**D**	N	N
9	78	F	ICU	2	N	**S**		Y	N
10	89	F	ED	2	N		**c**	Y	N
11	62	M	ICU	2	N	K		N	N

ED = emergency department; ICU = intensive care unit; OR = operating room.

*As documented in the electronic records of this regional transfusion service.

Antibodies in bold reflect those that were active on the day of the uncrossmatched RBC transfusion.

hemoglobin were not ordered on this patient. The patient died within 1 week of the uncrossmatched RBC transfusion. The reactive eluate in the setting of a decline in hemoglobin after uncrossmatched RBC transfusion, the increased bilirubin, and the worsening renal function are highly suggestive of immune-mediated hemolysis caused by the transfusion of incompatible uncrossmatched RBC units.

Several patients had clinically insignificant antibodies. There were two patients who had cold autoantibodies detected on the day of the uncrossmatched RBC transfusion, whereas a patient with four active clinically significant antibodies also had a history of a cold autoantibody that was not reacting on the day of the RBC transfusion (Patient 4 in Table 1). Two other patients had histories of anti-A_1 and warm autoantibodies, respectively, and another patient had a warm autoantibody along with anti-E that were both active on the day of the transfusion (Patient 7 in Table 1).

In total, four patients developed antibodies after receipt of uncrossmatched RBCs. Two patients developed antibodies within a short time after the uncrossmatched RBC transfusion; a 27-year-old D– male patient received two units of uncrossmatched D+ RBCs, and anti-D was detectable on an antibody screen 10 days later. This patient had a negative antibody screen 5 days after the RBC transfusion, and did not receive additional D+ RBC units between the uncrossmatched units and the detection of anti-D. A 30-year-old man developed anti-E 16 days after receipt of 18 uncrossmatched RBC units. His last negative antibody screen was 13 days after the uncrossmatched RBC transfusions, and the only addtitional RBCs he received were one crossmatched unit 5 days before the anti-E was detected, suggesting that the stimulus for the production of the antibody was the uncrossmatched RBCs themselves. Neither of these patients had a history of RBC transfusion or historical antibodies in the electronic records of the regional transfusion service; furthermore, neither patient demonstrated biochemical or clinical evidence of hemolysis after detection of the antibody. In the remaining two patients, the antibodies were detected on screens that were more remote from the time of the uncrossmatched RBC transfusion compared with the two patients described above; a 19-year-old male patient with no previous transfusion or antibody history on file at the transfusion service developed anti-Jk^a 30 days after the uncrossmatched RBCs. He had a negative antibody screen 4 days after the uncrossmatched RBC transfusion, and the anti-Jk^a was subsequently detected on his next screen. He received multiple crossmatched RBCs in the interval between the uncrossmatched RBCs and the detection of the anti-Jk^a. Similarly, a 59-year-old woman with a history of anti-K developed anti-D and anti-E 62 and 72 days, respectively, after the uncrossmatched RBC transfusion (Patient 6 in Table 1). This patient had five negative antibody screens before the anti-D and -E were detected, and the last negative screen was 47 days after the uncrossmatched RBC transfusion. She continued to receive multiple D+ RBCs in between the uncrossmatched RBCs and the detection of anti-D. As with the two patients described earlier, neither of these patients

had biochemical or clinical evidence of hemolysis after the uncrossmatched RBC transfusion.

Discussion

In our cohort, only 11 of 218 (5%) of the patients who received uncrossmatched RBCs had either a history of a clinically significant antibody or one that was active on the day of the transfusion. It is important to consider not only the patients with clinically significant antibodies that were active on the day of the RBC transfusion but also the patients with a history of a clinically significant antibody because evanescence is complicated and it is difficult to predict when or whether it will occur for a given antibody. Furthermore, recent data suggest that previously acquired antibodies seem to evanesce at a lower rate than newly formed antibodies.[13] Thus, antibodies that had been detected previously might still be present at a titer on the day of the uncrossmatched RBC transfusion sufficient to cause the immune-mediated hemolysis of incompatible units. Using the data from the current study, the risk of hemolysis after uncrossmatched RBC transfusion can be viewed in several ways. If only the patients with clinically significant antibodies that were active on the day of the uncrossmatched RBC transfusion are considered, then the risk is 1 of 7 (14%). Considering patients with either historical or active clinically significant antibodies, then the risk is 1 of 11 (9%). However, as frequently no immunohematologic information on a bleeding patient is available at the time the decision is made to use uncrossmatched RBCs, perhaps the most clinically relevant rate of hemolysis is 1 of 218 (0.5%), with the denominator including all of the patients in this study. These figures are similar to those from the study by Goodell et al. in which of the 265 uncrossmatched RBC transfusion episodes, 17 of the recipients had clinically significant antibodies and 1 patient had at least an exacerbation of an underlying immune-mediated hemolytic reaction.[4] Thus the evidence from the current study and that from the previous studies suggests that the use of uncrossmatched RBCs in the setting of a life-threatening hemorrhage is a relatively safe bridge until crossmatched RBCs become available.

As crossmatched RBCs are typically only matched for ABO and D, the use of uncrossmatched RBCs should not result in a higher incidence of anamnestic antibody responses or delayed hemolytic reactions compared with use of crossmatched RBCs. This is particularly true when the recipient's transfusion history is unknown or if there is no documentation of a historical antibody. That "new" antibodies in this study were detected relatively quickly after the uncrossmatched transfusions in two recipients without a history of antibodies suggests that these antibodies were possibly the product of a secondary immune response to D and E, respectively. Although the appearance of the anti-D could have been avoided had D– RBCs been transfused to the 27-year-old male recipient, O– RBC inventory pressures on the day of the uncrossmatched RBC transfusion required the use of D+ RBCs. Given that he did not receive other D+ blood products before the anti-D was detected, there is little doubt that the uncrossmatched RBCs were the stimuli for the reappearance of the anti-D. In the absence of any immunohematologic history, the sensitization to E in the other male recipient could not have been avoided by routine clinical practice. It is unclear whether the antibodies in the two other patients were stimulated by the uncrossmatched RBCs or by the additional RBCs that these patients received before their respective antibodies were detected.

The main limitation of this study is that we were not able to determine whether the patients with clinically significant antibodies on the day of the uncrossmatched RBC transfusion actually received RBCs that were incompatible with their antibody. Thus the observed rates of hemolysis in this study might be artificially low if the uncrossmatched RBCs were lacking the cognate antigen(s). In fact only D– uncrossmatched RBCs were transfused to two of the patients with active anti-D, although one of those recipients also had an active anti-K on the day she received uncrossmatched RBCs. Likewise, it is also possible that the recipient with the historical anti-Jk^a who had an antibody screen performed 14 days after receipt of the uncrossmatched RBCs was not reexposed to the cognate antigen; hence, the antibody did not reappear. Another limitation of the study is that because of the retrospective nature, the recipients were not specifically monitored for hemolysis after the transfusion of uncrossmatched RBCs; had each recipient of uncrossmatched RBCs been carefully followed after his or her transfusion with serial measurements of the biochemical markers of hemolysis and antibody screens, then perhaps evidence of hemolysis would have been detected in more patients. Furthermore, one patient who received uncrossmatched RBCs during resuscitation for septic shock died within a few hours of the transfusion; although no mention of an acute hemolytic event was made in the clinical notes of the resuscitation, a more thorough assessment was not possible; hence, the current rate of hemolysis might be slightly higher than reported. With the exception of the four recipients who produced an antibody after receipt of uncrossmatched RBCs, the number and results of subsequent antibody screens among the patients who did not have a known active or historical antibody on the day that they received their uncrossmatched

RBC transfusions were not recorded; thus we cannot derive the rate at which delayed serologic or hemolytic reactions occurred after the transfusion of uncrossmatched RBCs. This rate is not expected to be higher among recipients of uncrossmatched RBCs compared with recipients of crossmatched RBCs in our system because we do not routinely provide extended antigen-matched RBCs to most recipients.

Although hemolysis after the transfusion of uncrossmatched RBCs is an uncommon event that occurred in less than 1 percent of all recipients of uncrossmatched RBCs, close clinical and laboratory monitoring of recipients for evidence of hemolysis is warranted if a history of or active clinically significant antibodies are discovered on subsequent serologic investigation.

Acknowledgments

M.H.Y. thanks Dr. Jon Waters for thoughtful review and critical discussion of the manuscript.

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Case Report

Alloimmunization to Kell blood group system antigen owing to unmatched blood transfusion in a resource-poor setting

S. Malhotra, G. Kaur, S. Basu, R. Kaur, and G. Jindal

One of the major drawbacks of multiple blood transfusions in patients with thalassemia is the risk of development of alloimmunization to various red cell antigens within blood group systems such as Rh, Kell, Duffy, and Kidd. The problem is greater in developing countries because of lack of awareness and insufficient availability of specific typing antisera and antibody screening panels owing to financial constraints. It is of utmost importance to provide D, C, c, E, e, and K phenotype-matched blood in multiply transfused patients with thalassemia. This can prevent the development of antibodies against these clinically significant antigens, as antibodies can cause severe acute or delayed hemolytic transfusion reactions and create difficulties in providing a crossmatch-compatible packed red blood cell unit. The policy should preferably be adopted irrespective of financial constraints as it will pave the way for better transfusion practices and reduce the risk of adverse reactions in patients who are transfusion dependent for survival. ***Immunohematology* 2012;28:45–8.**

Key Words: alloimmunization, thalassemia, Kell blood group system, prophylactic matching

Transfusion therapy forms the backbone of treatment for patients with thalassemia, especially in thalassemia major in which it may have to be instituted during infancy. However, a major drawback of multiple blood transfusions in such a patient subset is the risk of development of alloimmunization to various red blood cell (RBC) antigens within blood group systems such as Rh, Kell, Duffy, Kidd, and others. Various studies from India estimate this risk to be less than 10 percent.[1,2] However, Michail-Merianou et al. reported the overall frequency of alloimmunization to be 18.9 percent among children with thalassemia and even higher, i.e., 25 percent, in children in whom transfusion therapy was initiated after their first year of life.[3] To minimize the occurrence of this adverse event, the British Committee for Standards in Haematology (BCSH) recommends routine and extended phenotyping of multiply transfused patients like those with thalassemia for ABO, D, C, c, E, e, and K antigens.[4] These antigens are immunogenically most potent and antibodies to these antigens are encountered in approximately two thirds of all cases of alloimmunization.[4] At our center, we strictly adhere to these BCSH guidelines. But in resource-poor centers, it may not always be possible to follow these guidelines, especially at peripheral centers, which may put the recipient at a higher risk of alloimmunization. Here, we report a 6-year-old child with thalassemia who was transfused with phenotype-matched blood since infancy and developed anti-K after receiving several transfusions outside our institution. To the best of our knowledge, there are only two case reports from India documenting this phenomenon.[5,6]

Case Report

A 6-year-old male child, blood group B, D+, had been diagnosed with thalassemia major at 13 months of age and was thereafter started on regular packed red blood cell (RBC) transfusions at 21-day intervals to maintain his hemoglobin at a level of 9 to 10 g/dL. His serum ferritin levels ranged between 720 and 790 µg/dL. At family screening, it was found that the parents were carriers of thalassemia trait and his two siblings were normal. The patient was being transfused with ABO, D, C, c, E, e, and K compatible packed RBCs. He had received more than 50 RBC transfusions, with an average of 13 to 14 transfusions per year. Two months before the incident reported here, his parents did not report for his regular transfusion, citing personal reasons. During this period, he received two packed RBC transfusions at a peripheral hospital. However, within 12 days of his last transfusion, he returned to our institution with complaints of increasing pallor and yellowish discoloration of the urine and eyes. An antibody screen was performed on the patient's blood sample along with an identification panel using commercially prepared RBCs (Dia Cell II, Bio-Rad, Haryana, India). The results of the antibody screen and identification panel confirmed the presence of anti-K in the patient's serum. An auto control was negative at both saline and anti-human globulin phases. No reactivity was seen with dithiothreitol (DTT)-treated serum. This was expected, as Kell antibodies are known to be denatured by DTT. An extended phenotype of the patient's RBCs revealed the phenotype as D+ C+ c– E– e+ K– k+ Jk(a– b+) Fy(a+b–) M+ N+ S– s+ and P1+. One PRBC

unit, group B, D+, and negative for E, c, and K, was issued to the patient and transfused uneventfully.

Discussion

In 1946, a new blood group antibody was identified in the serum of a woman, Mrs. Kelleher, that reacted with RBCs from her husband and her older child. The Kell blood group system has nearly 30 antigens; its two major antigens are K (Kell) and k (cellano). K is very immunogenic in stimulating antibody production, the sequence of immunogenicity being D > c > K > E > C > e.[7,8] Kell blood group antigens are well developed at birth, as K appears at 10 weeks of gestation and k at 7 weeks. Antibodies to K may be produced as a result of immune stimulation, i.e., during transfusion and pregnancy. Clinically, it is very significant as these antibodies can cause hemolytic disease of the fetus and newborn and hemolytic transfusion reactions. Table 1 shows the prevalence of Kell blood group system phenotypes in various ethnic populations.[8,9,10] Among north Indian blood donors, the prevalence of K is 5.68 percent.[9] Because of the low prevalence of K in the general population, anti-K may be missed during routine crossmatching but can be detected in an antibody screen.

Table 1. Prevalence of Kell blood group system phenotypes in various ethnic populations

Phenotype	Thakral et al[9] (%) (n=1240)*	Nanu et al[10] (%) (n=6334)†	Whites (%)[8]	Blacks (%)[8]
K+k+	5.68	4.04	8.8	3.5
K–k+	94.32	95.96	91	96.5
K+k–	0	0	0.2	<0.1
Kp(a+b+)	0.95	ND	2.3	Rare
Kp(a–b+)	99.05	ND	97.7	100

*North Indian blood donors.
†Selected north Indian population.

