## Contents

<table>
<thead>
<tr>
<th>Page</th>
<th>Section</th>
</tr>
</thead>
</table>
| 5    | Letter to the Readers  
Introduction to the 25th anniversary issues  
G.M. Meny |
| 6    | Invited Reviews: Milestones Series  
Milestones in *Immunohematology* 1984–2009  
D. Mallory |
| 9    | Original Report  
D+ platelet transfusions in D– patients: cause for concern?  
A.N. Bartley, J.B. Carpenter, and M.P. Berg |
| 13   | Case Report  
Autoantibody formation after alloimmunization inducing bystander immune hemolysis  
M. Mota, C. Bley, M.G. Aravechia, N. Hamerschlak, A. Sakashita, J.M. Kutner, and L. Castilho |
| 17   | Original Report  
Transfusion of rare cryopreserved red blood cell units stored at −80°C: the French experience  
T. Peyrard, B.N. Pham, P.Y. Le Pennec and P. Rouger |
| 22   | Original Report  
The polymorphism nt 76 in exon 2 of *SC* is more frequent in Whites than in Blacks  
A. Fuchisawa, C. Lomas-Francis, K. Hue-Roye, and M.E. Reid |
| 24   | Case Report  
Nonhemolytic passenger lymphocyte syndrome: donor-derived anti-M in an M+ recipient of a multiorgan transplant  
A.T. Makuria, A. Langberg, T. Fishbein, and S.G. Sandler |
| 28   | Original Report  
Development and validation of a fluorescent microsphere immunoassay for anti-IgA  
K.M. Rumilla, J.L. Winters, J.M. Peterman, E.A. Jedynak, and H.A. Homburger |
| 33   | In Memoriam  
Charles Salmon, MD (1925–2009) |
| 35   | Announcements |
| 37   | Advertisements |
| 41   | Instructions for Authors |
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Welcome to the 25th anniversary commemoration of *Immunohematology*. We will observe this event throughout the year by publishing a series of invited reviews titled “Milestones.” These reviews are written by distinguished, world-renowned immunohematologists who provide their perspectives on events in this field during the past 25 years.

Delores Mallory, the first editor-in-chief of *Immunohematology*, kicks off the “Milestones” series in the current issue by taking a look back at the evolution of the journal from the *Red Cell Free Press* newsletter to *Immunohematology*, an *Index Medicus*–cited, peer-reviewed journal.

In the next issue, Dr. Marion Reid will describe the changes in antigen-antibody detection assays that have occurred over the years, in “Milestones in Procedures and Techniques.” As Dr. Reid notes, during the 25 years of *Immunohematology*, there has been a “reversal of phenotype and genotype.” Red cells were typed with antisera to predict genotype and now DNA is tested to predict the phenotype.

Did you attend an Antibody Club meeting and hear a presentation from a guest speaker or review interesting cases? In an upcoming issue, several of these guest speakers and leaders in the field of blood group serology will be profiled in “Pioneers of Blood Group Serology in the United States in the Post-Antiglobulin Era” by one of the guest speakers, Steven R. Pierce. The “Milestones” series will conclude in the final issue of 2009 with “Milestones in Evolution of the Crossmatch—Impact of Serologic Methods on Clinical Practice,” by Dr. S. Gerald Sandler, the founding medical editor of *Immunohematology*.

Thank you very much to the readers, authors, editors, and peer reviewers who have participated in this venture for the past 25 years. I hope you enjoy the yearlong celebration and reflection on the success of both the journal and our profession.

Geralyn M. Meny, MD
Senior Medical Editor
*Immunohematology*
Milestones in *Immunohematology*

1984–2009

D. Mallory

The Beginning
The recognition of a need for a journal that publishes peer-reviewed articles in the field of immunohematology was the first milestone in the history of the journal. The American Red Cross (ARC) Reference Laboratory Committee had been publishing the *Red Cell Free Press* for a number of years, and many of the articles were of excellent quality. However, they were not peer-reviewed, and the circulation of the newsletter was limited to ARC laboratory personnel.

In 1983, it was decided to change the *Red Cell Free Press* from a newsletter to a peer-reviewed publication with a new name. Here is a quote from the last editorial: “An expanded Editorial Board will look for and edit material from several disciplines closely related to Immunohematology and blood group serology—education, HLA, immunology and any area that we feel will be of interest to our readers.” It went on to tell the readers and authors of one of the missions that the journal would repeat often, i.e., “Our Editorial Board has agreed to assist you in writing the articles in an attempt to encourage more of you to experience the great satisfaction of this type of contribution.”

Milestones

1984
*Immunohematology,* *Journal of Blood Group Serology and Education,* was founded in 1984 to help encourage new manuscripts and authors, and help it did! Extensive literature reviews were published in each issue, starting in 1984, and they continued until 2002. The reviews were both diverse and topic specific. Two examples were survival of chromium 51–labeled cells and the Lutheran blood group system.

Two issues were published in 1984, six in 1985, and then the journal found its niche, becoming a quarterly publication in 1986.

1988
The permanent look of the journal came about more than 20 years ago, when the cover, contents, page, color, and basic layout came into being. There have been a few changes, but the “look” is basically the same.

1989–1990
A harder cover added to the new look and it was decided that a subscription fee needed to be charged for the first time. However, students would still receive free issues, and free issues would also be sent to any state blood bank meeting as requested. The editors also went to the American Association of Blood Banks (AABB) annual meeting that year and gave out issues at the ARC booth.

*Immunohematology* received contributions in support of publication of the journal, from Baxter Fenwal, Inc. in 1989, and from Abbott Diagnostics, who contributed for the next 3 years. Ortho Diagnostics Systems, Inc., made the first of 16 yearly donations to support the publication of *Immunohematology.*

1991
*Immunohematology* was selected by the FDA, ISBT, International Committee for Standardization in Haematology, and WHO for publication of the meeting report on monoclonal reagents, and the Excerpta Medica database (Embase) began continuous coverage of publication of the journal.

1992
It was a very special year for the subscribers! An article written by Geoff Daniels, PhD, had a table listing all the high-frequency red cell antigens and the effects of certain enzymes and chemicals on these antigens (Vol. 8, No. 2). This table was made into an 11 × 17-inch poster and was handed out at the AABB meeting that year. It was subsequently sent out to the subscribers. Not many journals would think to do this valuable service for their readers!

It was announced in this and all subsequent issues that the journal is published on acid-free paper. For your edification, it has been from the start.

1994—Happy 10th Anniversary!
*Immunohematology* celebrated by sending subscribers a gift of a cardboard holder for the issues, another unique idea for the reader.

The contents of the final issue that year were outstanding. All papers paid tribute to the Rh blood system, with an editorial by Peter Issitt, PhD, seven original papers, and one on the first example of the red-cell phenotype, Rh:-32.

On a more mundane level, but of great interest to the foreign subscribers, *Immunohematology* at last offered the service of subscription by Visa and Master Card.
1995

The editorial board had met at the end of October 1994 and made major decisions about the direction of the journal. To that end, the editors actively pursued papers concerning WBC and platelet serology. The final issue of 1995 was dedicated to these topics, with three reviews and six original papers.

1996–1997

Publication of Immunohematology was transferred from Rockville, Maryland, to Philadelphia, Pennsylvania.

The first of the “Those Were the Days” articles was published in 1996 (Vol. 12, No. 2), and they continued until 2000. Since that time, “There are many blood bankers who have retired from the field or who will retire in the not-too-distant future, who have some wonderful memories that should not be lost or forgotten.” Such shared memories become part of our history.

The Web site was announced with all of the features, including availability of past issues, searches by topic, subscribing on line, letters to the editor, information for authors, the editorial board, related home page sites, and—finally—the editor’s direct e-mail. It was introduced at the AABB meeting with demonstrations at the ARC booth!

Of note, the first paper on PCR technology was published.

2000–2003

Dr. Phillip Sturgeon, then 80 years old (Vol. 17, No. 4), tells of developing the first multichannel colorimetric recorder autoanalyzer for blood typing. The picture of Dr. Sturgeon with the autoanalyzer is from 1963. Dr. Garratty’s introduction is a must-read of any of the articles published. Dr. Sturgeon is an amazing man and an inspiration for all of us.

Immunohematology is now publishing papers on PCR/DNA, flow cytometry, and other advanced testing technology, and is keeping pace with the needs of the readers. Immunucor, Inc. made a contribution to support the publication of the journal.

2004—Happy 20th Anniversary!

The four issues of 2004 contain 16 invited review papers, heralding a new direction for Immunohematology. Dr. Scott Murphy, who had become a senior medical editor in 2003, was a major factor in moving the journal toward the inclusion of more invited reviews.

In addition, the 2004 issues looked back at the 1984 issue, asking some of the original authors to comment. Two historically important articles from the Red Cell Free Press were republished, as were a poem and crossword puzzle.

Dr. S.G. Sandler, senior medical editor, medical editor, and founding medical director of Immunohematology, retired after 20 years, and Mary McGinniss, managing editor, retired after 19 years.

2005

In 2005, we received acceptance for citation by Index Medicus. This was a major milestone for Immunohematology and for the authors! In 2004, Dr. S.G. Sandler had agreed to apply for indexing and inclusion in Index Medicus. He took the application directly to the chairman and explained the purpose of the journal. It seemed that the reason we had not received acceptance in the past was that Index Medicus thought Immunohematology, Journal of Blood Group Serology and Education, was an immunology journal. Future and past authors can thank Dr. Sandler for this and many other contributions!

Ring in the new! Sandra Nance and Connie Westhoff took over as editors-in-chief. What a great start for the next 25 years.

A new format for presenting papers, Educational Forum, was introduced in 2007, which asks for “manuscript submissions that illustrate serologic and clinical case histories with a focus on the resolution process to include the progressive steps from the serology to clinical, from bench to bedside.” This new format brings the technologist and the clinician together in a better understanding of the patient and the environment that affects the immunohematologist.

The first issue of 2007 is dedicated to the memory of Dr. Scott Murphy, the senior medical editor, who lost a valiant and courageous battle with lymphoma. As a result of Dr. Murphy’s efforts, the journals from 2004 on would contain many more invited review articles. It is interesting to see a comparison of the content of the papers in the journal for the 20 years from 1988 through 2007. See Table 1.

Table 1. Content—20 years (1988–2007)

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<th>Published</th>
<th>Original</th>
<th>Invited Reviews</th>
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<tr>
<td>1988–1997</td>
<td>220 papers</td>
<td>180 (32 case reports)</td>
<td>40</td>
</tr>
<tr>
<td>1998–2007</td>
<td>220 papers</td>
<td>159 (7 case reports)</td>
<td>64*</td>
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*33 reviews in the four years between 2004 and 2007.

2008

The third issue is dedicated to Sandra Ellisor, who also lost a courageous battle with cancer. Sandy was a founding editor and remained on the editorial board until 2006, the year before she died. She had great vision, intelligence, and energy, which she dedicated to Immunohematology as well as to life!

The Past and the Future

Past

There are several people who deserve special recognition for their unequaled contributions and support to Immunohematology. Mary McGinniss was the managing editor from 1986 until 2004 and still reviews articles and helps authors! She had retired from NIH and had a very successful career in immunohematology, having written many original papers, and thus was eager to take on the challenge of edi-
Dr. Marion Reid was a member of the founders of and contributors to the Red Cell Free Press and was involved in and supported Immunohematology from the beginning. She has been on the editorial board, served as a reviewer, and contributed more papers than any other author. She is and always has been one of our most enthusiastic supporters.

Dr. S. Gerald Sandler was director at ARC National Headquarters when the idea was proposed to publish a peer-reviewed journal rather than a newsletter. He was always encouraging, always found the funds to support the publication, and always found a way when a way was needed. He edited every word of every issue of every volume from the beginning, 1984, until 2004. He wrote many articles then and now, is still a peer reviewer, and is on the editorial board. His policy has always been, “If you submit an abstract to a meeting, you will write a paper for publication.” (His staff got a lot of practice and often Immunohematology received a lot of articles submitted for consideration.) One of his finest contributions was the acceptance by Index Medicus for citation in 2005, including 8 years of back issues. Only Dr. Sandler could have done that! No other individual has had more impact on the quality or course of Immunohematology.

For 16 years Ortho Diagnostics Systems, Inc., contributed to the support of the publication of the third issue of each volume of Immunohematology. For years they distributed additional thousands of copies of the journal to their Bankers Club members in all parts of the world. It was a great show of support for education.

One also cannot forget the many people who made a difference in Immunohematology during the 25 years. Authors, staff, editors, reviewers, and financial supporters have worked for 25 years to bring the reader a unique and first-class publication. Of course, it is impossible to forget the readers who have continued to subscribe from 50 states and 21 countries, and are the raison d’être. Long may this continue to be the perfect combination!

Future

The journal’s editorial board never forgets the purpose and audience of the journal; it is and has been in the title. The editors and the editorial board are always looking for the “New, the True and the Interesting” for the readers and the authors. Therefore, on the 25th anniversary of Immunohematology, Journal of Blood Group Serology and Education, it is not at all difficult to predict that there will be many future milestones.

May the next 25 years be even better and may Immunohematology, Journal of Blood Group Serology and Education, always be the leader!

Delores Mallory, MT(ASCP)SBB
Emeritus Editorial Board

Attention: State Blood Bank Meeting Organizers

If you are planning a state meeting and would like copies of Immunohematology for distribution, please send request, 4 months in advance, to immuno@usa.redcross.org.

D. Mallory
D+ platelet transfusions in D– patients: cause for concern?

A.N. Bartley, J.B. Carpenter, and M.P. Berg

Patients whose RBCs are D– may produce anti-D if they are exposed to D on donor RBCs. Except in emergency situations, patients whose RBCs lack D are transfused with only D– RBCs. Platelets carry no Rh antigens, but platelet units may be contaminated by RBCs that could carry D when these units are collected from D+ donors. The purpose of this study was to determine whether our policy of allowing D+ platelets to be transfused to patients whose RBCs type as D–, without the use of prophylactic Rh immunoglobulin (RhIG), results in D alloimmunization. The transfusion records of all patients who received platelet transfusions from December 2004 to March 2007 were reviewed. Transfusion recipients were evaluated with pretransfusion ABO and D typings, and an antibody screen. Recipients were reevaluated in the same manner before subsequent transfusions. Transfusion records of 114 D– patients were analyzed. Overall, 104 patients received D+ platelets; 67 had repeat antibody screening after transfusion. No patients were shown to make anti-D after platelet transfusion. There was no evidence of D alloimmunization as a result of transfusion of D+ platelets in any D– patient during this study. The data do not support the practice of restricting D– patients to receiving only D– apheresis platelets, even among patients with chronic transfusion requirements. Prophylactic use of RhIG for D+ apheresis platelet transfusions in D– patients also appears to be unnecessary. *Immunohematology* 2009;25:9–12.

**Key Words:** platelets, Rh alloimmunization, transfusion

Patients whose RBCs are D– may produce anti-D if they are exposed to D on donor RBCs. At this institution, except in emergency situations, patients whose RBCs type as D–, are only transfused with D– RBCs. Platelets do not express Rh antigens, but platelet units may be contaminated with RBCs that could carry D when these units are collected from D+ donors. Some experts advocate restricting D– patients, particularly infants, small children, and women of childbearing age, to receipt of D– units for platelet transfusions or prophylactic use of Rh immunoglobulin (RhIG) when D– platelets are unavailable.¹ Current AABB standards require that transfusion services have a policy for the use of D+ RBC-containing components (including platelets) in D– recipients.² Because our donor population (and the American population in general) is 85 percent D+, we often do not have enough D– platelet units in our inventory to fully support D– patients who need chronic transfusion support. Therefore, our policy is to first issue D-matched platelets to all patients when possible, especially to children and women of childbearing potential, and to give mismatched platelets when matched platelets are unavailable. We currently use irradiated single-donor apheresis platelet units exclusively. In the past, pooled random donor platelets derived from whole blood collections were used on rare occasions. A grossly bloody (red-tinged) D+ platelet unit would not be given to a D– patient without first discussing the situation with the patient’s clinician. (Typically, those units are reserved for D+ patients.) Such units are extremely rare in our inventory. It is this institution’s standard policy not to give RhIG to D– patients who receive D+ platelets.

The purpose of this study was to determine whether our policy of allowing D+ platelets to be transfused to D– patients without the use of prophylactic RhIG resulted in D alloimmunization.

**Materials and Methods**

We retrospectively reviewed the transfusion records of all patients who received platelet transfusions at University Medical Center (Tucson, AZ) from December 26, 2004, to March 29, 2007. Transfusion recipients were evaluated with pretransfusion ABO and D typings and an antibody screen. All patients who were D– and were transfused with D+ platelets were included in the study regardless of disease status. The data were later censored for patients who only received D– platelets, had no repeat testing after transfusion, died shortly after transfusion, or had repeat testing performed less than 5 days after transfusion. ABO group, D type, and an antibody screen test were performed on all patients before transfusion and again every 3 days if additional transfusions were requested. ABO and D antigens were detected using commercially available reagents following the manufacturer’s instructions. D– patients who were pregnant were also tested with another reagent to detect weak D. The antibody screen test to detect the presence of anti-D was performed by tube method using LISS-IAT and a three-cell screen. For some patients beginning in February 2006, ABO and D typings and four-cell antibody screens were performed by the Galileo instrument (all reagents previously listed and the Galileo instrument were supplied by Immucor/Gamma, Norcross, GA).

Patient age, clinical diagnosis, source of platelets, dates of transfusion, number of units transfused, results of antibody testing, and duration of the period between an incompatible transfusion and the date of the last antibody screen were recorded. Institutional Review Board approval was obtained for all data collection in this study.
Results

One hundred fourteen D– patients received 1085 platelet transfusions during this study. Overall, 104 of the 114 D– patients received D+ platelets. Eighteen of the 104 recipients of D+ platelets had no repeat antibody screen after transfusion, 9 had antibody screens performed less than 5 days after transfusion, and 10 others died within days of receiving the transfusion. Sixty-seven patients who received a total of 866 platelet transfusions had repeat antibody screening after D platelet transfusions. Thirty patients were female and 37 were male. The mean age was 46.5 years (median, 52 years; range, 0.58 months to 90 years), including 13 pediatric patients, who were 0.58 months to 16 years of age. The mean number of transfused platelet units per patient was 12.9 (median, 6; range, 1 to 109). Six of the 67 patients received random donor platelets: 4 received one pool, 1 received five pools, and 1 received three pools. Clinical diagnosis at the time of transfusion varied, including hematologic disorders or malignancies (n = 31), trauma (n = 10), heart conditions (n = 15), organ failure or transplant (n = 9), and other malignancies (n = 2). None were shown to make anti-D.

Currently, the entire platelet inventory at this institution is irradiated; thus all patients receive only irradiated platelet units. During this study, most platelet transfusions given were prestorage leukoreduced apheresis units. Random donor platelets given earlier in the study were not leukoreduced. Only 8 patients of the 114 examined received a total of 14 pools of platelet concentrates prepared from whole-blood donors according to the buffy coat method. Each pool contained five platelet units and was considered the equivalent of one apheresis platelet transfusion. Seven of these 8 patients had an antibody screen performed after the transfusion. Of these 7, 1 had only one antibody screen performed less than 5 days after the transfusion. Therefore only 6 patients receiving 12 random donor platelet transfusions were included in the study population.

It is not possible to estimate the quantity of RBCs in the platelet transfusions administered in this study, but a published report that counted the residual RBCs in similar apheresis platelet units suggested that these units contain $2.7 \times 10^7$ RBCs per apheresis unit or fewer than 90 RBCs per microliter. None of the 67 included patients were obstetric patients, so pregnancy status was not known. As a consequence, none of the patients were tested for weak D.

Three recipients were found to have anti-D that were not thought to be related to platelet transfusions, as described later. A pregnant woman received prenatal RhIG, and afterward she was found to have a positive antibody screen attributable to anti-D before transfusion of platelets. She was not included in the final patient group because her last antibody screen was done the day after her last platelet transfusion. Another woman presented with anti-D and anti-C before any transfusion at this institution. Finally, a D– boy received D+ granulocytes donated by family members, along with prophylactic RhIG. An antibody screen performed 3 months later was positive for true (i.e., not infused) anti-D, despite the use of RhIG.

Discussion

There is little agreement as to whether RhIG immunoprophylaxis is necessary in D– patients receiving apheresis platelet transfusions. This is especially true in infants and women of childbearing age, and few conclusive studies have been published as to the necessity of immunoprophylaxis in these patients. Our data suggest that RhIG immunoprophylaxis is not necessary in D– patients receiving D+ apheresis platelet transfusions. After transfusing more than 800 D-incompatible platelet units during a 27-month period at our institution, we detected no cases of primary alloimmunization. Ten D– patients received only D– platelets. Of the 67 patients included in the final analysis, 65 had subsequent antibody screens that were negative, thus demonstrating no new antibody formation in response to the transfusions. Two patients did have positive antibody screens that demonstrated anti-D, but these antibodies could easily be explained by causes other than the platelet transfusions. Most patients received few platelet transfusions (the median number of transfusions was six), but 5 patients received more than 50 platelet transfusions. Of these, only the patient who also received granulocyte transfusions made anti-D.