In India, the incidence of alloantibodies in patients with thalassemia is 7 to 10 percent. Table 2 summarizes the various studies reporting the incidence of alloimmunization to Rh and Kell antigens in various thalassemia populations. Pradhan et al. found 7 patients among 100 patients with thalassemia to have RBC alloantibodies. Among these, three had anti-D and one each had anti-C, anti-C and anti-D, and anti-Fy^a. Another case demonstrated the presence of naturally occurring anti-N and anti-Le^b with no patient showing a Kell antibody.[1] Recently, Gupta et al. reported development of alloantibodies in 11 thalassemia patients among 116 patients, with 3 patients showing anti-K. The most common antibody identified was anti-E in 4/116 (3.4%), followed by anti-K in 3/116 (2.6%), anti-Kp^a in 2/116 (1.7%), and anti-C^w in 2/116 (1.7%) patients.[2] Thakral et al. reported the development of anti K in a transfusion-dependent child with thalassemia.[5] Arora et al. also recently reported the development of alloimmunization to both Rh and Kell system antigens (anti-C, -K) in a young patient with thalassemia.[6] In both these cases, the patient received ABO- and D-matched, crossmatch-compatible blood, but phenotyping for C, c, E, e, and K was not performed.

Our patient had been receiving ABO, D, C, c, E, e, and K antigen-matched blood since he was diagnosed at 13 months of age. However, after having received two transfusions at a peripheral center, the patient developed alloantibodies to K, which, in turn, led to clinical symptoms of anemia, jaundice, and decreased transfusion intervals as a result of the shortened survival of transfused cells. This can be attributed to transfusion of K-unmatched blood. At the peripheral center, an antibody screen was not performed, and crossmatch compatibility was tested at the saline phase only. This was because of a lack of expertise and adequate facilities.

Michail-Merianou et al. did not find a statistically significant difference in alloimmunization between unmatched blood (compatible for ABO and D) versus better-matched blood (compatible for ABO, D, C, c, E, e, and K) in transfusion recipients (23.4% vs. 14.3%).[3] No significant difference was seen between unmatched and better-matched policies among children in whom transfusion therapy was started before 12 months of age (9% vs. 5.2%). However, the difference was statistically significant when transfusion therapy was started after 12 months of age (38.7% vs. 18.9%, respectively). Among the alloimmunized recipients, nearly one fourth showed alloantibodies to K in an unmatched group vs. no one in the better-matched group. Michail-Merianou et al. thus concluded that the earlier transfusion therapy is initiated, the less is the likelihood of alloimmunization, probably owing to development of immune tolerance to repeated blood transfusions (induced by an immature immune response in children). Also, the frequency of transfusions is less in such patients. The authors strongly recommended prophylactic phenotype matching for at least Rh and K antigens as a transfusion policy for children with thalassemia when transfusion therapy is started after 12 months of age. It is the view of these authors that if transfusion therapy is started before 12 months, the better-matched policy appears unnecessary.[3]

In a study from Iran involving 313 children, 12 alloantibodies were seen in 9 children (2.87%), the most common being anti-D followed by anti-C and anti-E.[11] In another study, among an Arab population, 57 (30%) of 190 patients developed RBC alloantibodies, with anti-K being

Table 2. Studies showing the incidence of alloimmunization to Rh and Kell antigens in various thalassemia populations

Authors, country	Prevalence	Extended Rh alloantibodies	Kell alloantibodies	Others
Pradhan et al., India[1]	7%	Anti-D: 3%; anti-C,-D: 1%; anti-C: 1%	None	Anti-Fy^a: 1% anti-N,-Le^b: 1%
Gupta et al., India[2]	9.5%	Anti-E: 3.4%; anti-C^w: 1.7%	Anti-K: 2.6%; anti-Kp^a: 1.7%	None
Thakral et al., India[5]	Case report		Anti-K	None
Arora et al., India[6]	Case report	Anti-C	Anti-K in same patient	None
Merianou et al., Greece[3]	19.2% (23/120 showed a total of 50 antibodies)	Anti-C, anti-c, anti-C^w, and anti-E: 28%	Anti-K: 20%	Anti-Fy^a,-Fy^b: 10%; anti-Jk^a,-Jk^b: 16%; anti-M: 6%; anti-S: 6%; anti-s: 2%; anti-Le^a: 10%; anti-Le^b: 2%
Sadeghian et al., Iran[11]	2.9% (9/313 showed a total of 12 antibodies all of Rh system)	Anti-D: 88.9%; Anti-C: 33.3%; and Anti-E: 11.1%	None	None
Ameen et al., Kuwait[12]	30% (57/190 showed a total of 142 antibodies)	Anti-D: 21.1%; anti-C: 15.8%; anti-c: 8.8%; anti-E: 45.6%; anti-C^w: 8.8%	Anti-K: 72%; anti-Kp^a: 5.3%	Anti-Fy^a: 3.5%; anti-Fy^b: 3.5%; anti-Jk^a: 10.5%; anti-Jk^b: 1.8%; anti-S: 5.3%; anti-Le^a: 12.3%; anti-Le^b: 3.5%; anti-Lu^a:1.8%
Singer et al., United States[13]	22% (14/64 showed a total of 19 antibodies)	Anti-E: 21%; anti-c: 10%	Anti-K: 31%; anti-Kp^a: 5.2%	anti-Jk^b, anti-M, anti-Le^a, anti-I, anti-HTLA: 5.2% each
Spanos et al., Italy[14]	18% (220/1200 showed a total of 470 antibodies)	anti-D: 4.3%; anti-C: 4.7%; anti-C^w: 5.7%; anti-c: 4%; anti-E: 14%; anti-e: 1.3%	Anti-K: 28.5%; anti-Kp^a: 1.3%	anti- M: 2.1%; anti-N: 0.4%; anti-S: 4.9%; anti-s: 0.4%; anti-Jk^a: 3.8%; anti-Jk^b: 4.3%; anti-Le^a: 4.3%; anti-Le^b: 1.7%; anti-Fy^a: 2.8%; anti-Fy^b: 1.3%; anti-P1: 1.3%; anti-A$_1$: 0.9%; anti-Sd^a: 0.6%; anti-Lu^a: 0.4%; anti-H: 0.2%; anti-Di^a: 0.2%

the most common clinically significant alloantibody in 41 (72%) patients followed by anti-E in 26 (45.6%).[12] A report describing patients with thalassemia who were predominantly of Asian descent found that 14 (22%) of 64 patients became alloimmunized, and erythrocyte autoantibodies developed in 16 (25%) of the 64 patients, thereby causing severe hemolytic anemia in 3 of 16 patients.[13] In both these studies, the authors observed that a higher rate of alloimmunization is seen among patients with thalassemia transfused with non-leukocyte-depleted blood compared with leukocyte-depleted blood. It has been hypothesized that the release of immune-stimulatory antigens and soluble biological mediators from the residual leukocytes sensitizes the immune system of recipients and causes development of alloantibodies to foreign antigens. Martelli et al., using specialized techniques such as enzyme-linked immunosorbent assay, flow cytometry, and immunofluorescence, observed that nuclear matrix protein is released from apoptotic white blood cells during cold storage of RBC units. They suggested that this phenomenon might lead to antibody response in multiply transfused patients.[15] Further, it was observed that the rate of alloimmunization was higher in patients who had undergone splenectomy as compared with those who had not undergone this operation. It was postulated that patients without spleens are more prone to develop both alloantibodies and autoantibodies, and that antibody formation is prevented in the patients with intact spleens because of filtration of antigens in the spleen. In another study from Italy, 5.2 percent of the patients (74 of 1435 patients) had clinically significant RBC alloantibodies against Rh, Kell, Kidd, and Duffy system antigens, in decreasing order of frequency.[16]

The provision of adequately matched, crossmatch-compatible PRBCs to multiply transfused patients like those with thalassemia remains a major challenge in developing countries. Although these patients are at a considerable risk of developing antibodies to various foreign RBC antigens, this transfusion complication is underreported or underestimated. Thakral et al. attributed this to the relative lack of awareness, insensitive crossmatch techniques, and unavailability of antigen panels.[5] In addition, the RBC panels used for antibody detection and identification may not represent the true antigen prevalence within our Indian population. The present case emphasizes the importance of providing phenotype-matched blood and implementation of routine screening for alloantibodies to K in multiply transfused patients with thalassemia, as these can cause severe acute or delayed

hemolytic transfusion reactions. The policy should preferably be adopted irrespective of financial constraints as it will pave the way for better transfusion practices and reduce the risk of adverse reactions in patients who are transfusion dependent. In addition, once the patient develops alloantibodies, a special immunohematology report indicating the nature of the antibody should be provided to the patient for all future transfusions.

The adoption of such a policy in multiply transfused patients in developing countries will definitely lead to added expenses on the part of transfusion services. However, when compared with the risk of alloimmunization and the resulting complications of transfusion therapy, the benefits of such a policy outweigh the added financial burden. Once alloantibodies are formed, the risk of adverse transfusion reaction increases, which can increase the hospital stay of patients and decrease their transfusion interval. Further, multiple crossmatches may be required, which again means an increased cost levied on the patient and the hospital service. Hence, the implementation of such a stringent policy would ultimately lead to more systematic follow-up of patients and a better quality of life.

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Review

Cartwright blood group system review

M.R. George

The Cartwright (Yt) blood group system consists of two antigens, Yt^a and Yt^b, that result from point mutations in the acetylcholinesterase gene on chromosome 7q. Yt^a is a high-incidence antigen, whereas its antithetical antigen, Yt^b, shows much lower incidence. Anti-Yt^a and anti-Yt^b are relatively rare. Anti-Yt^a is more commonly found in individuals of Jewish descent. Cartwright antibodies are rarely clinically significant; however, cases of in vivo hemolysis have been reported, suggesting that clinical significance should be interpreted on a case-by-case basis. ***Immunohematology* 2012;28:49–54.**

Key Words: Cartwright, Yt, AChE

History

Anti-Yt^a and thus the Yt^a antigen of the Cartwright blood group system were first described in 1956 by Eaton and colleagues.[1] This discovery in the early 1950s came on the heels of the identification of three other antibodies against high-incidence antigens (anti-Tj^a, -Vel, and -U). This new antibody was noted to agglutinate the majority of red blood cells (RBCs) of whites and was independent of all other known blood group systems. The antibody was first identified in a woman with a history of four pregnancies complicated by severe hemorrhage at delivery. She received several units of blood after her last delivery. Several years later, she required more transfusions. RBC units were weakly incompatible with her serum by the indirect antiglobulin test (IAT). Blood samples from eight laboratory staff members were also tested with the patient's serum and found to be incompatible. Transfusion was deferred until compatible blood could be obtained. The patient was discharged to home, and her hemoglobin level recovered without transfusion.

Serologic investigations performed on the patient's serum against a panel of RBCs of known phenotype demonstrated no agglutination in saline or albumin, slight but easily dispersible agglutination with trypsin-treated cells, and no reactivity with papain-treated cells. Owing to the weak and variable reactivity, Eaton and colleagues[1] decided to rule out a mixture of antibodies, and repeatedly adsorbed the serum with a number of strongly and weakly reacting cells. Six adsorptions were performed with equal volumes of strongly reacting cells at 37°C. After 1 hour, the antibody titer was reduced from between 32 and 128 to between 2 and 4. The sera after adsorption tested against a panel of cells indicated that only a single antibody was present. The antigen to which this antibody was reacting was provisionally named Yt^a. Eaton and colleagues[1] assumed that the antithetical antigen in this system, if discovered, would be called Yt^b. Having excluded the presence of more than one antibody, the researchers noted that the observed differing strengths of reactivity could represent a dosage effect, with weak reactors symbolizing heterozygotes. Forty-four members of the laboratory staff were tested. Of those 44, 5 reacted weakly. If those 5 represented heterozygotes, it was calculated that a negative would be encountered in every 250 to 300 random blood samples (0.348%). The goal of these initial investigations was to obtain compatible blood. A total of 1051 group O donors were tested with the patient's serum at two dilutions, 1 in 4 and 1 in 8, by the IAT. Tests were interpreted by the same staff members and were graded as strong, weak, and intermediate. Four negatives were identified and confirmed by repeat testing. The incidence of negatives was calculated as 0.38 percent. Extensive serologic testing demonstrated the independence of this new blood group system from ABO, Rh, and MNS systems. Yt^a was identified on the RBCs of six infants at birth; reactivity was weaker than on adult cells. Further proof of this new blood group system was provided through the identification of additional cases of anti-Yt^a by Race and Sanger,[2] Bergvalds and colleagues,[3] and Dobbs.[4] In 1963, Yt^a was demonstrated to be separate from the Lutheran blood group system by Allen et al.[5] using a detailed family pedigree starting with a mother of six children who was homozygous for both Yt^a and Lu^a. Given the significant genetic recombination as evidenced by the varied phenotypes in the children, it was clear that Yt and Lu were not related. The same pedigree demonstrated Yt to be separate from the Rh system.

Giles identified and reported the expected antithetical antigen, Yt^b, in 1964.[6,7] The patient was known to have anti-Fy^b and an additional unidentified antibody. This antibody was isolated by adsorption and elution and tested against random blood samples. Nineteen of 229 samples demonstrated reactivity. Six known Yt(a–) RBC samples were tested against an eluate containing only the unknown antibody in the patient's serum. All reacted positively, with presumptive identification of anti-Yt^b, adding further evidence of the discovery of the antithetical antigen, Yt^b. A second example of anti-Yt^b was

presented by Ikin et al.[8] in a case report of a patient with paroxysmal nocturnal hemoglobinuria (PNH) with aplastic bone marrow. He had been receiving six units of blood two or three times a year since 1957. This group found Yt^b in about 8 percent of random samples, and the incidence of individuals with the Yt(a+b–) phenotype was reported to be as high as 91.4 percent. Given these statistics, it seemed likely that other cases of anti-Yt^b may have passed unnoticed. In 1968, Wurzel and Haesler[9] found another anti-Yt^b in a 76-year-old man who had received many transfusions.

Nomenclature

The blood group system was named after the patient, Mrs. Cartwright, in whom it was first identified. Eaton and colleagues[1] developed the system's nomenclature by using the last letters of the patient's first and last name (Y and T), since her initials, A and C, already represented well-known blood group antigens. They adopted the idea of using a superscript letter to denote individual antigens within the system, starting with Yt^a and assuming that the antithetical antigen in this system, if discovered, would be called Yt^b. The nomenclature across various systems is denoted in Table 1.[10–16]

Table 1. Nomenclature

System Name		System Symbol			Gene Symbol		
Traditional/ISBT	ISBT number	Traditional	ISBT	ISBT number	Traditional	ISBT	ISGN
Yt or Cartwright	011	Yt	YT		*Yt*	*YT*	*ACHE*
		Yt^a	YT1	011.001			
		Yt^b	YT2	011.002			

ISBT = International Society of Blood Transfusion
ISGN = International System for Gene Nomenclature

Genetics/Inheritance

The original cases of anti-Yt^a were identified in patients of Jewish ancestry. The National Blood Group Reference Laboratory in Jerusalem identified 14 patients of 4474 referrals with anti-Yt^a, which was higher than expected based on prior European data. Once anti-Yt^b became available, this group undertook a survey of Yt groups using both anti-Yt^a and anti-Yt^b on Israeli Jews. They tested 264 blood samples selected at random from the population and found that Yt^b and anti-Yt^a are relatively frequent among Israelis. These discoveries prompted further testing using the Yt system as a useful genetic marker in examining potential variability among different Jewish ethnic groups.[17–19] This same Israeli group looked at 1683 blood samples from Israeli Jews, Arabs, and Druse to determine the incidence of the Yt blood group phenotype and allelic frequencies. These populations had an overall incidence of the Yt(b+) phenotype ranging from 24 to 26 percent, versus a general European incidence of 8 percent. A null phenotype, Yt(a–b–), was not identified. These findings suggested that if blood group antibodies against a high-incidence antigen were detected in an individual of Jewish, Arab, or Druse descent, the probability of anti-Yt^a would be high. Few ethnic groups have been tested for the incidence of Yt blood group system phenotypes and for allelic frequencies; however, individuals of Jewish descent and other Mid-Eastern ethnicities tended to have a lower incidence of Yt^a and a higher incidence of Yt^b. Yt^b was not identified in a random sampling of 70 Japanese individuals.[17] The incidence of the Yt(a+b–) phenotype in American blacks and white Canadians was found to be essentially the same as determined in studies of Europeans, as reported by Wurtzel and colleagues[20] in 1968 and Lewis and colleagues[21] in 1987. Overall, Yt^a occurs in approximately 99.7 percent of American and European blood donors, whereas the antithetical Yt^b is found in 8.1 percent of these donors. Yt(a–b–) appears to be transient, and a true inherited Yt(a–b–) phenotype would be extremely rare.[22] The percent occurrences of general phenotypes in Americans and Europeans are summarized in Table 2.[23]

Table 2. Yt Phenotypes

		% Bloods Reacting With	
Phenotype	% Occurrence	Anti-Yt^a	Anti-Yt^b
Yt(a+b–)	91.9	99.7	
Yt(a+b+)	7.8		8.1
Yt(a–b+)	0.3		

Molecular Basis

Even after the Cartwright antigen system was well characterized serologically, the chromosomal location was not known until 1991. In 1987, Lewis et al.[21] looked at linkage between Yt and 42 chromosomally assigned loci, but relationships were not clear. By 1989, Yt was believed to be loosely linked to the Kell system.[24] Linkage was shown to occur between the Yt and Kell loci at a recombination fraction of 28 percent. So when Kell was provisionally assigned to chromosome 7q, the placement of Yt seemed to be the next step. Based on their previous work with loci on chromosome

7, Zelinski and colleagues were able to demonstrate linkage to *COL1A2* and *D7S13* genotypes through paternal meiosis. Because it showed no recombination, Yt was provisionally assigned to chromosome 7, particularly within 7q22.1–q22.3.[25–28] Further studies of chromosome 7 also localized acetylcholinesterase (AChE) to 7q22. AChE plays a crucial role in cholinergic neurotransmission, and is found in the RBC membrane, where its function remains largely unknown. AChE undergoes extensive posttranslational modification, which may leave room for genetic variability.[29–32] A study by Spring et al.[33] provided evidence that Yt blood group antigens are located on erythrocyte AChE. This study identified immune precipitates of human anti-Yt^a and -Yt^b of the same molecular weight as AChE from radioiodinated erythrocytes of appropriate Yt phenotype. The immune precipitates obtained with anti-Yt^a and -Yt^b contained AChE activity. These results indicated that the Yt antigens were derived from an inherited polymorphism of AChE, and in addition to Zelinski's provisional assignment of Yt blood group locus to chromosome 7, gave provisional identification of the location of the *ACHE* gene. Further corroborating the link between Yt and AChE, Bartels et al.[34] discovered that the Yt blood group polymorphism is caused by point mutations in the *ACHE* gene. Further genetic characterization demonstrated that the *YT(ACHE)* gene consists of six exons distributed over 2.2 kbp of genomic DNA. The molecular basis of the Yt antigens is summarized in Table 3.[11]

Table 3. Molecular basis of the Yt antigens

	Yt^a	Yt^b
Amino acid	His 353	Asn 353
Nucleotide	C at bp 1057 in exon 2	A at bp 1057 in exon 2
Additional features	Nucleotide at position 1431 C>T in exon 3 also differentiates Yt^a from Yt^b but does not alter the encoded amino acid. A second silent mutation in exon 5 does not correlate with the Yt polymorphism.	Nucleotide at position 1431 C>T in exon 3 also differentiates Yt^a from Yt^b, but does not alter the amino acid. A second silent mutation in exon 5 does not correlate with the Yt polymorphism.

Biochemistry

Early studies of the Kell blood group antigen system led to some biochemical parallels between this system and the Cartwright system. Studies of the effect of dithiothreitol (DTT) on Kell and other RBC antigens led to the discovery that all Kell blood group antigens as well as Yt^a are completely denatured after treatment with DTT. Other sulfhydryl reagents did not result in Yt^a denaturation. Yt^a was denatured within the same DTT concentration range as Kell, which led Branch and colleagues[35] to speculate that a biochemical relationship existed between the two systems. They concluded that Yt^a requires at least one disulfide bond for maintenance of antigen integrity, implying a protein backbone. These findings paved the way for further biochemical characterization of the Cartwright antigen system.[36,37] The protein structure of the antigens within the Cartwright system helps to explain their serologic properties. Effects of enzymes and chemicals on Yt antigens are summarized in Table 4.[11,38]

Table 4. Effects of enzymes and chemicals on Yt antigens

Enzyme/chemical	Yt^a	Yt^b
Ficin/papain	Sensitive (variable)	Sensitive (variable)
Trypsin	Resistant	Resistant
α-Chymotrypsin	Sensitive	Sensitive
Pronase	Sensitive	Sensitive
Sialidase	Resistant	Resistant
DTT 200 mM/50 mM	Sensitive/weakened	Sensitive/weakened
Acid	Resistant	Presumed resistant

Additionally, Levene and Harel[39] noted that 2-amino-ethylisothiouronium (AET)-treated RBCs, which their laboratory used to detect antibodies to high-incidence antigens, may be helpful in the identification of Cartwright antibodies. Because disulfide bonds are a requirement for Cartwright antigen integrity, AET can reduce these bonds and eliminate reactivity.

In tandem with the molecular studies of the Cartwright antigen system, once a protein backbone was suspected, the study of patients with PNH led to the discovery that the Yt antigens likely resided on a phosphatidylinositol (PI)-anchored protein, as the complement-sensitive (PNH III) erythrocytes of these patients failed to express these antigens. However, the relatively normal constituent of complement-insensitive erythrocytes from the same patients expresses these antigens normally. Other high-incidence antigens showed varying levels of reactivity.[40,41] Human RBCs express a wide variety of PI-anchored proteins, including decay accelerating factor (DAF; CD55), membrane inhibitor of reactive lysis (MIRL; CD59), lymphocyte function associated antigen-3 (LFA-3; CD58), AChE, oxidized nicotinamide adenine dinucleotide (NAD+) glycohydrolase, JMH protein (p76), and others. DAF is known to bear the Cromer blood group antigens. A study in 1993 by Rao and colleagues[22] demonstrated that Yt^a resides on erythrocytes' AChE. This study used RBCs from an individual with a previously uncharacterized Yt(a–b–) phenotype as well as normal Yt(a+) cells to serologically and

biochemically evaluate the relationship between Yt^a and PI-linked erythrocyte proteins. Their work demonstrated that the Yt(a–b–) RBCs expressed normal amounts of all PI-linked proteins except for AChE, which added further proof of the Yt blood group system stemming from AChE.[40] Yt^a is weakly expressed on cord RBCs, whereas Yt^b shows the same level of expression on cord cells as on adult RBCs.[11–13]

In the late 1970s and early 1980s, a number of studies were undertaken to investigate the presence or absence of RBC antigens on various white blood cell fractions. Historically, there was no consensus in the literature regarding this topic.[42,43] In 1984, using flow cytometry, Dunstan[44] demonstrated that Yt^a was not detected on lymphocytes, monocytes, or neutrophils.

Antibodies

The discovery of the Yt blood group system was dependent on the identification of the antibodies directed against the antigens within the system. There are only two antigens in the Yt blood group system, Yt^a and Yt^b, and therefore only two possible antibodies. Both anti-Yt^a and -Yt^b are of the IgG class and are optimally detected at the IAT phase. The incidence of the Yt (a–) phenotype, and hence anti-Yt^a, is higher in populations of Jewish descent. The first cases of anti-Yt^a were found in Jewish women who were sensitized by pregnancy or blood transfusion.

Eaton et al.[1] were the first to describe anti-Yt^a and hence Yt^a. Race and Sanger[2] reported three additional cases of anti-Yt^a in their book *Blood Groups in Man* in 1962. An additional case of anti-Yt^a was reported by Bergvalds and colleagues[3] in 1965. Their case was that of a Jewish woman who presented for induction of labor at 40 weeks' gestation. She had one previous pregnancy in which no antibodies were identified. She had no history of abortions, miscarriages, or previous blood transfusions. Late in her pregnancy, she was found to have a rising anti-D titer and a second unidentified antibody. After serologic investigation pointed to anti-Yt^a, a blood sample was sent to the Blood Group Reference Laboratory in London, England, where the presence of anti-Yt^a was confirmed. Additionally, the patient's RBCs were tested against anti-Yt^b, demonstrating that her phenotype was Yt(a–b+) as expected.

In 1968, Dobbs et al.[4] reported clinical experience in three cases of anti-Yt^a. Over 3 years this group identified anti-Yt^a in three patients: two were pregnant Jewish women with no evidence of hemolytic disease of the newborn (HDN); the third was a Jewish male patient with pure RBC aplasia who had been sensitized by multiple transfusions. Given the rarity of Yt(a–) blood and the severity of his anemia, this patient was transfused with several units of Yt(a+) blood with no evidence of untoward reaction. Chromium RBC survival studies were performed using an aliquot of transfused incompatible Yt(a+) blood. The study showed a RBC half-life of 30 days (normal is 27 to 30 days), so it appeared that this incompatibility had no effect on RBC survival in this patient. The patient required numerous transfusions and eventually underwent splenectomy and a regimen of steroids.

Clinical Significance

The clinical significance of Cartwright system antibodies is debatable. There are two main laboratory diagnostic tests to assess clinical significance: the monocyte monolayer assay (MMA), which is used in vitro, and the ^{51}chromium labeling study, used in vivo.[45,46] Most data regarding the clinical significance of these antibodies have been anecdotal through case studies. Despite evidence that anti-Yt^a and -Yt^b are capable of crossing the placenta, their presence does not necessarily result in HDN, which may in part be explained by the fact that Yt^a generally shows weak expression on cord blood cells. Anti-Yt^a does not generally cause transfusion reactions; however, moderate delayed reactions have been reported. The first evidence of clinical significance of anti-Yt^a came from Bettigole et al.[47] Their group demonstrated rapid in vivo destruction of Yt(a+) RBCs in a patient with anti-Yt^a. They conducted an in vivo RBC survival study by transfusing 10 mL of chromium-tagged Yt(a+) RBCs. The half-life of the transfused cells at 12 minutes was estimated to be 3 days. Only about 13 percent of the original radioactivity was detected in vivo, despite 91 percent effectiveness of chromium labeling in vitro. Several years later, Göbel et al.[48] and Ballas and Sherwood[49] identified additional examples of anti-Yt^a with rapid in vivo destruction of Yt(a+) RBCs.

Although chromium studies[50,51] are useful, some antibodies have demonstrated a change in perceived clinical significance as detected with long-term chromium-labeling studies. AuBuchon and colleagues[52] described an anti-Yt^a that initially did not appear to decrease RBC survival; however, 12 weeks after transfusion of Yt(a+) RBCs, the alloantibody was characterized as IgG1 and repeat radiolabeled RBC survival studies demonstrated significant shortening of RBC lifespan as followed for 7 days. This particular case demonstrated a two-component survival curve in which initial rapid destruction is followed by slower removal of remaining cells. The authors attribute this to uneven antigen distribution, exhaustion of antibody, effect of RBC age, or blockade of

macrophage receptors for the complement system. Previous reports suggested the homogeneity of the Yt blood group system. However, an allogeneic anti-Yt^a in a Yt(a+) individual was reported by Mazzi et al.[53] Both parents of the patient typed as Yt(a+). The patient's serum was adsorbed with his father's RBCs. Subsequently, the patient's serum was tested with his mother's RBCs, giving a positive reaction. Although all three parties tested Yt(a+), this pattern of reactivity suggests that a variant Yt^a was present in the propositus and his father that was distinct from the normal Yt^a found in the mother. Overall, recommendations for management of patients with anti-Yt^a must be based on the individual case. A 14-month study of five such patients conducted by Mohandas and colleagues[54] suggested considerable variability in these patients' tolerance of Yt(a+) RBC transfusions.

Little has been reported about the clinical significance of anti-Yt^b. Yt^b is expressed normally on cord blood cells; however, little is known about anti-Yt^b in pregnancy, and therefore its potential to cause HDN is unknown. Generally, anti-Yt^b is not believed to cause transfusion reactions. However, Levy et al.[55] both performed ^{51}chromium-labeling studies in vivo and used MMA studies as an in vitro estimate of clinical significance. Their study found slightly abnormal MMA results and evidence of decreased RBC survival as measured by the ^{51}chromium-labeling study. This finding was similar to that reported by Baldwin et al.,[56] which examined RBC antibodies of questionable clinical significance (anti-McC^a, -JMH, -Kn^a, and -Hy).