Although it is known that D is not expressed on platelets, it is present on RBCs that contaminate units of platelets. This contamination varies depending on the method by which the platelets are prepared. The dose of RBCs required to induce an immune response to D varies among individuals and is influenced by many factors. Studies attempting to quantify the immunogenic dose are limited in size and number. One early trial tested the immune response in healthy volunteers who were treated with small doses of D+ blood. Volumes as low as 0.05 mL were found to be immunogenic in some susceptible individuals. Additional studies on platelet transfusions from the 1970s and 1980s showed that alloimmunization resulting from incompatible platelet transfusions based on older methods of platelet preparation (pooled random donor and early apheresis methods) ranged up to 19 percent in patients with malignant disease. Platelet concentrates prepared by early apheresis methods as used in that study contained up to 3 mL of RBCs.

By current methods, whole blood–derived platelet concentrates contain 0.3 to 0.5 mL of RBCs, and platelet concentrates derived from modern apheresis methods contain trace RBC contamination (0.0002 to 0.007 mL). The rate of seroconversion after low-dose exposure is unknown, but anecdotal evidence suggests it is much lower than what has been observed with larger transfusions. Therefore, contaminant RBCs in modern apheresis platelet units are present in quantities that may be insufficient to incite an alloimmune response. Atoyebi et al. studied serology for the presence of anti-D after D-incompatible platelet transfusions in patients with hematologic and nonhematologic...
disease. The patients were transfused with platelets from either whole blood or apheresis methods or both. None of the patients were given prophylaxis with anti-D. Of the patients without hematologic disease, 68 percent received apheresis platelets, with the remainder receiving pooled platelets. Of those with hematologic disorders, 75 percent received apheresis platelets. Thirteen percent of patients without hematologic disease and no patients with hematologic disease formed anti-D. They concluded that the risk of alloimmunization after D-incompatible platelet transfusion using concentrates prepared by modern technical methods appeared to be small in patients with hematologic diseases, but was significant in immunocompetent patients.

A later case report by Haspel et al. described a D– infant who received two D-mismatched whole blood–derived platelet units at 17 weeks of age and was subsequently found to have anti-D on retesting 13 months later. The infant was exposed to less than 0.6 mL of D+ RBCs in these units. Another study by Molnar et al. examined all D-incompatible platelet transfusions administered to pediatric oncology patients during a 1.5-year period, which included 42 patients with various diagnoses. No cases of D alloimmunization were detected. They concluded that D immunoprophylaxis is generally unnecessary in pediatric oncology patients receiving D-incompatible single-donor platelets not visibly contaminated by RBCs. As well, a smaller study by Cid et al. examined 22 immunosuppressed patients receiving D-incompatible pooled platelet transfusions. None of them developed detectable anti-D after a median follow-up of 8 weeks. Thus, in studies that used only prestorage leukoreduced apheresis platelet units, there is no evidence that D– patients developed anti-D when transfused with D+ platelet units.

The most important reason for administration of prophylactic RhIG in D– women or girls with childbearing potential who are receiving D+ transfusions is to prevent HDN with a D+ fetus in future pregnancies. Secondarily, it reduces the need for additional pretransfusion testing owing to positive antibody screens in patients who would otherwise have made anti-D. The clinical practice guidelines of the American Society of Clinical Oncology on platelet transfusions in patients with cancer state, “anti-D prophylaxis should be considered for Rh-D-negative children and for women of childbearing age.” They give this recommendation a “Grade D,” however, which is categorized as “little or no systematic empirical evidence.”

Although generally not life threatening, the complications of RhIG administration include infection and hemotoma formation. Some patients experience low-grade fever, chills, headache, myalgias, flushing, or nausea and vomiting. Depending on the method of production (i.e., cold alcohol fractionation), RhIG may be contaminated with other plasma proteins, including IgA, which may put IgA-deficient patients at risk for developing hypersensitivity reactions, or with aggregates of IgG polymers, which are capable of activating the complement cascade. The formulations created using ion-exchange chromatography have been associated with hepatitis C virus outbreaks in the past. All formulations now include additional virus inactivation steps in their processing to minimize the risk of such outbreaks. One formulation also contains high levels of maltose, which can interfere with blood glucose testing, giving falsely high readings.

In the past, concern had been raised about the levels of thimerosal, a mercury-containing compound, in vials of RhIG. Since 2001, all of the brands of RhIG sold in the United States have been made without thimerosal, so concerns about this compound should no longer be an issue. The risks and benefits of RhIG administration should be discussed with the patient’s clinician. Based on our observations, we propose that there is little benefit to the patient of administering RhIG for platelet transfusions given to D– men or D– women who are incapable of conceiving. This is especially true when quality control measures are taken to ensure the RBC content of apheresis platelets is less than the average quality control dose of less than 0.1 mL RBCs per adult therapeutic dose.

We found no evidence of D alloimmunization caused by transfusion of D+ platelets in any D– patient during this study. Some limitations to our study include small sample size, retrospective analysis, incomplete knowledge of immune status, and lack of serial antibody screening in all patients. A prospective study of sensitization to D by exposure of D– patients to D+ platelets is needed in the future. However, our data do not support the practice of restricting D– patients to D– apheresis platelets, even among patients with chronic transfusion requirements. This restriction may further lead to continued unnecessary D– platelet inventory supply problems. Prophylactic use of RhIG for D+ apheresis platelet transfusions in D– patients also appears to be unnecessary. We have adopted and use this policy in our own patient population; however, we realize other institutions with different patient populations and platelet inventories may prefer a more conservative approach. We invite others to perform additional studies and to examine their current policies.

References

Angela N. Bartley, MD (corresponding author), John B. Carpenter, MD, and Mary P. Berg, MD, Department of Pathology, 1501 N Campbell Ave, P.O. Box 245108, Tucson, AZ 85724-5108.

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**Free Classified Ads and Announcements**

*Immunohematology* will publish classified ads and announcements (SBB schools, meetings, symposia, etc.) without charge. Deadlines for receipt of these items are as follows:

**Deadlines**
- 1st week in January for the March issue
- 1st week in April for the June issue
- 1st week in July for the September issue
- 1st week in October for the December issue

E-mail or fax these items to immuno@usa.redcross.org or (215) 451-2538.
Autoantibody formation after alloimmunization inducing bystander immune hemolysis

M. Mota, C. Bley, M.G. Aravechia, N. Hamerschlak, A. Sakashita, J.M. Kutner, and L. Castilho

The development of RBC autoantibodies resulting from or associated with allogeneic blood transfusions is not an easily determined complication of RBC transfusions. This report discusses one patient who developed RBC autoantibodies in association with an allogeneic blood transfusion and alloimmunization leading to a temporary bystander immune hemolysis. A 72-year-old woman was hospitalized as a result of severe anemia and received two units of ABO- and D-compatible RBCs. She had a history of two pregnancies 40 years before, but no history of RBC transfusion, and her antibody screen was negative. On the tenth day after transfusion her hemoglobin dropped, and alloanti-c was identified in her serum and eluate. At this time she received another two units of compatible blood according to her phenotype (group O, R\(\text{R}_{1}\), K:-1). After 48 hours, she developed joint pain, pyrexia, and hemoglobinuria, and her Hb dropped from 9.2 g/dL to 5.3 g/dL. The direct antiglobulin test was positive, an IgG autoantibody was present in the eluate, and the antibody investigation revealed the presence of anti-JK\(^{a}\) in addition to the previously identified alloanti-c. Her genotype was determined, and, based on the findings, two additional units were selected, found to be compatible, and transfused without incident. Transfusions were discontinued, and she was treated with IVIG and corticosteroids. Her Hb increased to 9.7 g/dL, and the patient made an uneventful recovery. It was concluded that transfusion of incompatible RBCs induced the formation of an autoantibody in this patient, resulting in lysis of bystander RBCs. The need for additional blood transfusion was successfully avoided by treatment with IVIG, steroid therapy, and rituximab. *Immunohematology* 2009;25:13–16.

Key Words: bystander hemolysis, autoantibody, alloimmunization, RBC transfusion

The presence of alloantibodies in chronically transfused patients and pregnant women is a well-recognized complication of RBC transfusions and pregnancies and, as such, is unrelated to the concomitant autoantibodies. In contrast to alloimmunization, the risk of autoimmunization resulting from or associated with the development of alloantibodies is poorly understood; however, RBC autoimmunization and the development of autoimmune hemolytic anemia (AIHA) should be recognized as a complication of allogeneic RBC transfusion.\(^1\)

Recent studies of patients with multiple transfusions, such as those with sickle cell anemia, have drawn attention to the association between autoimmunization and alloimmunization.\(^2\)–\(^5\) RBC autoimmunization has also been described in both animal and human experimental models in which introduction of incompatible RBCs induced both alloantibody and autoantibody formation.\(^6\)–\(^10\)

The development of AIHA concurrent with or shortly after alloimmunization from blood transfusions has also been reported, and one possible mechanism to explain this phenomenon is that alloantibody binding to transfused RBCs could lead to conformational changes in antigenic epitopes that then stimulate production of an autoantibody.\(^7\) The term *bystander immune hemolysis* is applied when AIHA occurs after exposure to alloantigens.\(^11\)

This phenomenon, which has been recognized in the medical literature for many years, seems to receive little attention although it is clinically very important and it touches on some basic immunologic principles that might have broader implications than has been previously realized.\(^12\)

We report one case of severe, life-threatening bystander immune hemolysis associated with the development of an autoantibody in association with allogeneic blood transfusions and alloimmunization.

Case Report

A 72-year-old white woman with a history of coronary artery disease, hypertension, diabetes mellitus, and angiodysplasia was hospitalized as a result of severe anemia, weakness, chest discomfort, dyspnea, and acute lower gastrointestinal bleeding. On physical examination, the patient was pale, tachycardic, and normotensive with normal oxygen saturation by pulse oximetry. Her admission laboratory evaluation revealed an Hb of 7.9 g/dL, Hct of 24%, reticulocyte count of 3.1%, WBC count of 4,53 x 10\(^3\)/\(\mu\)L, platelet count of 343 x 10\(^3\)/\(\mu\)L, blood urea nitrogen of 21 mg/dL, and creatinine of 0.7 mg/dL. She received two units of ABO- and D-compatible blood. No RBC alloantibodies were detected, and her RBCs did not react in the DAT before this transfusion. No symptoms occurred during the transfusion. Ten days later, the patient was readmitted with slight signs and symptoms of hemolysis, and there was no evidence of blood loss. The clinical investigation revealed no history of previous RBC transfusion, but she had a history of two pregnancies 40 years ago. The patient’s Hb level was 8.2 g/dL, LDH was 1509 IU/L, nonconjugated bilirubin was 1.5 mg/dL, and hemoglobinemia was 8.9 mg/dL; alloanti-c was identified in her serum and eluate. Clinical and serologic results demonstrated a delayed hemolytic transfusion reaction (DHTTR) attributable to anti-c. At this time she received two additional units of compatible blood according to her phenotype (group O, R\(\text{R}_{1}\), K:-1). After 48 hours, she
exhibited generalized musculoskeletal pain, pyrexia, and hemoglobinuria, and her Hb dropped from 9.2 g/dL to 5.3 g/dL. The DAT was 4+; a warm IgG autoantibody that reacted with her RBCs and all panel cells by the IAT was present in the serum and eluate. A differential absorption method revealed the presence of the known alloanti-c coexisting with a newly identified alloanti-Jk^b in her serum. Molecular analysis was performed, and she received two units of compatible blood according to her genotype (RHD, RHCE*C/C, RHCE*e/e, KEL*2/2, JK*A/A, FY*A/B, GYPB*S/s) without clinical or laboratory response. A clinical diagnosis of AIHA was made, and treatment was started on day 8 after the diagnosis with IVIG (0.4 g/kg for 5 days) and IV methylprednisolone (0.5 g for 5 days). Her Hb increased to 9.8 g/dL, but a chest discomfort persisted. She received two RBC units, and 48 hours after the transfusion her Hb dropped to 7.7 g/dL. Transfusions were discontinued, and she was treated with rituximab (500 mg one time weekly for 1 month). After 2 months, there was no evidence of hemolysis, her Hct remained stable, and the laboratory tests revealed alloanti-c and alloanti-Jk^b in her serum. No RBC autoantibody was detectable.

Materials and Methods

Sero logic studies

Direct and indirect antiglobulin tests were performed by hemagglutination in tubes and in gel cards (DiaMed AG, Cressier sur Morat, Switzerland). Antisera and reagent RBCs were obtained from a variety of commercial companies (DiaMed AG; Gamma Biologicals Inc., Houston, TX; Fresenius Hemocare, São Paulo, Brazil). Eluate was performed using Gamma Elu Kit II (Gamma Biologicals Inc.) from patient RBCs. Differential absorptions were performed on the patient’s serum to remove the autoantibody, allowing the identification of the alloantibodies.

Molecular studies

DNA was extracted from blood samples by using a kit (Easy DNA kit, Invitrogen, Carlsbad, CA) according to the manufacturer’s recommendations. Allele-specific PCR (AS-PCR) and PCR-RFLP were used for RH, KEL, FY, JK, and Ss genotyping. The primers and the amplification conditions used for genotyping have been previously published. After the first hemolytic episode, the patient’s RBCs were weakly reactive in the DAT (tube and gel) and alloanti-c was identified in the eluate, but after the second hemolytic episode, the DAT became strongly positive. The eluate from the posttransfusion sample demonstrated the presence of a warm IgG autoantibody that reacted with her RBCs and all panel cells, including all c− panel cells, by the IAT. Her serum was positive with all RBCs tested, and allogeneic adsorptions using selected RBCs with the same patient’s antigen profile according to the predicted phenotype from genotype results (RHD, RHCE*C/C, RHCE*e/e, KEL*2/2, JK*A/A, FY*A/B, GYPB*S/s) were performed to identify underlying alloantibodies. After allogeneic adsorptions on patient’s serum, alloanti-c and alloanti-Jk^b were identified.

After the third transfusion episode, even receiving two units of compatible blood according to her genotype, the DAT was still strongly positive (4+), and a panagglutinin was found in the eluate, suggesting the presence of the autoantibody.

RBC units

A total of three transfusion episodes, during which six different RBC units were transfused, were analyzed retrospectively. In the first transfusion episode, crossmatches performed with serum from a pretransfusion sample and two units of ABO- and D-compatible RBCs were found to be repeatedly negative with PEG-IAT. In the second transfusion episode, crossmatches performed with the absorbed serum of the posttransfusion sample and two units of compatible blood according to her phenotype (group O R, K:-1) were negative, and in the last transfusion episode, crossmatches performed with the absorbed serum of the posttransfusion sample and two units of compatible blood according to her phenotype (group O R, K:-1, Jk(b−)) were also negative. RBCs from the first donor unit were found to be c+ and Jk(b+), but the other five RBC units were c− and Jk(b−). Retrospectively, the transfusion that was most probably responsible for the immune response–induced alloantibody and autoantibody formation could be traced back to the first transfusion episode, when the c+ and Jk(b+) RBC units were administered.

Discussion

This case demonstrates the development of AIHA after alloimmunization from blood transfusion and hemolytic reaction. The autoimmune hemolysis was initially associated with the appearance of an alloantibody; anti-c was detected in an eluate prepared from the patient’s RBCs after the first DHTTR. However, 10 days after the last compatible transfusion, the DAT became strongly positive and an eluate prepared from the patient’s RBCs indicated the presence of a warm IgG autoantibody, suggesting that it was responsible for the bystander hemolysis. The nadir levels of Hb and Hct during acute hemolysis were substantially lower than the

Results

Patient’s samples

Before the first hemolytic transfusion reaction, the alloanti-c was not detected; most probably the antibody titer was very low and the antibody was undetectable by serologic techniques. After the second hemolytic transfusion reaction following the transfusion of compatible R R1 RBC units, alloanti-c and alloanti-Jk^b were found.

The two alloantibodies were identified in the serum samples with PEG-IAT, GEL-LISS, and GEL-PAPAIN.

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pretransfusion levels of Hb and Hct, indicating the destruction of autologous RBCs. Based on these observations, we believe that there is clinical evidence of autoimmune hemolysis in the present case.

The development of RBC autoantibodies and AIHA after transfusion was first described by Dameshek, in 1965, when he observed a number of patients who exhibited a positive antiglobulin test after several transfusions. He suggested that some of these reactions had the appearance of an autoimmune reaction, possibly because of cross-reacting antibodies. However, he stated that because the stimulation of exogenous antigen is required to set off the reaction and it is not self-perpetuating, such reactions are actually alloimmune reactions resulting in a temporary state of pseudoautoimmunity.

There is a significant body of literature on the development of RBC autoantibodies and AIHA after transfusion and it is of interest to keep in our minds the possibility of autoimmunity and bystander immune hemolysis. Different reports describe the temporary presence of autoantibodies and AIHA, with either spontaneous resolution in weeks or months or complete response to initial therapy. Some reports describe acute fatal hemolysis, and only a few describe AIHA or autoantibodies persisting for an indefinite time. Data from animal experiments show the induction of RBC autoantibodies in mice immunized with rat RBCs. The mice developed autoantibodies, as demonstrated by a positive IAT and DAT. Some mice exhibited AIHA. It is of great interest that persistence of the positive DAT required that the mice be repeatedly immunized with rat RBCs. After immunization was interrupted, the serologic tests returned to normal. When the mice were immunized again with rat RBCs, there was a burst of autoantibody production, and then after 4 to 5 weeks, autoantibody production ceased.

There are several published reports on humans. Lalezari et al. published a report of a 40-year-old woman who had a partial D phenotype and had been transfused with several units of D+ blood, and 10 years later, received three additional units. She had a high titer of anti-D, and this anamnestic response was associated with the development of an autoantibody. The DAT, which had been negative before transfusion, became positive and remained so for 6 months. Chaplin et al. described 5 patients with sickle cell disease who exhibited AIHA. All patients had made alloantibodies in response to transfusion before they made autoantibodies. The patients with AIHA experienced severe anemia and had increased reticulocyte counts. All patients responded to steroid therapy. After discharge the DATs became negative. Argioli et al. reported 4 patients with thalassemia major in whom they diagnosed AIHA on the basis of an increase in transfusion requirement, associated with the presence of RBC autoantibodies. All the patients were treated with IVIG therapy, and 3 responded as indicated by normalization of the blood consumption. Wodzinski et al. described an extraordinary case of paroxysmal cold hemoglobinuria (PCH) after an ABO-incompatible transfusion. The authors suggested that the ABO HTR triggered or exacerbated an autoimmune response manifesting as PCH. Dameshek and Levine reported a case in which successive transfusions resulted in extreme, almost fatal, hemolysis. In contrast to their later conclusions, Dameshek and Levine at that time suggested that an irreversible autohemolytic process had been established by the multiple transfusions and that this report was published well before the description of autoadsorption or alloadsorption of sera containing autoantibodies to detect the presence of alloantibodies, which could have been the cause of the hemolysis.

Young et al. drew attention to the association between autoimmunization and alloimmunization that occurs commonly but only manifests obvious clinical ill effects on rare occasions. The phenomenon deserves much more attention because it involves basic immunologic mechanisms that have broad implication. Our case reinforces that RBC autoimmunization and the development of AIHA could be recognized as an adverse effect of allogeneic RBC transfusion. A strategy that minimizes exposure to allogeneic blood transfusion would reduce risks of RBC autoimmunization and development of new alloantibodies.

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Transfusion of rare cryopreserved red blood cell units stored at –80°C: the French experience

T. Peyrand, B.N. Pham, P.Y. Le Pennec, and P. Rouger

The technology allowing freezing of RBC units has been available for many decades. The high-glycerol method for RBC storage at –80°C is predominantly used. Several studies have shown satisfactory results regarding the in vitro viability and function of cryopreserved RBCs. RBC freezing is nowadays mostly encountered in rare blood programs and military deployments. Preservation time of frozen RBCs appears to be virtually indefinite, but most countries apply a 10-year outdate. There is no mandatory time restriction in France. The National Rare Blood Bank currently includes 962 (17.5%) RBC units aged 10 years or more and 153 (2.8%) aged 20 years or more. Since 1994, 1957 RBC units have been thawed and transfused, among which 118 were aged 10 years or more and 8 were aged 20 years or more. Discarding RBC units older than 10 years may be highly sensitive for very rare blood groups, e.g., U–, of which approximately 30 percent of the cryopreserved units are aged 10 years or more. However, the lack of nucleic acid testing for HIV and HCV may be problematic for old RBC units drawn from donors who were not subsequently tested for these markers, which is now mandatory in most countries. Regarding the 118 transfused RBC units older than 10 years, no evidence of hemolysis of thawed RBCs and no transfusion reaction, clinical or biologic hemolysis, or transfusion ineffectiveness was reported, either by any of the parties involved in the transfusion supply of rare RBC units or through the French hemovigilance program, which requires a mandatory report of any transfusion reaction. It has recently been suggested to extend the 10-year restriction in some countries. Considering our experience and observational data, we may consider it safe and efficient to transfuse rare frozen RBC units older than 10 years. An international consensus for RBC cryopreservation time should ideally be established. *Immunohematology* 2009;25:17–21.