In summary, the Cartwright blood group system is a small system that demonstrates interesting demographic patterns. The expression of Yt antigens is dependent on intact AChE and a PI backbone. Although antibodies to this system are generally considered clinically insignificant, case reports have demonstrated decreased RBC survival. It would be advisable to consider the clinical circumstances surrounding each case of anti-Yt^a or -Yt^b in making a judgment on the clinical significance of the antibody. In emergency or urgent transfusion, most patients receiving Yt-incompatible blood have fared well; however, the various case reports discussed previously suggest that some cases have demonstrated decreased cell survival such that Yt-compatible blood would be preferable.

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Case Report

Posttransplant maternal anti-D: a case study and review

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Plasma from a 35-year-old, D– woman was found to have anti-D, -C, and -G at 5 weeks' gestation and again at 8 weeks' gestation, when she presented with a nonviable intrauterine pregnancy. The anti-D titer increased with a pattern that suggested it was stimulated by the 8-week pregnancy. Six years before this admission, the patient's blood type changed from group O, D+ to group O, D– after a bone marrow transplant for aplastic anemia. Three years after transplant, the antibody screen was negative. After the patient was admitted for the nonviable pregnancy, the products of con-ception were found to be D+ by DNA testing for *RHD*. There were no documented transfusions or pregnancies during the interval in which anti-D appeared. The timing of the alloimmunization was unusual. In a subsequent pregnancy, fetal D typing was performed by molecular methods. ***Immunohematology* 2012;28:55–9.**

Key Words: immunohematology, stem cell transplantation, transfusion medicine

Case Report

A 30-year-old, D+ woman with aplastic anemia underwent allogeneic bone marrow transplant (BMT) from her HLA-identical, D– brother in December 2003. The pretransplant antibody screen (by indirect antiglobulin test [IAT]) was negative both in donor and in host. The patient received red blood cell (RBC) and platelet transfusions from D+ donors before the BMT. RBC transfusions were restricted to D– components from the time of conditioning. The patient received three units of group O, D+ RBCs on Day –70 and five units of group O, D– RBCs between Day –30 and Day +12 (Table 1). She also received 30 units of single-donor platelets (SDP) between Day –70 and Day +12; these included SDPs from D+ and D– donors. The conditioning regimen consisted of cyclophosphamide and antithymocyte globulin (ATG). The patient initially received cyclosporine and methotrexate for graft-versus-host (GVH) prophylaxis. On Day +20, she still typed as D+ with a negative antibody screen. She required no further blood or platelet transfusions after posttransplant Day 12, and she was tapered off cyclosporine immunosuppression 9 months after transplant. The patient delivered a healthy, D– baby in 2006. During that pregnancy and at delivery, the patient typed as D– with a negative antibody screen by solid-phase technology. The cord blood typed as group O, D–, weak D–, and the father typed as group O, D–.

In January 2009, the patient presented with a pregnancy at 5 weeks' gestation and was found to have anti-D, -C, and -G with a titer of 128 against a D+, C+, G+ RBC sample. At 8 weeks' gestation, the patient presented with a nonviable intrauterine pregnancy, and the anti-D and -C titers were 1024 and 64, respectively. The patient's RBCs were negative by the direct antiglobulin test (DAT). As the G antigen is expressed in the presence of D or C or both, it is highly unlikely that the patient and thus the products of conception would express G because the D+G– and C+G– phenotypes are extremely rare and the D+C+G– phenotype has not been reported. After the patient was admitted for the nonviable pregnancy, the products of conception were predicted to be D+ by DNA testing for *RHD*. Polymerase chain reaction (PCR)–based assays predicted the following phenotypes: mother D+ C+ E– c+ e+ by buccal swab and D– C– E– c+ e+ by peripheral blood white blood cells; father D– C– E– c+ e+; and conceptus D+ C+ E– c+ e+.

In October 2009, the patient became pregnant for the third time. Fetal *RHD* typing, performed at 18 weeks' gestation from amniotic fluid, predicted a fetal phenotype of D– C– E– c+ e+. An analysis of variable number of tandem repeats was used to confirm that the results were not affected by maternal cell contamination of the amniocyte sample. At that time, the anti-D and -C titers were 2048 and 64, respectively.

Materials and Methods

At the hospital laboratory in 2006, the maternal antibody screens were performed on an automated analyzer (ABS 2000, Immucor Inc., Norcross, GA) using a four-cell screen by solid phase technology (Capture-R Ready Screen IV, Immucor Inc.). Cord blood typing on the newborn was performed by manual tube testing, including weak D testing. In 2009, the maternal antibody screen was performed on a newer analyzer (Galileo, Immucor Inc.) using a solid phase two-cell screen (Capture R Ready Screen II, Immucor Inc.). At the New York Blood Center immunohematology laboratory, the anti-D, -C, and -G specificities were confirmed using manual tube

Table 1. Condensed case description including transplant Day 0 (December 2003), transplant Day +20 (December 2003), full-term pregnancy (2006), pregnancy at 5 weeks' gestation (January 2009), nonviable pregnancy at 8 weeks' gestation (January 2009), and subsequent pregnancy (2010)

	Date					
Case description	2003	2003	2006	2009	2009	2010
Patient Rh phenotype	D+C+E–c+e+	D+	D–	D–	D–C–E–c+e+	D–
Antibody screen	Negative	Negative	Negative	Positive	Positive	Positive
Anti-D titer	—	—	—	128	1024	2048
Anti-C titer	—	—	—	—	64	64
RBC transfusion	3 units, group O D+, Day –70	5 units, group O D–, Day–30 to Day +12	—	—	—	—
SDP transfusion	30 units (Day–70 to Day +12)	—	—	—	—	—
Fetal gestational age	—	—	40 weeks	5 weeks	8 weeks (nonviable)	18 weeks (viable)
Fetal source material	—	—	Cord blood	—	Products of conception	Amniotic fluid
Fetal Rh phenotype	—	—	D–	—	D+C+E–c+e+	D–C–E–c+e+

RBC = red blood cell, SDP = single-donor platelet.

methods including a sequential adsorption/elution procedure. PCR-based assays for *RHD, RHCE*C/c,* and *RHCE*E/e* were performed on the products of conception, maternal and paternal buccal epithelial cells, and maternal blood sample. At a reference laboratory during the third pregnancy (early 2010), amniotic fluid was used for genomic DNA testing by allele-specific gene amplification.

Table 2. Chimerism results by fluorescence in situ hybridization (donor XY, host XX) to show percent engraftment of the bone marrow transplantation in the patient (host)

Time	Source	Cells analyzed	Interpretation
Day +30	Marrow	497 XY, 3 XX	99.4% donor, 0.6% host
Day +100	Marrow	495 XY, 5 XX	99% donor, 1% host
Day +365	Blood, unsorted	497 XY, 3 XX	99.4% donor, 0.6% host
Day +365	Blood, T cells	96 XY, 4 XX	96% donor, 4% host

Results

Table 1 contains pertinent features of the case in a timeline beginning with the BMT and associated transfusions, followed by a total of three pregnancies. In summary, Table 1 depicts the patient's change in type from D+ to D– after the BMT, the negative maternal antibody screen at the time of the healthy full-term delivery, the rising anti-D titers during the nonviable pregnancy, and the molecular test results from the second and third pregnancies. Details of the case history have been described earlier in this report.

Table 2 summarizes the results of chimerism testing by fluorescence in situ hybridization (FISH) during the year after the BMT. The patient's chimerism results indicated a tiny percentage of host-type lymphocytes in the peripheral blood a year after transplant. The posttransplant specimens did not show mixed-field reactivity in the patient's RBC typing.

Discussion

The timing of this patient's alloimmunization to D was unusual. If it was caused by the nonviable pregnancy, the alloimmunization occurred unusually early in gestation. If it was caused by peritransplant exposure to D, it is unusual that the antibody did not appear soon after immunosuppression was tapered. Posttransplant formations of anti-D are uncommon because the donor-related primary immune response usually starts about 6 months after the BMT; after this period, if recipient bone marrow is 100 percent of donor origin, residual D+ recipient RBCs are no longer detectable.[1] Anti-D was identified in one of seven D+ recipients of a D– graft, who was also exposed to D+ blood components before and after the nonmyeloablative transplant.[2] In one case, anti-D appeared only when immunosuppression was discontinued, 2 years after the BMT.[3] Our patient's plasma contained no anti-D 3 years after the BMT, yet anti-D was present 6 years after transplant. In one study of D– hosts with D– bone marrow donors, the alloimmunization rate by D+ blood components was less than 5 percent,[4] but some authors suggest prophylaxis with intravenous anti-D immunoglobulin if D+ platelets must be used.[1] Our patient received SDPs from both D+ and D– donors, and only D– RBCs were given from the start of conditioning for the BMT. There were no

documented transfusions or pregnancies during the interval in which anti-D appeared (between late 2006 and early 2009).

The patient's chimerism results by FISH showed that her engraftment status was almost completely donor, and her RBCs typed as donor without mixed-field reaction. RBC chimerism was not excluded by more sensitive methods. For example, flow cytometry was not performed to look for D+ cells.

Based on the analogous scenario of minor ABO mismatched transplants, we would expect this patient to have a low risk of alloimmunization for the following reasons: the regimen was myeloablative; the source was marrow, not blood stem cells; and GVH prophylaxis included both cyclosporine and methotrexate. On the other hand, passenger lymphocyte–mediated hemolysis has been reported to be more common in recipients of related donor versus unrelated donor transplants.[5] The conditioning regimen for aplastic anemia is somewhat less aggressive than myeloablative regimens for other clinical indications and may be considered submyeloablative. Low-intensity conditioning regimens may leave the host's antigen-presenting machinery relatively intact, possibly resulting in more potent stimulation of donor memory B cells.[6]

The maternal anti-D titer increased from the 5-week gestation sample to the 8-week gestation sample, suggesting the antibody was stimulated by very early pregnancy. Few D– women form anti-D that is IgG during their first uncomplicated pregnancy. The average rate reported for this is 0.9 percent, range 0.3 to 1.9 percent. The risk of D immunization in a D– woman is considered to be 1 to 2 percent during a pregnancy with a D+ (ABO compatible) fetus, increasing to 14 to 17 percent during delivery.[7] During normal pregnancy, transplacental hemorrhage (TPH) can occur as early as at 4 weeks after fertilization, or 6 weeks after last menstrual period (LMP). This is the time when fetal and maternal circulations in the placenta have been formed and when the vascularization of the villi and the pumping action of the fetal heart begin. At this stage of pregnancy, TPH of 0.004 mL has been detected.[7]

Bergström et al.[8] found Rh antigens on the RBCs of a 10-mm fetus, obtained approximately 38 days after conception or 52 days after LMP. The embryo was found in a 47-year-old woman, with systemic lupus erythematosus (SLE), who was undergoing hysterectomy because of a cervical myoma. The embryo had a yolk sac and was obtained within intact membranes. Microscopic examination of the suspension showed almost exclusively nucleated megaloblasts of fetal type. The fetal material was found to be D+. Theoretically, early abortion could induce anti-D immunization in the D– woman. However, there is no evidence in the literature to show that spontaneous abortion occurring in the first trimester can cause anti-D immunization.[7,9] In this patient, the high-titer, IgG maternal response is unexpected given the 8-week gestation without trauma.

The occurrence of anti-D detected in D– individuals who have never been exposed to D+ RBCs through either pregnancy or tranfusion is rare. Six cases have been reported in the literature. These include a never-pregnant 26-year-old woman, three women who delivered D– babies, and two male patients.[9] The authors excluded alloimmunization after intravenous drug use with shared needles and administration of intravenous immunoglobulin. They suggest other possible mechanisms: occult pregnancy followed by undetected abortion; the transfer of D+ RBCs from mother to D– fetus resulting in alloimmunization (the grandmother theory); anamnestic response induced by infection or malignancy; or exposure to an unidentified antigen similar to D.

Most recipients of allogeneic hematopoietic stem cell transplantation (HSCT) suffer from secondary infertility owing to gonadal damage from myeloablative conditioning. However, the use of nontotal body irradiation and nonablative protocols means that more patients may preserve their fertility. Hence, the number of pregnancies involving HSCT recipients is increasing.[10] Furthermore, in the case of aplastic anemia, the post-HSCT outlook for fertility is more favorable than for other conditions that require more aggressive regimens. Indeed, a 1998 study found that at least half of the female patients transplanted for aplastic anemia preserved or regained the ability to become pregnant.[11] For hemolytic disease of the fetus and newborn (HDFN), previous pre-HSCT pregnancy history cannot predict the risk. This is because both the RBC and immune system of the pregnant HSCT recipient have switched to those of donor origin. Fortunately, the incidence of post-HSCT anti-D is low, and clinical hemolysis is rare.[1] Au and Leung[10] state that it is uncertain whether the anti-D titer may increase as a result of anamnestic exposure during post-HSCT; therefore, frequent monitoring may be prudent.

When the patient in our study became pregnant again in October 2009, early fetal genotyping from maternal plasma was desired but was limited by the absence of a reliable internal control, given that the bone marrow donor was male. Fetal D typing from maternal plasma has been performed for several years[12,13] and is now available in the United States[14] using real-time quantitative PCR. Fetal DNA represents between 3 and 6 percent of total free DNA in maternal plasma,[15] and separating fetal DNA from maternal DNA is problematic.[16] *SRY* is a Y chromosome–specific sequence that can be amplified to confirm that fetal DNA has been successfully detected in the case of a male fetus. If the *SRY* gene is used as the control,

cell-free fetal D typing is inconclusive in 14 percent of cases, namely those in which there is a D– female fetus detected. In these cases, the absence of the *RHD* gene in the fetus cannot be verified because there is no internal control to confirm the presence of free fetal DNA in the specimen. Thus, amplification of *SRY* is only effective as a control when the fetus is male.[14] But in this patient, maternal plasma would likely contain traces of male DNA from her bone marrow donor, so *SRY* could not be used as a control. Indeed, Minon et al.[17] reported an unusual false-positive fetal *RHD* result using DNA derived from maternal plasma from a solid-organ recipient. Tests on DNA isolated from the plasma of a D– pregnant woman predicted a D+ male fetus, whereas DNA isolated from amniocytes gave a D– result. The woman, who had received a kidney transplant from a D+ male, delivered a D– girl.