**Key Words:** cryopreservation, biopreservation, RBC, rare blood, transfusion, hematology, blood banking, freezing, frozen blood, transfusion safety

Humans have experimented with blood transfusions for more than 300 years and attempted to preserve human blood since the early 1900s. The first modern approach to blood storage, with a citrate-glucose solution, was stimulated by World War I. Long-term storage of RBCs using glycerol as a cryopreservative was initially implemented in the early 1950s. Two methods have been described: the low-glycerol method, for freezing units in liquid nitrogen at –196°C, and the high-glycerol method for storage of RBCs at –80°C. The technology that is currently predominantly used is the high-glycerol technique, which was initially developed in the 1960s and early 1970s by the American Red Cross Research Laboratory (Washington, DC) and the U.S. Naval Blood Research Laboratory (Boston, MA).

The technology allowing freezing of RBC units has been available for more than four decades. In addition to long-term storage, RBC cryopreservation has other advantages, which are mainly related to the necessary washing procedure for glycerol removal once units are thawed. Indeed, washing of thawed RBCs eliminates most cell debris, WBCs, platelets, cytokines, residual plasma, and free hemoglobin. However, RBC cryopreservation has never reached the expected popularity; it is still infrequently used, especially because of the labor-intensiveness and expense of the procedure, as well as the RBC loss through the process and limited shelf life of thawed RBC units. As a result, stockpiling of frozen inventory RBCs is today mostly encountered in rare blood program management and military deployments.

**Organization of the Rare Blood Program in France**

French regulations stipulate that a blood group is regarded as rare if its prevalence is 4 in 1000 or less (i.e., ≤1 in 250) in the general population. A national rare blood donor database was implemented in our country in the late 1960s to ensure the transfusion and obstetric safety of patients with rare blood phenotypes. A national rare blood bank was set up in the early 1980s. Potential rare blood donors from the national database are highly encouraged to donate blood on a routine basis for the National Rare Blood Bank. This facility is located in Créteil (a few kilometers from Paris) and is comanaged by the National Institute of Blood Transfusion (INTS, Paris) and the French Blood Establishment (EFS Ile de France, Paris). The confirmation of the rare blood type, registration of new people with a rare blood group in the national database, and selection and delivery agreement of rare blood units are exclusively carried out by the National Reference Laboratory for Blood Groups (CNRGS, Paris), a department of the INTS, Paris. To date, 9508 individuals (patients and donors) are enlisted in the national registry of people with rare blood phenotypes or genotypes.

Rare blood units from the National Rare Blood Bank are frozen according to the Cohn method, using glycerol in an ionic medium to achieve a final glycerol concentration of 40 percent (wt/vol), the so-called high-glycerol method. RBCs are subsequently stored at a mean temperature of –80°C.
(range ~65° to ~90°C). Since late 2005, the Haemonetics Automated Cell Processor (ACP) 215 closed-circuit instrument (Haemonetics Corp., Braintree, MA) has been systematically used to glycerolize and deglycerolize human RBCs, allowing for a 7-day shelf life for thawed units when stored at 2° to 6°C in a saline-adenine-glucose-mannitol (SAGM) additive solution. Other RBC units are handled with the Cobe 2991 Cell Processor (COBE BCT, Lakewood, CO), with a 24-hour shelf life for thawed units resuspended in SAGM. According to the French current rules, the quality control of the thawed RBC units consists in the hemoglobin measurement (~235 g/unit), hematocrit determination (50% to 80% for RBC units handled in an open circuit, 40% to 70% for those handled in a closed circuit), extracellular hemoglobin measurement (~1.2% of total hemoglobin), and extracellular glycerol measurement (~1 g/unit). Both open-circuit and closed-circuit technologies have been validated. It was shown for the open-circuit and closed-circuit procedures that the post thawing mean extracellular hemoglobin was 0.21 percent (0.06% to 0.57%) and 0.16 percent (0.11% to 0.25%), and the extracellular glycerol was 0.14 g (0.02 to 0.73 g) and 0.15 g (0.11 to 0.19 g), respectively. Random sampling is used for the routine quality control of the thawed units according to the NF-ISO-2889 standard.

The National Rare Blood Bank currently includes 5503 cryopreserved blood units, from 1630 blood donors. From 2001 to 2005, an annual average of 161 rare blood units were transfused (i.e., 2.53 per 10^6 inhabitants per year) for an annual average of 28 patients and 70 transfusion episodes; 149 rare blood units were transfused in 2006 and 236 in 2007.

The Preservation Time of Rare Blood Units

Rare blood, by its very nature, is considered a rare resource, and its long-term storage is necessary. The preservation time of frozen RBC units appears to be virtually indefinite. However, the question of a maximum period of validity has often been raised. In September 1987, the Food and Drug Administration in the United States approved the change from a 3- to a 10-year outdate period for glycerol-frozen RBCs stored at ~80°C. Most other countries, e.g., China and South Africa, currently apply this 10-year outdate. The European guidelines from the Guide to the Preparation, Use and Quality Assurance of Blood Components state that storage of frozen RBC units is possible for at least 10 years, provided that an adequate temperature can be guaranteed (~60° to ~80°C for the high-glycerol cryopreservation procedure). The Technical Manual of the AABB, 16th edition (2008), states that “frozen RBCs must be stored at temperatures colder than ~65°C and will expire after 10 years. Rare frozen units may be used beyond the expiration date, but only after medical review and approval that are based on the patient’s needs and the availability of other rare compatible units.”

Several studies have been performed to investigate the in vitro recovery and quality of deglycerolized RBCs. Valeri et al. reported that RBCs frozen with 40 percent to 45 percent (wt/vol) glycerol could be stored at ~80°C for up to 37 years, with mean in vitro freeze-thaw-wash recovery values of 75 percent, without affecting the RBC 2,3 DPG level and with hemolysis after 24 hours of postwash storage at 4°C usually less than 1 percent. All units tested were sterile. Moreover, frozen RBC units that were stored for as long as 21 years and subsequently thawed, washed, and stored at 4°C for 24 hours have been shown to demonstrate an in vivo 24-hour posttransfusion survival value greater than 75 percent. In 2004, a similar study was performed to investigate the in vitro quality of RBCs frozen with 40 percent (wt/vol) glycerol at ~80°C for 14 years, deglycerolized with the Haemonetics ACP 215 instrument, and stored at 4°C in AS-1 or AS-3 for up to 3 weeks. Acceptable results were achieved (mean in vitro recovery value of 80 ± 7%), similar to those previously described, although deglycerolized RBCs in AS-1 exhibited significantly higher hemolysis than those in AS-3 after storage at 4°C for 7 to 21 days. Another study in 2004 examined RBC units that had been cryopreserved in 40 percent (wt/vol) glycerol and stored at ~80°C for up to 22 years. Postthaw RBCs had acceptable mean freeze-thaw-wash recovery and normal oxygen transport function, RBC morphology, RBC indices, methemoglobin, and osmotic fragility. Interestingly, it was also shown that the in vitro viability and function of cryopreserved RBCs was not dependent on the length of frozen storage or postthaw storage at 4°C, but was influenced by the length of storage at 4°C before cryopreservation: The more quickly the RBC units are frozen, the better are the in vitro viability and function of the thawed RBCs.

Despite the conclusions from these different studies advocating the possibility of storing RBCs well over 10 years, the 10-year outdate has remained since 1978 in the United States and is still largely applied in rare blood banks throughout the world.

Storage and Transfusion of Frozen RBCs Beyond 10 Years: The French Experience

Age of frozen RBC units and thawed transfused RBC units: statistical data

Between 1994 and 2007, a mean of 628 rare blood units per year were frozen in the National Rare Blood Bank. After blood donation, RBC units are frozen as rapidly as possible, ideally within 7 days. However, freezing blood up to the 42-day maximum period of validity is still possible for exceedingly rare types. Since 2004, 13.2 percent of RBC units have been frozen within 7 days of blood donation, 88.2 percent within 14 days, and 97.0 percent within 21 days.

Despite European recommendations, no official mandatory time limit regarding the validity period of rare frozen RBC units exists in France. The National Rare Blood Bank...
currently includes 962 (17.5%) RBC units aged 10 years or more and 153 (2.8%) aged 20 years or more (Fig. 1). The average age of rare frozen RBC units is 6.1 years (maximum 33.5 years). Since 1994, 1957 rare RBC units have been thawed and transfused, among which 118 (6.0%) were aged 10 years or more and 8 (0.4%) aged 20 years or more (Fig. 1). The average age of rare transfused RBC units is 3.6 years (maximum 23.8 years).

Discarding rare blood units older than 10 years may be, ethically speaking, highly sensitive, as there are usually no alternative resources for the specified patients. If we decided to discard all rare blood units aged 10 years or more, this would represent the destruction of 17.5 percent of the current French rare frozen RBC stock, which could be very problematic for some rare blood phenotypes. For example, 240 Vel− (VEL−:1) rare RBC units are now cryopreserved, among which 64 (26.7%) are aged 10 years or more. For the Kp(b−) rare specificity (KEL−:4), 75 (32.8%) are aged 10 years or more. For the U− rare specificity (MNS−:3,−4,−5), which is quite frequently requested in France for patients of Afro-Caribbean ancestry suffering from sickle cell disease,3−7 113 (29.7%) are aged 10 years or more.

Long-term preservation of rare RBC units and nucleic acid testing in blood donors

A specific problem for long-term preservation of rare RBC units concerns testing for mandatory markers for transfusion-transmitted diseases according to the current national regulations. In France, the current mandatory and systematic serologic markers are anti-HIV-1/anti-HIV-2, anti-HCV, AgHBs and anti-HBc for HBV, and anti-HTLV-I/anti-HTLV-II. In addition, nucleic acid testing (NAT) for HIV and HCV has been required since July 2001. For either serologic markers or NAT, RBC units drawn before their respective official implementation date are considered valid, provided that a blood donation or blood sample has been subsequently tested, according to a so-called retroactive process. In our country, however, no testing is performed for that purpose on frozen samples from a biologic resource center. Presently, 4562 (82.9%) of the RBC units stored within the National Rare Blood Bank have been tested for all legal mandatory markers, including NAT, either in real terms or retroactively. RBC units confirmed by a retroactive NAT testing are considered fully valid for transfusion according to the French regulations.21 Another issue is the 941 (17.1%) rare RBC units for which the corresponding donors have never been subsequently tested for NAT. RBC units aged 10 years or more represent 54.9 percent of these 941 blood donations. These RBC units may also be used for transfusion, provided there is no alternative solution.21 Our current policy, however, is to progressively discard such units that were drawn from donors who were never subsequently tested for HIV NAT and HCV NAT. Nevertheless, this attitude requires a very special care for RBC units with an exceedingly rare phenotype, e.g., Jr(a−b−) (JK−:1,−2), Hr− (RH−:18), Hr− (RH−:34), Hy− (DO−:4), or Jr(a−). There are no specific rules in France about an optimal inventory for a given rare blood type. However, the following criteria are usually taken into account when a decision has to be made about the destruction of rare RBC units from a donor who was never tested for NAT: alive or deceased blood donor; prevalence of the rare blood phenotype within the general population; and number of available frozen RBC units from other donors with the same rare blood group, including D, C, E, c, e, K, Fy a/Fy b, Jk a/Jk b, S/s antigen match. If we consider the example of the Jr(a−) rare blood group, nine Jr(a−) blood donors are known in France, and the current stock of Jr(a−) rare blood units is 15, among which 6 units (40%) are aged 10 years or more. HIV and HCV NAT have never been performed in five of these nine Jr(a−) donors, which affects 9 (60%) of the 15 Jr(a−) blood units. As a result, it does not seem reasonable to discard 60 percent of the Jr(a−) RBC units from the national stock, considering the highly sensitive ethical aspect of such a decision.