In the current case, cell-free fetal D testing was not performed because of the absence of a reliable internal control, given that the bone marrow donor was male. Instead, fetal *RH* genotyping was performed on DNA isolated from amniocytes. A commercial system in development in the United States, which involves mass spectrometry, will include other genes to be used as controls.[18–20] Tests using other fetal identifiers are also in development outside the United States.[21]

Fetal RBC phenotype may appear to exclude maternal contribution in scenarios such as donor egg or maternal host of HSCT (Table 3). Finally, this case raised the possibility that maternal anti-D might impact early gestational outcomes, although no such relationship exists in the literature. In this patient, two pregnancies with D– fetuses progressed without complication, whereas the pregnancy with a D+ fetus was nonviable.

Table 3. Scenarios in which fetal red blood cell predicted phenotype appears to exclude maternal contribution

Scenario	Explanation
Mother group O, father group A, offspring group B	Donor egg
	Maternal stem cell transplant
Mother group O, father group AB, offspring group AB	Donor egg
	Maternal stem cell transplant
	Cis AB

Conclusions

In this case, maternal anti-D alloimmunization was detected 6 years after BMT, at the time of an early nonviable pregnancy. The 8-week pregnancy may represent a secondary alloimmunization event after a primary event of graft immune cells responding to residual host RBCs, but the timing is unusual. Fetal D typing from maternal plasma is now available in the United States using real-time quantitative PCR. Genes other than *SRY* must be used as fetal identifiers to provide a reliable internal control for female D– fetuses. In a mother who has had a transplant, DNA from the bone marrow donor can be amplified. This is particularly problematic when the mother's type has changed from D+ to D–.

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REPORT

Should blood donors be routinely screened for irregular antibodies?

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Alloantibody reactivity is approximately 0.3 percent in blood donors worldwide. The present study established total alloantibody and clinically significant alloantibody (CSAA) frequencies in all Colombian Red Cross National Blood Bank donors (almost all donors were Colombian). The probability of these alloantibodies reacting with a specific antigen in the general population was also determined, focusing on male CSAA data because routine practice in this blood bank is to discard female plasma components to avoid transfusion-related acute lung syndrome. Total blood donor population between 2007 and 2009 was 60,309 (55.4% male and 44.6% female). Cells I and II were used for alloantibody screening following the Autovue protocol. Positive samples were identified by red blood cell (RBC) panels (Panel A, Panel B, and Panel C, Ortho Clinical Diagnostics, Raritan, NJ). Alloantibody and CSAA frequency were established for both sexes. The database for RBC antigens estimated for the Colombian population was used for calculating the probability of antigen-antibody reaction from donors in this blood bank. Total alloantibodies (438) and CSAA frequency (138) were significantly higher in women than men ($p < 0.01$). Seventy-four percent of CSAA found in women came from the Rh blood group system. Calculated probability of generating antigen-antibody reaction using plasma only from male donors was estimated as 20.55 episodes for every 100,000 donations, and the probable number of events per year was 1.48. Meanwhile, considering all blood components from male and female origin, the calculated probability of antigen-antibody reaction was 123.54 episodes for every 100,000 donations and 28.67 probable events per year. The data presented here do not represent strong support for the routine screening of alloantibodies in blood donors. ***Immunohematology* 2012;28:60–6.**

Key Words: blood donor, alloantibody screening, alloantibody identification, blood bank, blood recipient

Blood component transfusion can produce a series of immunologic effects, the most serious ones being alloimmunization, allergic reactions, febrile reactions, and immunosuppression.[1–3] Red blood cell (RBC) antigen immunogenicity is the likelihood of an antibody being generated in a blood recipient. It depends on exposure route, the recipient's medical condition (inflammation, immunosuppression),[4] and history of previous alloimmunization (which promotes new alloimmunization).[5,6] Once exposed to an antigen, the immune system can develop antibodies, called *regular* when they are generated against the antigens of the ABO blood group system and *irregular* or *alloantibodies* (AAs) when they are generated against other RBC blood group systems.[7]

AA screening is a test performed by blood banks and transfusion services for reducing minor incompatibility.[7] AA seroprevalence in blood donors around the world has been estimated as being 0.3 percent (0.2%–0.8%).[7] Some reports from Latin America have shown similar values: 0.19 percent[8] in Costa Rica and 0.34 percent in Brazil.[9] Colombian regulations[10,11] make this test mandatory for all units of blood collected, without taking prevalence in national blood donors into account.

A previous study on a Colombian population[12] found 0.39 percent RBC AA prevalence, almost half of which were considered clinically significant alloantibodies (CSAAs) because they were related to hemolytic transfusion reactions (HTR) or shortened RBC survival.[12,13] The CSAAs found, in order of frequency, were anti-D, -E, -K, -M, -S, and -k. On the other hand, a 1993 study of 30,259 men involved in paternity suits from all regions of Colombia[14] documented RBC antigen prevalence for the eight most frequent blood group systems; the authors concluded that RBCs presented a predominantly Caucasian phenotype. Likewise, the Colombian National Institute of Health issued a consolidated report in 1999 regarding 338,063 blood units that were classified for ABO and D blood group antigens.[15] Both series are summarized in Table 1.

The present paper has two components: the first is a description of RBC AA frequency in Colombian Red Cross National Blood Bank (CRCNBB) blood donors in Bogota (2007–2009). A selection of CSAAs was also taken and organized by sex. The second component is the calculation of the likelihood of generating HTR or reduced RBC survival, using data from Table 1.

Study Design and Methods

Study Population

Total donations received between 2007 and 2009 were 60,309 (55.4% male and 44.6% female).

Table 1. Relevant red blood cell antigen frequency found in Colombian males and Colombian blood donors*

System	Phenotype	Frequency in males (%)	Frequency in blood donors (male and female) (%)
ABO (ISBT 001)	A	26.86	26.00
	B	9.24	7.30
	AB	1.83	1.40
	O	60.65	56.20
Rh (ISBT 004)	D	94.18	91.16
	CCdEE	0.00	
	CCdEe	0.00	
	CCdee	0.01	
	CcdEE	0.00	
	CcdEe	0.01	
	Ccdee	0.38	
	ccdEE	0.00	
	ccdEe	0.17	
	ccdee	5.19	
Kell (ISBT 006)	K+k–	0.06	
	K+k+	3.63	
	K–k+	96.29	
Duffy (ISBT 008)	Fy(a+b–)	30.86	
	Fy(a+b+)	39.04	
	Fy(a–b+)	26.61	
	Fy(a–b–)	3.46	
Kidd (ISBT 009)	Jk(a+b–)	26.52	
	Jk(a+b+)	42.66	
	Jk(a–b+)	29.24	
	Jk(a–b–)	1.56	

*Data obtained from Sandoval C et al.[14] and Beltran M et al.[15]

Screening Assay and RBC AA Identification

A retrospective search was conducted for whole-blood donations made between January 2007 and July 2009 to establish RBC AA prevalence in our donor population. AA screening was done using the Autovue system with the column agglutination method (polyclonal anti-IgG -C3d) and a 2-cell screen (Surgiscreen, Ortho Clinical Diagnostics, Raritan, NJ). AAs were identified in units having positive AA screening, using panels (Panel A, Panel B, and Panel C, Ortho Clinical Diagnostics). The following AAs were identified using those panels: anti-Le^a, -Le^b, -D, -C, -c, -E, -e, -Fy^a, -Fy^b, -K, -k, -Jk^a, and -Jk^b.

Nomenclature

We defined the following terms:

- Alloantibodies against low-prevalence antigens: those with a positive screening test but having a negative identification test result with panel A.
- Cold antibodies (CAs): antibodies reactive below 37°C.
- Nonspecific IgG antibodies (NIAs): those units having a positive screening test and complete agglutination in all cells from panel A.
- Non–clinically significant alloantibodies (NCSAAs): those not related to disease, HTR, or reduced RBC survival.
- Minor incompatibility: hemolysis after destroying a percentage of the patient's RBCs by antibodies present in a plasma unit.

Calculating Minor Incompatibility Likelihood for a Single Transfusion

The likelihood of generating an antibody-antigen reaction in a single plasma transfusion from male donors was established using RBC antigen frequencies estimated previously for Colombian people[14] and the frequency of AAs found in our blood donors. This calculation was performed using only male donors because our standard practice is to discard female fresh-frozen plasma units without taking into account the donor's history of pregnancies to reduce the incidence of transfusion-related acute lung injury (TRALI).[16,17] International reports suggest a 10 to 20 percent prevalence of antibodies to leukocyte antigens in female blood donors with a history of pregnancy and 1 to 5 percent in male donors as the main risk factor for TRALI (odds ratio, 15),[16] but it is not a regular practice in CRCNBB to perform leukocyte antibody screening. Moreover, as the demand for plasma components can be met with only male donors, this is a standard CRCNBB policy. Antigen-antibody reaction probability was calculated as being the likelihood of finding an AA in plasma donors and the corresponding RBC antigen in the recipient. Calculations were made as follows: the found antibody frequency was divided by the total number of donors, and the result was then multiplied by 100,000 transfusions to yield the antibody probability. Antigen-antibody reaction probability was calculated by multiplying antibody probability by 100,000 transfusions to yield the antigen likelihood in the population. The antigen probabilities we used in this calculation were those in Table 1.

Statistics

The χ^2 test was used to determine the significance of sex-related AA frequency with a significance level of 95 percent. AAs and RBC antigens are expressed as percentages of the total population.

Results

During the period being studied (2007–2009) there were 60,309 whole-blood donations, and 438 AAs were found (0.73%), 66.7 percent of them in female blood donors and 33.3 percent in male donors. The CSAAs were 140 (31.96%) and NCSAAs were 300 (68.5%) of those identified. Our results showed that the three AAs most frequently found in blood donors corresponded to NCSAAs; the most common AA found in blood donors (anti-Le^a) had similar sex distribution, as did anti-S (not significant). The other AAs had a clear tendency to be more prevalent in women, most of them being classified as CSAAs, such as anti-D or -C (Table 2).

Table 2. The frequency of alloantibodies in the donor population and their relationship to sex

Alloantibody	Number	% Total	Females	% F*	Males	% M*	p value
Anti-Le^a	137	31.28	68	49.64	69	50.36	
AALFAs	73	16.67	48	65.75	25	34.25	<0.1
Cold antibodies	61	13.93	34	55.74	27	44.26	
Nonspecific IgG	13	2.97	11	84.62	2	15.38	<0.1
Anti-Le^b	14	3.20	8	57.14	6	42.86	
Anti-D†	53	12.10	50	94.34	3	5.66	<0.05
Anti-E†	25	5.71	20	80.00	5	20.00	<0.05
Anti-K†	23	5.25	13	56.52	10	43.48	
Anti-M†	13	2.97	6	46.15	7	53.85	
Others†	26	6.0	22	100.00	4		<0.001
Total	438	100.00	280	63.93	158	36.07	<0.05

*F = female; M = male.

†Indicates alloantibodies considered clinically significant alloantibodies. The probability value represents the difference by sex.

AALFA = alloantibodies against low-prevalence antigens.

There were 12 cases in which two AAs were found in the same donor during the period being studied. Six of them were male donors having the following combinations: one anti-Le^a/CA; three anti-Le^a,-Le^b; one anti-Le^a/NIA, and one anti-Le^a, -Jk^a. Six female donors had the following combinations: three anti-D,-C, one anti-c,-E, and two anti-D,-E. It is worth noting that all AAs found in these women belonged to the Rh blood group system (ISBT 004).

Probability of Generating Antigen-Antibody Reaction in a Recipient

Tables 3 to 5 present the calculated probability of an antigen-antibody reaction, using fresh-frozen plasma (male donors only), platelets, and cryoprecipitate, respectively.

Discussion

Screening for AAs in every blood donor is mandatory for all Colombian blood banks but not in countries such as the Netherlands, United Kingdom, or United States.[7,18,19] In these countries there is a policy of specific screening in certain populations considered at risk, such as multiply-transfused patients or intrauterine transfusions. Although not required, most if not all blood centers in the United States are routinely screening blood donations for serum or plasma AAs. This may be because U.S. donor centers try to identify those donors having AAs of high enough titer for use in typing antisera in serologic investigations. The supposed aim of mandatory screening in Colombia, according to the regulations,[10,11] is to reduce the risk of AAs that may potentially cause early and delayed HTR in blood recipients, as well as shortened RBC survival. However, the evidence for serious adverse outcomes resulting from the transfusion of AAs other than ABO and Rh blood group antibodies contained in donor units to individuals that would, by chance, have the corresponding antigen(s) is lacking in the literature. Additionally, one has to consider that after transfusion these AAs would be diluted, making it unlikely that they would cause the recipient a detectable problem (Tables 3–6). Also, it is important to take into account that most if not all blood is now transfused as RBCs, having very little plasma.

The results in our donor population showed a 0.70 percent AA prevalence, which is higher than other reports.[7,8,9,13] Of the AAs reported, a third (0.2%) were considered CSAAs (anti-D, -C, -c, -E, -M, -S, -Fy^a, -Fy^b, -Jk^a, and -Jk^b).[12,13] As expected, in female donors the AA frequency was three times as high as in males,[7,20] mainly reflecting the presence of anti-D. Previous results have shown that RBC AA origin is related to history of previous transfusions and pregnancy.[13,20] Likewise, 80 percent of CSAAs were found in female donors; 65% of them belonged to the Rh blood group.[21,22]

Similarly, anti-K was the third CSAA found in our blood donor population; there was no statistical significance between sexes in frequency. This AA is the primary cause of hemolytic disease of the newborn (HDN) and HTR in other countries.[23–25] Nevertheless, the estimated frequency of dominant homozygous and heterozygous phenotypes in Colombia is close to 3.7 percent.[14] That is, we found a frequency of 3.8 cases per 10,000 patients for every unit transfused, which together with the previous considerations about the antigen-antibody reaction reinforces the low probability of experiencing these events. On the other hand, anti-M is rarely associated with HDN or HTR,[26,27] even when its expression

Table 3. Estimated probability of antigen-antibody reaction based on 60,309 donations screened for alloantibodies, using plasma only from only male donors for calculations

AA	Number of tested units	Ab frequency × 100,000 units	Ag probability per 100,000 Colombian inhabitants	AAR probability × 100,000 units	Probable number of events per year	Units required for generating 1 AAR	Cases per year
Anti-D	7152	4.97	0.9421	4.69	0.34	21,338	1 each 2.98 years
Anti-K	7152	16.58	0.0369	0.61	0.04	163,439	1 each 22.85 years
Anti-E	7152	8.29	0.4132	3.43	0.25	29,191	1 each 4.08 years
Anti-M	7152	11.61	0.8653	10.04	0.72	9957	1 each 1.39 years
Anti-S	7152	3.32	0.5372	1.78	0.13	56,133	1 each 7.85 years
Total				20.55	1.48		

AA = alloantibody; Ab = antibody; Ag = antigen; AAR = antigen-antibody reaction.

Table 4. Estimated probability of antigen-antibody reaction based on 60,309 donations screened for alloantibodies, using platelets from both male and female donors

AA	Number of tested units	Ab frequency × 100,000 units	Ag probability per 100,000 Colombian inhabitants	AAR probability × 100,000 units	Probable number of events per year	Units required for generating 1 AAR	Cases per year
Anti-D	5228	87.88	0.9421	82.79	4.33	1208	1 each 0.23 years
Anti-K	5228	38.14	0.0369	1.41	0.07	71,060	1 each 13.59 years
Anti-E	5228	41.45	0.4132	17.13	0.9	5838	1 each 1.11 years
Anti-M	5228	21.56	0.8653	18.65	0.98	5361	1 each 1.02 years
Anti-S	5228	6.63	0.5372	3.56	0.19	28,066	1 each 5.37 years
Total				123.54	6.47		

AA = alloantibody; Ab = antibody; Ag = antigen; AAR = antigen-antibody reaction.

Table 5. Estimated probability of antigen-antibody reaction based on 60,309 donations screened for alloantibodies, using cryoprecipitate from both male and female donors

AA	Number of tested units	Ab frequency × 100,000 units	Ag probability per 100,000 Colombian inhabitants	AAR probability × 100,000 units	Probable number of events per year	Units required for generating 1 AAR	Cases per year
Anti-D	1317	87.88	0.9421	82.79	1.09	1208	1 each 0.92 years
Anti-K	1317	38.14	0.0369	1.41	0.02	71,060	1 each 59.95 years
Anti-E	1317	41.45	0.4132	17.13	0.23	5838	1 each 4.44 years
Anti-M	1317	21.56	0.8653	18.65	0.25	5361	1 each 4.07 years
Anti-S	1317	6.63	0.5372	3.56	0.05	28,066	1 each 21.31 years
Total				123.54	1.64		

AA = alloantibody; Ab = antibody; Ag = antigen; AAR = antigen-antibody reaction.

Table 6. Estimated probability of antigen-antibody reaction based on 60,309 donations screened for alloantibodies, using red blood cell units from both male and female donors

AA	Number of tested units	Ab frequency × 100,000 units	Ag probability per 100,000 Colombian inhabitants	AAR probability × 100,000 units	Probable number of events per year	Units required for generating 1 AAR	Cases per year
Anti-D	23,204	87.88	0.9421	82.79	19.21	1208	1 each 0.05 years
Anti-K	23,204	38.14	0.0369	1.41	0.33	71,060	1 each 3.06 years
Anti-E	23,204	41.45	0.4132	17.13	3.97	5838	1 each 0.25 years
Anti-M	23,204	21.56	0.8653	18.65	4.33	5361	1 each 0.23 years
Anti-S	23,204	6.63	0.5372	3.56	0.83	28,066	1 each 1.21 years
Total				123.54	28.67		

AA = alloantibody; Ab = antibody; Ag = antigen; AAR = antigen-antibody reaction.

begins in erythroid precursors.[26] The global frequency of M is 75 percent; hence, 25 percent of the population lacks M; such individuals are able to generate anti-M when they are exposed to the antigen. However, the frequency of anti-M was only 0.02 percent in this population, reflecting perhaps a low immunogenic power of this antigen.

Regular Antibodies Compared With Irregular Antibodies

Old transfusion practices used whole blood from universal group O donors, and the regular antibodies in the plasma fraction of whole blood usually were not associated with significant antigen-antibody reactions. It would thus be inconsistent to think that an irregular antibody having a lower serum concentration than a regular antibody can produce transfusion reactions in a recipient, given the percentage of plasma present in a unit of RBCs (which is less than 5%[28] and is diluted in preservative solution), which is the most transfused component. Otherwise, transfusion of group O blood to group A or B patients would be considered unacceptable.[29] Although these antibodies are transfused, they can adsorb to antigens located in different organs (e.g., endothelium, liver) because their expression is not limited to RBCs, reducing the chances of HTR or exposure to circulating RBCs.

To test this hypothesis, a study was conducted that determined regular antibodies' agglutination capacity in supernatant from packed RBC units obtained from our blood bank. We were unable to demonstrate any agglutination despite including an indirect antiglobulin test for regular antibodies in the RBC supernatant whose ABO groups were known (data not shown). The data shown in Tables 3 to 5 reflecting calculations of the probability of antigen-antibody reaction, along with the traditional practice of transfusion, showed a low probability as a result of transfusion of plasma components, as has been shown in other studies.[30–32] Then, even if transfused (fresh-frozen plasma, RBC units, platelets, or cryoprecipitates), these antibodies will be unlikely to cause recipient hemolysis because they will be diluted in the recipient's circulation.[33–36]

As shown in Tables 3 through 6, the likely number of antigen-antibody reactions per year was significantly higher in the calculations involving the components obtained from women than those from men. If the analysis is performed separately for men and women for the transfusion of RBC units (Table 6), the probable number of events per year for anti-D, -K, -E, -M, and -S is 23.99 cases per year (women) versus 4.68 cases per year (men). However, as mentioned previously, because the content of plasma in RBC units is very low and the dilution in the intravascular compartment is high, the overall effect may be negligible.

Special Considerations

With respect to the resistance to irregular antibody screening in platelet donors, it is necessary to emphasize that the problem of rejection and platelet alloimmunization is attributable mostly to the presence of anti-HLA antibodies, antineutrophil antibodies, and antiplatelet glycoprotein antibodies,[37] which are found in 30 percent of women not transfused but with a history of pregnancy,[38,39] and 66 percent of women with a history of transfusion and pregnancy.[40] Not all of them correspond to RBC antibodies, and therefore they are not routinely screened for in blood banks. Therefore, performing a routine RBC antibody screen is of little value. Additionally, although transfused plasma could have RBC antibodies, they are distributed throughout the available intravascular fluid and eventually join a fraction of these RBC receptors. However, because there is a much larger number of RBCs than RBC antibodies, the rate of antibody-antigen reaction is limited by the antibody provided, which has been processed at temperatures between 18° and 23°C, which reduces its activity.[41,42] Thus, the proposal to remove the screening of irregular antibodies will neither increase the number of cases of HTR nor reduce RBC survival in this population.

In emergency transfusion situations and massive transfusions such as patients with hemorrhagic shock who need immediate blood transfusion, there is no time for the blood bank to perform antibody screening tests; under these circumstances the risk of transfusing group O uncrossmatched RBCs is very low and is lower than the risk of the patient's death if a blood transfusion is delayed.[43]

These data, taken together with that presented by other authors,[7,12,13,18,19,44] demonstrated the low frequency of such AAs in the blood donor population and called into question this measure's usefulness regarding its cost as a routine procedure.

Conclusion

The data presented above do not represent strong support for the routine screening for AAs in blood donors. It is thus proposed that the Colombian blood donor population should not be subjected to routine AA screening; instead, we suggest that all Colombian blood banks adopt the Colombian Red Cross policy of discarding female plasma, thus withdrawing from the market four of five units that could theoretically contribute

to antigen-antibody reaction. AA screening in donors is only useful if the recipient's phenotype has been ascertained. This should only be done for those who need long-term blood transfusions and who clearly have high CSAA frequency.[44] AA screening is not clinically significant in patients who are only transfused once. Colombia is a country having limited resources, and public health assets should be adjusted to the population. Evidence-based strategies for redistributing existing resources should therefore be implemented. These would avoid annual resources being inappropriately used on screening that does not prevent HTR reactions or shortened RBC survival in 99.96 percent of cases.

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REPORT

Cryopreservation of red blood cell units with a modified method of glycerolization and deglycerolization with the ACP 215 device complies with American and European requirements

J. List, M. Horvath, G. Weigel, and G.C. Leitner

Current red blood cell (RBC) glycerolization with the ACP 215 device is followed by volume reduction of the glycerolized RBCs before freezing. We investigated a modified method of glycerolization and deglycerolization which eliminates the final centrifugation step that reduces glycerolized RBC supernatant. A total of 37 RBC units collected from healthy volunteers were analyzed. After removal of the supernatant, RBCs were glycerolized using the high glycerol method and stored at –80°C. After deglycerolization, RBCs were preserved with either SAG-M or AS3 and stored for at least 10 or 14 days, respectively. Quality of stored RBCs was assessed by measuring osmolarity, blood cell counts, free hemoglobin, adenosine triphosphate (ATP), hemolysis, and glucose. The overall RBC mass recovery after deglycerolization was 86 ± 7.6 percent, and the osmolarity was 336 ± 23 mOsml/kg H_2O. The hemolysis for stored components at the end of their shelf life was 0.21 ± 0.08 percent for AS3-preserved RBCs and 0.25 ± 0.08 percent for RBCs preserved with SAG-M. On expiration, 32 percent of initial ATP values were measured in AS3-preserved RBCs vs. 62 percent in SAG-M–preserved RBCs. This modified method of glycerolization and deglycerolization meets the quality requirements of the European Council and the AABB standards. The prolonged storage of thawed RBCs enables optimized transfusion management for patients with rare blood groups. ***Immunohematology* 2012;28:67–73.**

Key Words: cryopreservation, intracellular purines, hemolysis, high glycerol method, red blood cell units, ACP 215

Cryopreservation of red blood cells (RBCs) still remains an important method for maintaining an inventory of rare RBC units and managing special transfusion circumstances. The influence of RBC distress on the quality of RBCs during the collection process and the extent of storage lesions during liquid storage (4°C ± 2°C) is well known.[1,2] It has been shown previously that the length of centrifugation and especially the gravitational force play an important role in the impairment of RBCs.[1] High gravitational forces or a long exposure time results in accelerated intracellular ATP degradation and consumption of glucose during storage as a result of increased membrane repair processes. Subzero storage of RBC units reduces erythrocyte metabolism and allows a frozen storage period of at least 10 years.[3] However, RBC damage during the collection procedure may affect the quality of deglycerolized RBC units during postthaw storage. A variety of freeze and thaw protocols have evolved so far. The methods differ with regard to cryoprotectants, storage temperature, and thawing and washing procedures.[4] In Europe, predominantly the high-glycerol method (HGM) is used because of the ease of storage in a –80°C freezer without the need for highly technical equipment. The low-glycerol method (LGM) requires a storage temperature of –190°C, which can only be achieved in liquid nitrogen. Currently, RBC units that undergo the HGM are centrifuged at least three times: during the collection process, before glycerolization to achieve a hematocrit (Hct) of at least 75 percent, and before freezing to remove the supernatant glycerol.[5] In this prospective study we investigated a modified freeze and thaw protocol by eliminating the last centrifugation step in conventionally collected and apheresis-derived RBC units. The quality of RBCs after thawing was assessed by analyzing osmolarity, recovery of RBC mass and hemoglobin content, intracellular ATP, hemolysis, and glucose at defined time points.

Donors and Methods

Study Design

The aim of this study was to evaluate a modified method of glycerolization and deglycerolization of RBC units with the automated device ACP 215 (Haemonetics Corp, Braintree, MA), which eliminates the highly manual step of supernatant reduction before freezing. This change in manipulation is compensated by a modification in the deglycerolization process as shown in Table 1. The dilution volume and the

dilution rate are the crucial points. They were calculated by the scientific board of Haemonetics. The objective was to reduce the osmolality of the glycerolized and frozen components to values of conventionally cryopreserved units before deglycerolization and to avoid osmotic shock of RBCs during the deglycerolization process.

Table 1. Comparison of variables using the current and postthaw modified ACP 215 washing procedures

	Current ACP 215 configuration	Study ACP 215 configuration*
Hypertonic solution volume (mL)	50	50
Hypertonic solution flow rate (mL/min)	150	150
Saline dilution volume no. 1 (mL)	340	600
Saline dilution flow rate (mL/min)	60	110

*Modified washing volumes

Each manipulation step bears the risk of bacterial contamination or artificial damage of the component, which may be of particular interest in certain hemoglobin abnormalities.[6,7] A simplified method reduces the risk of component impairment and is also time sparing, which is certainly an advantage in daily routine. Second, glycerolized RBCs have a large volume that is difficult to handle in routine blood banks as the centrifuge buckets are normally not designed for such high volumes.

To evaluate the applicability of this simplified method to different production methods, conventionally collected as well as apheresis-derived RBCs were investigated. A major requirement for managing stockpiling of cryopreserved RBC units is the shelf life after deglycerolization. We included two preservation solutions (SAG-M, AS3) in our postthaw investigation to compare their suitability. In consideration of previous studies, which showed hemolysis above the threshold of 0.8 percent in SAG-M–preserved deglycerolized units within 1 week, the postthaw shelf life was set at 10 days for SAG-M units vs. 14 days for AS3 units.[8,9]

Finally, we assessed the compliance of these modified RBC units with requirements for cryopreserved RBC concentrates, as defined by the Council of Europe (CE) and the AABB standards.[3,10] In brief, hemolysis should stay less than 0.8 percent at the end of shelf life, and the minimal required content of hemoglobin (Hb) is 36 g/unit.[10]

Donors

RBCs were collected from routine donors of the Austrian Red Cross Blood Service by standard whole blood (WB) collection (n = 17), and from volunteer donors of the Department of Blood Group Serology and Transfusion Medicine (Medical University of Vienna) who qualified for multicomponent collection (MCC). RBC double units were collected by two automated collection devices (MCS+, Haemonetics, n = 6) and (Cymbal, Haemonetics, n = 4) according to the technical manual. The study was approved by the local ethics committee, and the donors gave written informed consent.