Rare RBC transfused units older than 10 years and the French hemovigilance data

Between 1994 and 2007, 118 RBC units older than 10 years were transfused. For every transfusion episode that involved an RBC unit aged 10 years or more, the CNRGS medical staff questioned the National Rare Blood Bank to learn whether a macroscopic hemolysis of the RBC supernatant was visible after the thawing-washing procedure. In addition, no extracellular hemoglobin measurement was performed. Between 1994 and 2007, 118 RBC units older than 10 years were transfused. For every transfusion episode that involved an RBC unit aged 10 years or more, the CNRGS medical staff questioned the National Rare Blood Bank to learn whether a macroscopic hemolysis of the RBC supernatant was visible after the thawing-washing procedure. In addition, no extracellular hemoglobin measurement was performed.
hemoglobin rise in the recipient was compatible with the one expected and whether there was any reported clinical or biologic transfusion reaction. Regarding the transfusion of the 118 units that were older than 10 years, no evidence for hemolysis of thawed RBCs and no transfusion reaction, clinical or biologic hemolysis, or transfusion ineffectiveness was reported that could be related to long-term storage beyond 10 years.

Transfusion of RBCs older than 10 years has always been legal in France. As a consequence, it did not appear necessary to perform a full prospective or retrospective study justifying the possible use of such RBCs. However, we may consider our observational data about the transfusion safety and efficiency of RBC units older than 10 years reliable for two main reasons.

The first reason lies in the close communication among all parties involved in the transfusion supply of rare RBC units, probably much stronger and closer than that of a standard blood transfusion. Indeed, every single rare RBC unit, delivery of which is exclusively authorized by the CNRGS, is strictly time-followed. The patient is carefully and closely monitored, and the transfusion outcome is subsequently discussed between the medical staff in charge of the patient and the CNRGS staff.

The second reason is the presence of an active hemovigilance program in France since 1994, the rules of which are clearly defined according to the French regulations.22–24 The report of every adverse transfusion reaction by anyone within the transfusion chain is strictly mandatory, whatever the intensity, seriousness, and imputability of the reaction. A transfusion reaction is defined in France as an “unexpected or undesired event, due or susceptible to be due to the transfusion of a blood product.”25 The precise list of the clinical or biologic manifestations that may occur during or after a blood transfusion and that must lead to a hemovigilance report is legally stipulated.23-24 Any unexpected or undesired clinical manifestation has to be reported. These include hemoglobinuria; positive direct antiglobulin test; rise in bilirubin; and failure of expected hemoglobin increment, estimated through the hemoglobin measurement performed within 24 hours after transfusion.

Between 1994 and 2007, no transfusion reaction was reported for any of the 118 rare RBC units older than 10 years that were transfused. As a result, our observational data may provide new arguments for the safe and efficient use of very long-term cryopreserved RBC units.

Conclusions

Between 1994 and 2007, 118 rare blood units aged 10 years or more (6.0%) and 8 (0.4%) aged 20 years or more were transfused in France. The maximum age of a rare RBC transfused unit was approximately 24 years. No transfusion reactions potentially linked to the long-term preservation of blood units older than 10 years were reported, either by the medical staff involved in the careful monitoring of the patients or through the French hemovigilance program, which requires a strict mandatory report of every transfusion adverse reaction, whatever its intensity or imputability. It has been quite recently suggested to extend the 10-year restriction for frozen RBC units in the United States.14 Based on our experience and observational data, we may consider it to be safe and efficient to transfuse rare frozen RBC units older than 10 years. Such a policy allows keeping a larger stock of frozen RBC units for the rarest phenotypes, to urgently meet the national or even international requests for rare blood. However, the question of a maximum time limit for rare frozen blood storage is still raised. Indefinite storage, theoretically possible, may be difficult because of ever-growing quality management requirements and constraints. The period of validity of rare frozen RBC units should ideally be the same for all rare blood banks throughout the world. This point deserves consideration by the ISBT Working Party on Rare Donors to establish an international consensus.

Acknowledgments

The authors thank the staff of the CNRGS (National Institute of Blood Transfusion, Paris, France) and the National Rare Blood Bank (French Blood Establishment, Créteil, France) for their high commitment to the French Rare Blood Program. The authors are also grateful to Sophie Maréchal-Françon (National Institute of Blood Transfusion, Paris, France) for reading the manuscript.

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The polymorphism nt 76 in exon 2 of SC is more frequent in Whites than in Blacks

A. Fuchisawa, C. Lomas-Francis, K. Hue-Roye, and M.E. Reid

The Scianna blood group system comprises seven antigens encoded by alternative forms of SC. The SC gene also has two polymorphisms in the leader sequence, at nucleotides 54 (C/T, silent) and 76 (C/T, 26His/Tyr) in exon 2, which are not involved in expression of blood group antigens. The nucleotide change at position 76 has an NlaIII restriction enzyme site; thus, DNA samples from 100 Caucasians and 100 African Americans were analyzed for the SC nucleotide 76 change. DNA from Caucasian and American donors was tested by polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) using the restriction enzyme NlaIII. In selected samples, sequencing of exon 2 was performed. PCR-RFLP results for samples from 100 donors (mostly Caucasian) and 100 African American donors (400 alleles) showed the nucleotide 76T variant had a prevalence of 25 percent in Whites and 5 percent in African Americans. In 11 samples (2 C/C, 3 C/T, and 6 T/T) sequencing of exon 2 confirmed the presence of the expected nucleotides at position 76. The allele frequency in Caucasians was 0.75 for nt76C and 0.25 for nt76T. In African Americans, the frequencies were, respectively, 0.95 and 0.05.

**Key Words:** alloantibodies, blood groups, ERMAP, ethnic diversity, human polymorphisms, Scianna

The Scianna (Sc) blood group system (ISBT 013) comprises seven antigens that are encoded by alternative forms of SC (or ERMAP).1–3 Sc1 and Sc2 have an antithetical relationship and are, respectively, high- and low-prevalence antigens. Sc3 is expressed on all RBCs except those from individuals with the Scnull phenotype. Sc4 (Rd) is a low-prevalence antigen, and Sc5 (STAR), Sc6 (SCER), and Sc7 (SCAN) are high-prevalence antigens whose antithetical antigens have not been reported. The molecular bases associated with the antigens are known. In addition, two nucleotide (nt) polymorphisms (nt54C>T, a silent change; and nt76C>T, predicted to encode His26Tyr) are in the sequence of nucleotides that encode the leader sequence of the SC glycoprotein, which is not present on the RBC.1,2 We report the analysis of DNA samples from 100 donors (mostly Caucasians) and 100 African Americans for the prevalence of the SC nt76 change.

**Materials and Methods**

**Blood samples**

Samples from 100 random (mostly Caucasian) donors and 100 donors who had either the homozygous mutated FY-GATA box (n = 20) or self-identified as being African American (n = 80) were obtained under Institutional Review Board–approved protocols and tested.

**Sequence analysis of genomic DNA for SC**

Genomic DNA was isolated from whole blood (QIAamp DNA Blood Mini Kit, QIAGEN, Inc., Valencia, CA). The regions of SC that included and flanked exons 2, 3, and 4 were amplified separately using the oligonucleotide primers listed in Table 1. The primers were synthesized by Life Technologies, Inc. (Gaithersburg, MD). Five microliters of DNA per reaction were amplified by 5 U of Taq DNA polymerase (HotStarTaq, QIAGEN Inc.) in a 50-μL reaction mixture containing 2.5 mM MgCl2, 1× PCR buffer, 0.2 mM dNTPs, and 100 ng of forward and reverse primer. Amplification was achieved during 35 cycles with a final extension time of 10 minutes. The PCR was performed using 62°C as the annealing temperature. The PCR products were sequenced in the Laboratory of Microchemistry at the New York Blood Center using an ABI 373XL sequencer (Applied Biosystems, Inc., Foster City, CA), and ABI Big Dye reagents with BD Half-Term (GenPak, Stony Brook, NY). The PCR products were directly sequenced using the forward and reverse primers that were used for PCR amplification.

**Table 1. Primer sequences and expected products**

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequences 5′ to 3′</th>
<th>Region amplified (amplicon size)</th>
<th>RFLP using NlaIII</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scd2F</td>
<td>cagccgcttgtgctgtctc</td>
<td>Exon 2 (161 bp)</td>
<td>Wild type: 121 bp, 40 bp</td>
</tr>
<tr>
<td>Scd2R</td>
<td>ggacaggcgaggaagagg</td>
<td>Exon 2 (161 bp)</td>
<td>Variant: 161 bp</td>
</tr>
<tr>
<td>Scd3F</td>
<td>cctcccagtggccctgtc</td>
<td>Exon 3 (149 bp)</td>
<td>Not applicable</td>
</tr>
<tr>
<td>Scd3R</td>
<td>cccactacgtccttgg</td>
<td>Exon 4 (188 bp)</td>
<td>Not applicable</td>
</tr>
</tbody>
</table>

*Primer sequences are written in lowercase letters because they are located in the introns.

**PCR-RFLP analysis**

One hundred DNA samples mostly from Caucasians and 100 from African Americans were amplified for the region of SC that included and flanked exon 2, using the Scd2F and Scd2R oligonucleotide primer pair (Table 1). The PCR products (161 bp) were digested using the restriction enzyme NlaIII. The restriction enzyme site of NlaIII is present in the wild-type DNA with nt 76C and ablated in the variant nt 76T. The expected sizes for bands in the wild type were 121 bp and 40 bp.
and 40 bp, and for the variant, 161 bp (uncut). There is no restriction site to distinguish the wild-type nt54C from the variant nt54T.