Red Blood Cell Units

All RBCs (WB collections and apheresis-derived) were leukocyte-depleted, suspended in SAG-M, and stored for 6 days at 4°C ± 2°C before freezing. A prerequisite for further manipulation was the compliance of the RBC units with the requirements of the EC concerning Hb content/unit (40 g/unit) and leukocyte contamination ($\leq 1 \times 10^6$/unit).[11] RBC units were connected to 500-mL transfer bags by a sterile connecting device (SCD, Haemonetics) and centrifuged for 15 minutes at 2000 rpm (2858*g*) in a floor model, cooling centrifuge (Roto Silenta RP, Hettich, Vienna, Austria). Using a plasma extractor, supernatant was extracted to the transfer bag to achieve a Hct of at least 75 percent.[5] Finally, after drawing blood samples to determine the actual Hct, the remaining packed RBC units were connected to the 1800-mL freezing bag (EVA bag reference GSR 8000AU Macopharma, Werken, Belgium) using a sterile connecting device. RBC units were defined as small (RBC mass ≤130 g), typical (RBC mass >130 g and ≤180 g), or large (RBC mass >180 g).

Freezing Procedure

To achieve the final concentration of 40 volume-percent glycerol, the amount of added 57 gram-percent glycerol solution was calculated depending on the actual hematocrit of the units. The RBC units were glycerolized with the ACP 215 using the HGM (Haemonetics) according to protocol. RBC units and the glycerol solution were at room temperature. Calculation of the amount of added glycerol was done automatically by the device and was completed within 10 minutes. Finally, glycerolized RBC units were frozen by placing them into a −80°C freezer (Revco Freezer, Thermo Scientific, Langenselbold, Germany) and stored for at least 10 to 14 days. Glycerolized RBCs had a mean volume of 578 mL (standard deviation, 89 mL; range, 354 to 736 mL).

Thawing Procedure

After 10 days (SAG-M) and 14 days (AS3), frozen RBCs were thawed with a a 37°C pulsed-air chamber with infrared temperature control of RBC bag content (Sahara Thawer, Transmed Medizintechnik Gmbh, Bad Wünnenberg, Germany). The thawing procedure was completed when a

temperature of 34°C was reached. Deglycerolization was performed with the ACP 215 according to the manufacturer's protocol, using either the regular bowl set (LN235) for small and typical units (130–180 g of RBC mass) or the large bowl set (LN236) for large units with more than 180 g of RBC mass. The deglycerolization process included an initial deglycerolization step with hypertonic saline solution (12%) to facilitate the intracellular glycerol removal and five consecutive washing steps with a saline rinse solution (0.9% saline, 0.2% glucose-buffered solution bags, BIO-Wash, Haemonetics) to achieve a remaining glycerol content of less than 1 gram-percent. For this purpose we compared the color of the final wash fluid with the blocks of a color comparator (Hemolysis Color chart, Haemonetics) and measured the osmolarity of the component, as recommended by the AABB.[3,12] The entire deglycerolization process lasted an average of 69 minutes (standard deviation ± 4.5 min). The main difference between the standard and modified deglycerolization methods is shown in Table 1. Postdeglycerolization RBC units were resuspended either in SAG-M (Cymbal, n = 4; MCS, n = 6; WB, n = 8) or in AS3 (Cymbal, n = 4; MCS, n = 6; WB, n = 9) and stored at 4°C (± 2°C) for another 10 (SAG-M) or 14 (AS3) days.

Assessment of In Vitro Quality

Our aim was to assure that the RBC units treated with the new, modified method of glycerolization, freezing, and deglycerolization still met the required standards of the CE and the AABB.[3,10,12] Storage lesion was assessed by measuring volume (net weight), blood counts (RBC mass), Hct, Hb, glucose, pH, supernatant (free) hemoglobin (fHb), and intracellular ATP content. The samples were taken at defined time points: after collection, before glycerolization, after thawing, after deglycerolization, and during storage. Additionally, supernatant osmolarity was measured after deglycerolization. All samples were drawn under aseptic conditions on days 0 (day of deglycerolization), 3, 5, 7, and 10 (SAG-M), and on days 0, 5, 7, 10, and 14 (AS3) of storage.

Laboratory Analyses

The weight was measured by an electronic scale and calculated by the net weight of the component divided by weight density of the RBCs. The concentration of Hb in the RBC supernatant was analyzed (AU 5430, Olympus Diagnostika, County Clare, Ireland) by the photometric color method. The blood count was measured using an automated cell counter (Cell Dyn 3500CS, Abbott Diagnostics, South Pasadena, CA). Osmolarity of the RBC units was determined (OSMO AKRAY OM-6050, A. Menari Diagnostics, Florence, Italy). Glucose was measured (Olympus AU 5430, Olympus Diagnostika) with the enzymatic, hexokinase method. ATP was measured by high-performance liquid chromatography as described previously,[13] and pH was analyzed at 37°C (ABL 80, Radiometer Gmbh, Brønshøj, Denmark). All analyses were done according to the manufacturer's instructions. Hemolysis (%) was calculated as follows: $([fHb \times \{100-Hct\}]/Hb_{total})/1000$.

Statistical Analyses

Results were expressed as mean and standard deviation for descriptive purposes in the text. Because of the nonnormal distribution of data all comparisons were made by nonparametric statistics. Procedures were split into four subgroups by the nature of the blood collection method (WB or apheresis) and of the preservation solution used to resuspend deglycerolized RBCs (SAG-M or AS3). Asigned-rank test was done with Minitab (Wilcoxin, Minitab®15.1.0.0, Inc., State College, PA). Comparisons between the various collection methods within a group were done using the Kruskal-Wallis test. A probability value of less than 0.05 was considered significant.

Results

Red Blood Cell Collections

All collected RBC units (17 WB collections, 6 MCS+–derived double units [12 single RBC units], and 4 Cymbal-derived double units [8 single RBC units]) met the requirements of the CE and were included in the study.[11] No significant differences were found in component characteristics among the different collection methods. Values are detailed in Table 2. Thirty-three RBC units were defined as typical (130 g < RBC mass < 180 g), and four were defined as large (RBC mass > 180 g).

Red Blood Cells Deglycerolized

The volume of all deglycerolized RBC units was 305 ± 13 mL and ranged from 277 to 357 mL. The RBC mass in all units was greater than 140 mL, and Hb content/unit was greater than 36 g (Table 3). The overall Hb recovery in the 37 tested RBC units after deglycerolization was 75 ± 6.5 percent, and the overall RBC mass recovery was 86 ± 7.6 percent. Recovery of Hb and RBC mass was significantly lower in MCS+ and Cymbal than in WB-derived components. We measured a recovery of 72 percent Hb and 85 percent RBC mass for MCS+ and Cymbal components vs. 78 percent Hb and 90 percent RBC mass for WB-derived components ($p < 0.05$ for Hb), respectively. The overall osmolarity was 336 ± 23 mOsm/kg

H_2O after deglycerolization, which was well below the AABB requirements of 400 mOsm/kg,[8,12] and MCV returned from a mean of 134 femtoliters (fL; ± 4.3) during frozen storage to 99 fL (± 4.3), which represents 108 percent of initial values. The color comparator indicated a residual glycerol of less than 1 percent. Detailed values for both additive solutions are given in Table 4.

Red Blood Cells Stored in Either SAG-M or AS3

Hemolysis

The mean overall hemolysis for all stored components was 0.23 ± 0.08 percent at the end of shelf life, set by the study design. It increased during storage as expected, but remained less than the threshold of 0.8 percent in all units. Although it was slightly higher in SAG-M–preserved units, there was no significant difference between the preservation solutions (SAG-M, AS3) for Cymbal- and WB-derived RBC units. RBCs collected with the MCS+ showed significantly higher hemolysis when stored in SAG-M ($p < 0.05$). The significantly lowest hemolysis was measured in WB-derived RBCs irrespective of the preservation solution (Fig. 1).

Intracellular Adenosine Triphosphate Content and Glucose

The overall intracellular ATP was 48 ± 19 percent of initial values at the end of shelf life, which is considerably greater than the threshold of 10 percent residual ATP content.[14] During storage, ATP values decreased continuously to 32 percent (Day 14) of initial values for AS3-preserved units and to 62 percent (Day 10) for units stored in SAG-M, respectively ($p < 0.05$; Table 4, Fig. 2). On day 10, 40 percent of initial ATP values were measured in AS3-preserved units. Initial intracellular ATP content was significantly higher in WB collections than in apheresis-derived units (Table 2, Fig. 2; $p < 0.05$). The lowest intracellular ATP values and the highest purine degradation were measured in AS3-preserved, MCS+–derived RBCs ($p < 0.05$; Fig. 2A). The preservation solution had almost no impact on the course of ATP degradation in RBCs produced with Cymbal (Fig 2). Consumption of glucose was comparable irrespective of the collection method and preservation solution (Table 2).

Discussion

In this study we evaluated a modified RBC freeze and thaw method that eliminated the third centrifugation step. This procedure is not only simpler to perform, but it also may reduce RBC lesions by shortening exposure to centrifuge force. The impact of shear stress caused by centrifugation is

Table 2. Manipulation characteristics of RBC units collected with MCS+, with Cymbal, or conventionally (whole blood)*

Variable	MCS+ (n = 12)	Cymbal (n = 8)	WB (n = 17)
Volume (mL)	276 ± 20	256 ± 9	292 ± 14
Hct (%)	60 ± 2	63 ± 6	58 ± 5
Hb (g/dL)	19.8 ± 0.7	20.6 ± 2.0	19.3 ± 1.6
ATP (pg 10^6 erythrocytes)	118 ± 15	101 ± 17	146 ± 16†
Glucose (mg/dL)	457 ± 19	421 ± 18	488 ± 24
Hb/unit (g/unit)	55 ± 6	53 ± 5	56 ± 6
RBC mass (mL)	165 ± 17	161 ± 14	171 ± 19

*Values are expressed as mean ± standard deviation.
†$p < 0.05$.
ATP = adenosine triphosphate; Hb = hemoglobin; Hct = hematocrit; RBC mass = red blood cell volume; WB = whole blood.

Table 3. Component characteristics and recovery of deglycerolized RBC units collected with MCS+, with Cymbal, or conventionally (whole blood)*

Variable	MCS+ (n = 12)	Cymbal (n = 8)	WB (n = 17)
Recovered Hb content/unit (%)	73.5 ± 3.8	69.0 ± 7.7	78.0 ± 7.2
Hb (g/dL)	12.3 ± 0.6	12.5 ± 0.9	14.1 ± 1.4†
Recovered RBC mass (%)	85.5 ± 4.2	83.0 ± 8.4	90.0 ± 10.2†
Hct (%)	43.6 ± 3.08	45.2 ± 3.1	49.1 ± 5.06†
MCV (fL)	96.5 ± 2.4	102 ± 7.6	98 ± 3.1
Osmolarity (mOsm/kg H_2O)	336 ± 25	345 ± 23	332 ± 23

*Values are expressed as mean ± standard deviation.
†$p < 0.05$.
Hb = hemoglobin; Hct = hematocrit; MCV = mean cell volume; RBC = red blood cell; WB = whole blood.

Table 4. Osmolarity after deglycerolization on Day 0 and the course of hemolysis and adenosine triphosphate percent in SAG-M– and AS3-preserved deglycerolized red blood cells after deglycerolization and during storage until end of shelf life*

Day 0	SAG-M (n = 18) Osmolarity mOsm/kg H_2O 358 ± 12		AS3 (n = 19) Osmolarity mOsm/kg H_2O 316 ± 8	
	Hemolysis (%)	ATP (%)	Hemolysis (%)	ATP (%)
Day 0	0.14 ± 0.04	100 ± 12	0.14 ± 0.03	93 ± 18
Day 3	0.17 ± 0.06	86 ± 27	—	—
Day 5	0.17 ± 0.04	83 ± 15†	0.14 ± 0.03	73 ± 14†
Day 7	0.18 ± 0.05	72 ± 15†	0.16 ± 0.07	59 ± 14†
Day 10	0.25 ± 0.08	62 ± 12†	0.17 ± 0.05	40 ± 12†
Day 14	—	—	0.21 ± 0.08	32 ± 10

*Adenosine triphosphate (ATP) results are calculated in percent of the initial (preglycerolization) values; for comparison day 10 (expiration for SAG-M–preserved units) is highlighted.
†Statistical significance between SAG-M and AS3 with $p < 0.05$.

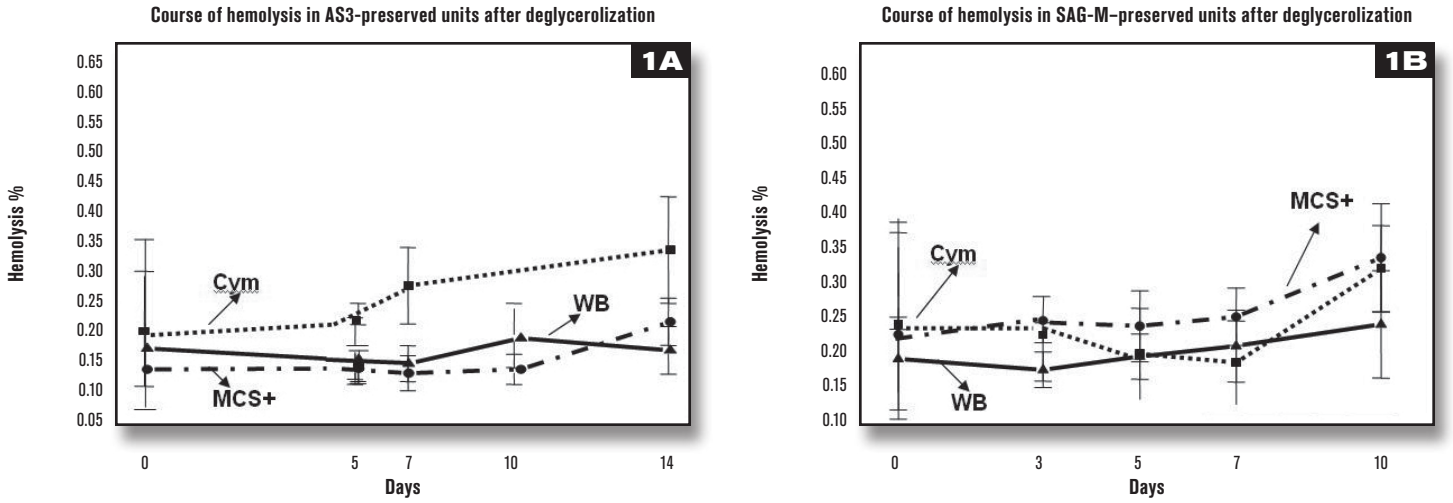

Fig. 1. Course of hemolysis during 14 days' postthaw storage for AS3-preserved (**A**) and SAG-M–preserved (**B**) components of deglycerolized red blood cell units collected with MCS+ (circles, dash-dotted line), with Cymbal (Cym; squares, dotted line), or conventionally (whole blood [WB]; triangles, solid line). Values are given in mean and standard deviation on Days 0 (immediately after deglycerolization), 3, 5, 7, and 10 and on expiration (Day 10 for SAG-M; Day 14 for AS3).