Results

PCR-RFLP analysis on random samples

PCR-RFLP analysis for nt76C/T in the 100 random (mostly Caucasian) donors resulted in 62 samples that were consistent with the expected digestion pattern of the wild type (76C), 11 samples that were consistent with the expected digestion pattern for homozygosity for the variant (76T), and 27 that were consistent with the expected digestion pattern for heterozygosity for the variant (76C/T). For the 100 samples from African American donors, 91 were consistent with the expected digestion pattern of the wild type (76C), 1 was consistent with the expected digestion pattern for homozygosity for the variant (76T), and 8 were consistent with the expected digestion pattern for heterozygosity for the variant (76C/T). These results are summarized in Table 2.

Sequence analysis of the random samples

Eleven of the random donor samples, which demonstrated either a homozygous change (n = 6), heterozygous change (n = 3), or wild type (n = 2), were sent for direct sequencing. Sequence analysis of the six samples homozygous for the variant were confirmed to be nt76T and also shown to be homozygous for the variant nt54T. Sequence analysis of the three samples heterozygous for the variant selected on the basis of the PCR-RFLP assay were confirmed to be heterozygous nt76C/T and also shown to be heterozygous for nt54C/T. Sequence analysis of the two wild-type samples selected on the basis of the PCR-RFLP assay were confirmed to be homozygous wild type for nt76C and shown to be also homozygous wild type for nt54C.

Discussion

The SC nt76C>T change (His26Try) is located in the sequence of nucleotides that encode the leader sequence, which is cleaved and not present in the RBC-bound ERMAP. The SC nt54C>T silent change is also in this sequence but does not give rise to or ablate a restriction enzyme site. In the 11 samples analyzed by sequencing, either both nt54 and nt76 changes were present or neither was present. Whether these are linked polymorphisms in the heterozygotes requires plasmid cloning of the PCR products to evaluate single sequences. In our study, the allele frequency in Caucasians was 0.75 for 76C and 0.25 for nt76T. In African Americans, the frequencies were 0.95 and 0.05, respectively.

Table 2. Results of PCR-RFLP using NlaIII

<table>
<thead>
<tr>
<th>Sample Source</th>
<th>nt 76C (W/W)</th>
<th>nt 76 C/T (W/V)</th>
<th>nt 76T (V/V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Random donors</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(mostly Caucasian)</td>
<td>62</td>
<td>27</td>
<td>11</td>
</tr>
<tr>
<td>African Americans</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n = 100)</td>
<td>91</td>
<td>8</td>
<td>1</td>
</tr>
</tbody>
</table>

V = variant; W = wild type.

Acknowledgment

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References


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Notice to Readers

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Nonhemolytic passenger lymphocyte syndrome: donor-derived anti-M in an M+ recipient of a multiorgan transplant

A.T. Makuria, A. Langeberg, T.M. Fishbein, and S.G. Sandler

Passenger lymphocyte syndrome (PLS) is a well-recognized complication that may follow a hematopoietic progenitor cell or solid-organ transplant. Typically, the syndrome presents as acute hemolysis of the recipient’s RBCs, which have become serologically incompatible with blood group antibodies formed by passively transfused donor-origin B lymphocytes. Most cases involve anti-A or anti-B. However, there are cases involving non-ABO serologic incompatibility, as well as cases in which the serologic incompatibility was not associated with clinical evidence of hemolysis. This report describes a case of passenger lymphocyte syndrome in an M+ recipient who developed anti-M after receiving a multiorgan transplant from an M- cadaver donor. Although the temporal events and serologic findings were consistent with a diagnosis of PLS, there was no evidence of in vivo hemolysis associated with the identification of a newly formed anti-M. This report includes a literature review of other case reports of PLS associated with non-ABO antibodies in solid-organ and hematopoietic progenitor cell transplant recipients. Immunohematology 2009:25:24–27.

Key Words: passenger lymphocyte syndrome, hemolysis, solid-organ transplant

Patients undergoing hematopoietic stem cell or solid-organ transplants may develop hemolytic reactions if viable B lymphocytes are passively transfused with the transplant and form antibodies to blood group antigens expressed on the recipient’s RBCs (passenger lymphocyte syndrome, PLS). Ideally, all transplant candidates would be matched with ABO-identical donors. However, because of the shortage of ABO-matched donors and because genes encoding human leukocyte antigens (HLAs) and controlling A and B blood group antigens segregate separately, ABO matching is often compromised to achieve an optimal HLA donor-recipient match.1

PLS was first observed in recipients of ABO-mismatched (nonidentical) bone marrow transplants in whom hemolysis was caused by anti-A or anti-B of donor origin.2,3 The syndrome continues to be observed involving anti-A or anti-B,4 but is recognized increasingly to be a potential complication of mismatched antigens of other blood group systems, as well as in recipients of solid-organ transplants.5

We report the first case, to our knowledge, of PLS caused by anti-M in an M+ recipient of a solid-organ or a hematopoietic stem cell transplant. Our patient did not exhibit clinical signs of hemolysis. The absence of clinical evidence of hemolysis in our patient is consistent with the usual clinical outcome for serologic incompatibility involving anti-M in the presence of M+ RBCs.

Case Report

A 44-year-old man with idiopathic small bowel motility disorder experienced severe liver failure associated with total parenteral nutrition therapy. His past history included a colon resection and correction of pseudo-obstruction, but no blood transfusions. He underwent a successful multi-organ transplant including stomach, small intestine, large intestine, pancreas, and liver from a cadaver donor and was discharged 6 weeks later. Immediate posttransplantation treatment consisted of tacrolimus (Prograf, Astellas Pharma US, Dearfield, IL) 3 mg/day, adjusted to maintain a blood level of 18 to 20 ng/mL; sirolimus (Rapamune, Wyeth Pharmaceuticals, Philadelphia, PA); 4 mg/day, adjusted to maintain a blood level of 5 to 10 ng/mL; and methylprednisolone (Solu-Medrol, Pfizer, NY, NY), 500 mg/day, and tapered.

The transplant recipient’s blood type was group A, D+. No blood group antibodies were detected in his plasma by a highly sensitive solid-phase antibody screen (ABS2000, Immucor, Norcross, GA) before transplantation. The organ donor’s blood type was group A, D+. During the peri-transplant period, the transplant recipient was transfused with 5 units of frozen plasma and 9 units of gamma-irradiated RBCs (leukocyte-reduced).

The patient did not require blood transfusions until posttransplant day 16, when the transfusion service performed the first posttransplant antibody screen (Table 1). On that day, the recipient’s indirect and direct antiglobulin test results were positive for the first time. Anti-M was detected in his plasma by an automated solid-phase antibody screen (Capture-R Ready-Screen 4, Immucor/Gamma, Norcross, GA; score = 3/7) and by a standard manual tube identification panel (Panocell-10, Immucor; Table 1). Anti-M was identified on his circulating RBCs by rapid acid elution (Gamma Elu-Kit II, Immucor). The serologic characteristics were consistent with a naturally occurring anti-M, i.e., an antibody that reacted selectively with M+ RBCs by immediate spin at room temperature, but not if the patient’s plasma and reagent RBCs were prewarmed to 37°C.6 Because alloimmunization to transfused M+ RBCs would occur only in an M-, not an M+, recipient, we considered the possibility of PLS caused by anti-M of donor origin. The diagnosis of PLS was confirmed when a sample of the organ donor’s RBCs, which was obtained through the organ retrieval service, was typed as M-. The organ donor’s hospital reported
a negative antibody screen on admission, but the method used (ProVue, Ortho Diagnostics, Raritan, NJ) rarely detects antibodies reacting at room temperature, which, typically, are not clinically significant for routine blood transfusions. A sample of the organ donor’s pretransplantation plasma was not available for a repeat antibody screen, this time at room temperature, as is required for detecting most examples of anti-M. The transplant recipient’s positive direct antiglobulin test result persisted until posttransplant day 45. During the entire posttransplantation period, there was no clinical evidence of hemolysis.

Table 1. Results of immunohematologic studies in recipient after transplantation

<table>
<thead>
<tr>
<th>Days after transplant</th>
<th>Anti- IgG/C3d (prewarmed)</th>
<th>Anti- IgG (prewarmed)</th>
<th>Anti-C3d (prewarmed)</th>
<th>Saline/RT</th>
<th>Anti-IgG + C3d (prewarmed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-1</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>M+ or M- RBCs: 0 (NT)</td>
</tr>
<tr>
<td>+16</td>
<td>1+</td>
<td>1+w</td>
<td>0</td>
<td>1+</td>
<td>M+ RBCs: 1+ (0)</td>
</tr>
<tr>
<td>+21</td>
<td>1+</td>
<td>1+</td>
<td>0</td>
<td>1+</td>
<td>M+ RBCs: 1+ (0)</td>
</tr>
<tr>
<td>+29</td>
<td>1+</td>
<td>1+</td>
<td>0</td>
<td>1+</td>
<td>M+ RBCs: 1+ (0)</td>
</tr>
<tr>
<td>+45</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1+*</td>
<td>M+ RBCs: 0 (NT)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>M- RBCs: 0 (NT)</td>
</tr>
</tbody>
</table>

NT = not tested; RT = room temperature.

Discussion

PLS is a well-described immune hemolytic syndrome after solid-organ, as well as hematopoietic progenitor cell, transplantation. It has been observed after transplantation of kidney, lung, heart-lung, liver, pancreas, and pancreas-spleen, as well as hematopoietic progenitor cell (HPC) transplantation. In our case, the patient received a cadaver stomach, small intestine, large intestine, pancreas, and liver. In this newly described technique, the patient’s duodenum and pancreas were retained, but because of the need to replace his stomach, the donor’s pancreas and intestine were also transplanted en-bloc. PLS, typically characterized by acute hemolysis caused by blood group antibody(s), occurs when donor-origin B lymphocytes are passively transferred with the transplant and subsequently form antibodies to the blood group antigens on the recipient’s RBCs.

Although most cases of PLS involve the ABO blood group system, PLS has been associated with serologic incompatibilities in other blood group systems. Table 2 summarizes a literature review of other reports of PLS involving non-ABO blood group antigens in hematopoietic progenitor cell and solid-organ transplant recipients. We were unable to find a prior report of PLS involving anti-M in a solid-organ transplant recipient. PLS, which may be regarded as a variant of graft-versus-host disease (GVHD), rarely occurs after blood transfusions today, because hospital transfusion services routinely exclude blood components from donors known to have alloantibodies and gamma irradiate blood components for transplant recipients to prevent transfusion associated GVHD.

The immune response in PLS may be primary or secondary (anamnestic). Hemolysis may be acute or delayed, or as in our case, there may be serologic incompatibility without hemolysis. The time of onset of hemolysis varies from days to weeks, but is most often observed between days 5 and 15 after hematopoietic progenitor cell or solid-organ transplants.