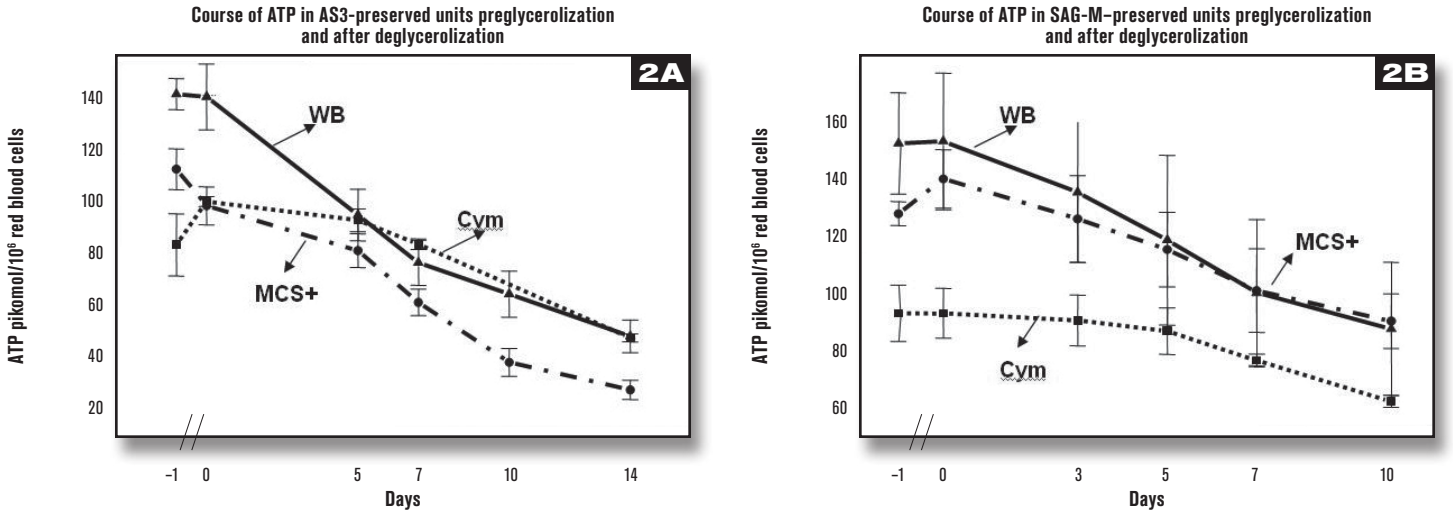

Fig. 2. Course of intracellular adenosine triphosphate (ATP) values during 14 days' postthaw storage for AS3-preserved (**A**) and SAG-M–preserved (**B**) components of deglycerolized red blood cell units collected with MCS+ (circles, dash-dotted line), with Cymbal (Cym; squares, dotted line), or conventionally (whole blood [WB]; triangles, solid line). Values are given in mean and standard deviation on Days minus (–) 1, which represents the initial value after collection and before glycerolization, on Day 0 (immediately after deglycerolization), on Days 3, 5, 7, and 10, and on expiration (Day 10 for SAG-M; Day 14 for AS3).

well known.[1,2,6,15] A major drawback of our study is the lack of a conventionally treated control group, but this study was conducted to provide evidence that the modified method is an alternative for all centers lacking the appropriate equipment, as we do. However, the *Technical Manual* of the AABB and the European guidelines provide clear requirements for frozen and thawed RBC units. Thus, we verified our results with the requirements of the international authorities and compared them with previous publications.

To remove glycerol almost completely after thawing, the washing procedure was modified (Table 1). The mean osmolarity of 336 mOsm/kg H_2O (threshold, 400 mOsm/kg H_2O), the mean MCV of 108 percent of initial values, and the color comparator (degree of hemolysis) indicated sufficient removal of glycerol.[8] In accordance with previous observations, we also found significantly higher osmolarity in SAG-M–preserved units than in AS3 RBCs ($p < 0.05$; Table 4), although no differences were seen among the three collection methods

(Table 3). This can be explained by the mannitol and sodium content in SAG-M causing higher osmolarity of the solution itself.[8] Interestingly, the highest recovery of RBC mass (90%) and total Hb (78%) after deglycerolization was measured in conventionally collected (WB) RBCs. Moreover, during further storage the lowest hemolysis was also measured in WB-derived RBCs, irrespective of the nature of preservation solution (Fig. 1). This can be best explained by the high ATP content found in WB RBCs before freezing (Table 2). ATP is known to power all energy-requiring processes, such as the Na+/K+ pump and membrane repair.[13,14,16] Thus, RBC membrane stability during collection and manipulation could obviously be best maintained in WB-derived RBCs, which may have led to less ATP degradation than in apheresis-derived components.

The composition of the additive solution also influences the extent of hemolysis and degradation of intracellular ATP during storage and treatment.[16–19] As described previously, SAG-M is superior to AS3 in maintaining ATP content and inferior to AS3 in avoiding hemolysis.[18] In our study, unlike in previous investigations, the choice of additive solution had a minor impact on hemolysis in our study (Table 4). Although it was higher in almost all SAG-M–preserved components, a significant difference between SAG-M and AS3 was only seen in MCS+–derived RBCs ($p < 0.05$; Fig. 1). Intracellular ATP was better maintained in SAG-M–preserved WB and MCS+–derived RBCs ($p < 0.05$) than in AS3 units throughout the entire storage period. ATP values in Cymbal RBCs were almost equal for both solutions (Fig. 2). Thus, we assume that the differences in hemolysis, ATP content, and ATP degradation of deglycerolized and stored RBCs cannot be ascribed to the additive solution alone but also to the initial production device.[20–22] As mentioned earlier, each manipulation of RBC units has a negative impact on RBC quality, resulting in increased hemolysis and enhanced loss of intracellular ATP as a result of increased membrane repair processes.[1,2,13] Despite all differences observed between the RBC manipulation devices and preservation solutions after deglycerolization, hemolysis remained less than 0.8 percent in all units. The highest hemolysis of 0.34 percent (± 0.09%; Fig. 1A) was measured in AS3-preserved Cymbal RBCs after 14 days of storage. In all other units hemolysis remained between 0.20 percent and 0.30 percent at the end of shelf life (10 days for SAG-M–preserved units and 14 days for AS3-preserved units; Fig. 1). ATP did not drop below the critical threshold of 10 percent of initial values in any of the RBC units.[14] These observations are in contrast to those in previous investigations that described a rise of hemolysis above the threshold of 0.8 percent in SAG-M–stored deglycerolized RBC units within 1 week.[8] Likewise, hemolysis in AS3-preserved units remained less than values described in the literature.[23–25] These findings may be attributable to the omitted centrifugation step.

Glycerol is known to lead to reduction of ATP and 2,3-diphosphoglycerate levels in glycerolized RBCs.[26] Although a repletion of ATP occurs after thawing and deglycerolization, a possible negative impact of ATP loss during subzero storage cannot be dismissed. However, we were able to show that the omission of the third centrifugation step had no negative effect on SAG-M– and AS3-preserved units, but rather may allow a prolonged storage of deglycerolized RBCs even in SAG-M–preserved components. This is important for the transfusion management of frozen and thawed components and minimizes the waste of RBCs.

In summary, all RBC units treated with the new method complied with the CE and AABB standards for cryopreserved blood components regarding hemolysis and ATP at expiration. The RBCs collected conventionally (WB) showed the best results irrespective of the additive solution. Thus, we conclude that this simplified method is a feasible, time-sparing, and user-friendly alternative to the established method. Additionally, based on the results of this evaluation, the modified procedure as well as the medical device equipment has been CE approved (Declaration of Conformity of Haemonetics Products 93/432EG). To what extent the length of subzero storage influences the quality of deglycerolized RBCs treated with this modified method has yet to be investigated in future studies.

Acknowledgments

The authors thank the staff for perfect technical assistance and appreciate the partial financial support from the Haemonetics Company (Braintree, MA).

Authors Jana List and Michaela Horvath contributed equally to the work of this paper.

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Letter to the Editors

I read "Challenging dogma: group A donors as 'universal plasma' donors in massive transfusion protocols" (*Immunohematology,* Volume 27, Number 2, 2011) with interest. Assumption *"(3) there has been no reported case of a minor reaction with group A plasma in either a group B or group AB patient..."* stated in the discussion section is of concern. "Probiotic-associated high-titer anti-B in a group A platelet donor as a cause of severe hemolytic transfusion reactions" *Transfusion* 2009;49:1845-1849 appears germane to this topic. This donor was male, and appears to have been eligible for plasma donation as well as platelet donation. Ingestion of bacteria in food, vaccine administration, and pregnancy are known to influence anti-A/B titers. Changes in environmental influences could alter the prevalence of high titer antibodies in blood components.

A review of 4 months of "trauma reds" (181 patients) at a level I trauma center in eastern North Carolina showed 20 percent of patients would be incompatible with group A plasma (46.5% O, 33.5% A, 16.5% B, 3.5% AB). The thought of infusing incompatible plasma with unknown anti-B titers to every fifth patient is not comforting (and not practiced).

The idea of testing and labeling "low titer" group A and group B plasma for emergency use is appealing. However, until a standard of care and standard titration process can be established, in the spirit of doing no harm, blood centers should seek group AB plasma donations with the same diligence as group O red cell donations.

Respectfully,

Michael Passwater, MT(ASCP)SBB,DLM

Annual Symposium Announcement

September 13, 2012: National Institutes of Health, Department of Transfusion Medicine, 31st Annual Symposium: Immunohematology and Blood Transfusion. The symposium is co-hosted by the American Red Cross and is free of charge, but advance registration is encouraged. **Contact** Karen Byrne, NIH/CC/DTM, Bldg.10/Rm 1C711, 10 Center Drive MSC 1184, Bethesda, MD 20892-1184, e-mail: KByrne@mail.cc.nih.gov or visit our Web site: www.cc.nih.gov/dtm >education

Specialist in Blood Bank (SBB) Program

The Department of Transfusion Medicine, National Institutes of Health, is accepting applications for its 1-year Specialist in Blood Bank Technology Program. Students are federal employees who work 32 hours/week. This program introduces students to all areas of transfusion medicine including reference serology, cell processing, HLA, and infectious disease testing. Students also design and conduct a research project. NIH is an Equal Opportunity Organization. Application deadline is December 31, 2012, for the July 2013 class. See www.cc.nih.gov/dtm > education for brochure and application. For further information **contact** Karen M. Byrne at (301) 451-8645 or KByrne@mail.cc.nih.gov

Manuscripts

The editorial staff of *Immunohematology* welcomes manuscripts pertaining to blood group serology and education for consideration for publication. We are especially interested in case reports, papers on platelet and white cell serology, scientific articles covering original investigations, and papers on new methods for use in the blood bank. **Deadlines** for receipt of manuscripts for consideration for the March, June, September, and December issues are the first weeks in November, February, May, and August, respectively. For instructions for scientific articles, case reports, and review articles, see Instructions for Authors in every issue of *Immunohematology* or on the Web at www.redcross.org/immunohematology. **Include fax and phone numbers and e-mail address with all manuscripts and correspondence.** E-mail all manuscripts to immuno@redcross.org

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Immunohematology will publish classified ads and announcements (SBB schools, meetings, symposia, etc.) **without charge.**

Deadlines for receipt of these items are as follows:

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1st week in January for the March issue	1st week in July for the September issue
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Contact the following programs for more information:

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

Program	Contact Name	Phone Contact	E-mail Contact	Web Site	Onsite or Online Program
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American Red Cross, Southern California Region	Catherine Hernandez	909-859-7496	Catherine.Hernandez@redcross.org	www.redcrossblood.org/socal/communityeducation	Onsite
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Revised May 2012

Immunohematology

Journal of Blood Group Serology and Education

Instructions for Authors

I. GENERAL INSTRUCTIONS

Before submitting a manuscript, consult current issues of *Immunohematology* for style. Double-space throughout the manuscript. Number the pages consecutively in the upper right-hand corner, 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.
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   a. In text, use superscript, Arabic numbers.
   b. Number references consecutively in the order they occur in the text.
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   b. Use short headings for each column needed and capitalize first letter of first word. Omit vertical lines.
   c. Place explanation in footnotes (sequence: *, †, ‡, §, ¶, **, ††).
8. Figures
   a. Figures can be submitted either by e-mail or as photographs (5 ×7″ glossy).
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   c. When plotting points on a figure, use the following symbols if possible: ○ ● △ ▲ □ ■.
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   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.

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A. All submitted manuscripts should be approximately 2000 to 2500 words with pertinent references. Submissions may include:

1. An immunohematologic case that illustrates a sound investigative approach with clinical correlation, reflecting appropriate collaboration to sharpen problem solving skills
2. Annotated conference proceedings

B. Preparation of manuscript

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   a. Capitalize first word of title.
   b. Initials and last name of each author (no degrees; all CAPs)
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      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)
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