In our patient, detection of anti-M in an M+ transplant recipient was the specific event that raised the possibility of PLS. The diagnosis was confirmed by demonstrating that the recipient’s RBC phenotype was M+, whereas the donor’s RBC phenotype was M−. An alternative interpre-

Table 2. Literature review of passenger lymphocyte syndrome involving non-ABO blood group antigens in hematopoietic stem cell and solid-organ transplant recipients

<table>
<thead>
<tr>
<th>Organ transplant (n)</th>
<th>Donor’s antibody(ies)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidney (2)</td>
<td>Anti-D</td>
<td>9</td>
</tr>
<tr>
<td>Kidney (1)</td>
<td>Anti-D</td>
<td>10</td>
</tr>
<tr>
<td>Kidney (1)</td>
<td>Anti-D</td>
<td>11</td>
</tr>
<tr>
<td>Kidney (1)</td>
<td>Anti-D</td>
<td>12</td>
</tr>
<tr>
<td>Kidney (1)</td>
<td>Anti-D</td>
<td>13</td>
</tr>
<tr>
<td>Kidney (1)</td>
<td>Anti-c</td>
<td>14</td>
</tr>
<tr>
<td>Liver (1)</td>
<td>Anti-D, -C, -k</td>
<td>4</td>
</tr>
<tr>
<td>Liver (1)</td>
<td>Anti-D</td>
<td>15</td>
</tr>
<tr>
<td>Liver (1)</td>
<td>Anti-D</td>
<td>16</td>
</tr>
<tr>
<td>Liver (1)</td>
<td>Anti-Jk¹</td>
<td>17</td>
</tr>
<tr>
<td>Liver (1)</td>
<td>Anti-K, -Fy¹</td>
<td>18</td>
</tr>
<tr>
<td>Pancreas (1)</td>
<td>Anti-D</td>
<td>19</td>
</tr>
<tr>
<td>Lungs (2)</td>
<td>Anti-D</td>
<td>4</td>
</tr>
<tr>
<td>Heart-lung (1)</td>
<td>Anti-D</td>
<td>20</td>
</tr>
<tr>
<td>Kidney and pancreas (1)</td>
<td>Anti-Fy¹</td>
<td>18</td>
</tr>
<tr>
<td>Bone marrow (1)</td>
<td>Anti-Le¹</td>
<td>21</td>
</tr>
<tr>
<td>Bone marrow (1)</td>
<td>Anti-D</td>
<td>22</td>
</tr>
<tr>
<td>Bone marrow (1)</td>
<td>Anti-D</td>
<td>23</td>
</tr>
<tr>
<td>Bone marrow (2)</td>
<td>Anti-D</td>
<td>3</td>
</tr>
<tr>
<td>Bone marrow (1)</td>
<td>Anti-D, -C, -E</td>
<td>3</td>
</tr>
<tr>
<td>PBPC (1)</td>
<td>Anti-Jk¹</td>
<td>24</td>
</tr>
<tr>
<td>PBPC (1)</td>
<td>Anti-Jk¹</td>
<td>25</td>
</tr>
<tr>
<td>PBPC (2)</td>
<td>Anti-Jk¹</td>
<td>26</td>
</tr>
<tr>
<td>PBPC (1)</td>
<td>Anti-Jk¹, -E, -Dp</td>
<td>27</td>
</tr>
</tbody>
</table>

n = number of cases
PBPC = peripheral blood progenitor cell
tation, although less likely given the temporal and clinical events, is that the M+ transplant recipient developed auto-anti-M, coincidentally, during the narrow window when one would anticipate onset of PLS. Case reports of these uncommon examples of auto-anti-M have been summarized by Issitt and Anstee. Many transfusion services have adopted serologic methods to avoid routine detection of room temperature, saline-reactive (only) “naturally occurring” alloantibodies, because they are considered to be clinically insignificant. In our case, anti-M was detected as a new antibody during a routine posttransplantation antibody screen. The antibody was detected using a highly sensitive solid-phase assay for routine antibody screens and a room temperature, saline immediate spin reading for the crossmatch. Anti-M in our patient was not associated with clinical evidence of hemolysis of his M+ RBCs. Absence of clinical evidence of hemolysis is the typical clinical finding for most, but not all, examples of anti-M when present in the plasma of patients who may be transfused with allogeneic M+ RBCs.

References


Addisalem T. Makuria, MD, Pathology Resident, Department of Pathology, Albert Langeberg, MT, Team Leader, Blood Bank, Thomas M. Fishbein, MD, Director Small Bowel/Pediatric Transplantation, Department of Surgery, and S. Gerald Sandler, MD, (corresponding author) Director Transfusion Medicine, Georgetown University Hospital, 3800 Reservoir Road, NW, Washington, DC 20007.
Development and validation of a fluorescent microsphere immunoassay for anti-IgA

K.M. Rumilla, J.L. Winters, J.M. Peterman, E.A. Jedynak, and H.A. Homburger

Anti-IgA may cause anaphylactic transfusion reactions in IgA-deficient individuals. Testing for IgG anti-IgA is useful to identify persons at risk. This report describes an immunoassay for anti-IgA that uses polyclonal IgA coupled to fluorescent microspheres as an immunosorbent. Anti-IgA is detected by phycoerythrin-labeled anti-IgG. The assay is calibrated in arbitrary units by use of a serum that contains anti-IgA. Dose-response studies with sera that contain anti-IgA showed positive responses at dilutions up to 32-fold greater than the dilution used to test patients’ samples. Inhibition studies with purified IgA and IgA-deficient serum showed no inhibition with IgA-deficient serum and complete inhibition with soluble IgA. Clinical tests performed in more than 90 assays had a CV of 13.6 percent for measurements of an internal positive control. The fluorescent immunoassay method is rapid, reproducible, and sensitive to low concentrations of IgG anti-IgA.

Key Words: microsphere immunoassay, anti-IgA, antibodies, validation

Selective IgA deficiency is the most common humoral immunodeficiency. The reported frequency varies widely in different populations. The frequency of IgA deficiency in Caucasians of Northern European ancestry is estimated to be approximately 1 in 600 with lesser frequencies reported in Asian populations. The definition of IgA deficiency is determined in part by the analytic sensitivity of immunoassay methods used to measure IgA. Commonly used nephelometric immunoassays are capable of detecting IgA at concentrations greater than 0.3 mg/dL, and 0.5 mg/dL is often cited as a cutoff concentration to define IgA deficiency in adults. A lower concentration, 0.05 mg/dL, is used to define IgA-deficient blood donors and blood products. Approximately one third of persons with IgA deficiency have detectable anti-IgA. The mechanisms responsible for development of anti-IgA in IgA-deficient individuals are unknown.

Anti-IgA has been reported to be associated with severe anaphylactic transfusion reactions in IgA-deficient patients, and antibodies of both the IgG and IgE isotypes have been described. Although it is assumed that IgE antibodies may be capable of eliciting anaphylactic transfusion reactions, not all investigators have been able to identify such antibodies, and the mechanism(s) by which anti-IgA produces anaphylactic reactions remains unclear. Although several studies suggest that IgG anti-IgA is associated with transfusion reactions, reported data also indicate that some patients with anti-IgA fail to react to IgA and reactions have been described in patients who have no demonstrable antibodies. Nevertheless, accurate identification of IgA-deficient persons with anti-IgA is important to investigate the possible etiology of immediate hypersensitivity transfusion reactions and to facilitate the optimal use of blood products obtained from IgA-deficient donors.

A variety of analytical methods have been used to detect anti-IgA, including double immunodiffusion with purified IgA paraproteins, hemagglutination, two-site immunometric assays, flow cytometric immunoassays, and enzyme immunoassays. In this report we describe the development, analytical validation, and clinical performance of a fluorescent microsphere immunoassay for IgG anti-IgA. The assay has excellent analytic sensitivity and reproducibility and a rapid turnaround time, and makes use of commercially available reagents and instrumentation.

Materials and Methods

Institutional Review Board approval was obtained for use of residual patients’ sera and to review the medical records of all patients tested as part of this study. Previously tested sera (n = 7) shown to contain IgG anti-IgA were available from the Division of Transfusion Medicine. These sera were from healthy blood donors who had fulfilled all donation criteria defined by the U.S. Food and Drug Administration and who had been found to be negative on blood donor infectious disease testing. The donors were identified by a routine screening process used in our collection center to identify IgA-deficient donors as previously described. None of the seven donors had previously been transfused, and therefore none had a clinical history of anaphylaxis to IgA. All represented selective IgA deficiency with anti-IgA. Serum from a patient with IgA deficiency, a history of previous anaphylactic transfusion reactions, and high levels of IgG anti-IgA was tested at serial dilutions (described later) and was used as the assay calibrator (1 in 1280 dilution) and internal control (1 in 640 dilution) in all subsequent assays. Serum samples from 133 healthy adults were tested to determine background binding of IgG to the IgA-coupled fluorescent microspheres (described later), and specimens submitted for testing in the clinical laboratory obtained from 724 patients suspected of having immediate hypersensitivity transfusion reactions were used to determine the frequency of IgG anti-IgA in patients. Additional clinical information for the 724 patients, beyond the fact that they were suspected of having an immediate hypersensitivity...
reaction to transfusion, was not available. The IgA status of the 133 healthy subject and 724 patient samples was determined by nephelometry using a Dade Behring BN II nephelometer (Siemens Healthcare Diagnostics, Deerfield, IL) and Dade Behring IgA reagents according to manufacturer’s instructions.

Preparation of IgA-Coupled Fluorescent Microspheres

Purified, human polyclonal IgA (Fitzgerald Industries International Inc., Concord, MA) was coupled covalently to fluorescent microspheres (SeroMap D microspheres, Luminex Corp., Austin, TX) by a modified carbodiimide method as follows: An aliquot of $50 \times 10^6$ microspheres was reacted in the dark for 20 minutes with 100 μL of 50 mg/mL N-hydroxysulfosuccinimide and 100 μL of 50 mg/mL 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide in a final volume of 1 mL of 100 mM monobasic sodium phosphate, pH 6.2. After activation, the microspheres were washed twice with 50 mM MES, pH 5.0 (2-[N-morpholino]ethanesulfonic acid), and 1 mg of purified IgA in 1.5 mL of MES, pH 5.0, was added. The microspheres were then incubated in the dark for 2 hours at room temperature. The IgA-coupled microspheres were washed, resuspended in 0.1 M PBS with 0.05% Tween 20, and incubated in the dark for an additional 30 minutes. After this incubation, the IgA-coupled microspheres were diluted 1 in 1600 in PBS with 1% BSA, 10% sodium azide, and 100 μL/L of protease inhibitor cocktail #P2714 (Sigma Chemical Co., St. Louis, MO) to a final concentration of 31,350 microspheres/mL.

Dose-Response and Dose-Inhibition Analyses of IgA-Coupled Fluorescent Microspheres

Maximum specific binding of IgG antibodies to the IgA-coupled microspheres was tested in a standard assay (described later) by incubating 100-μL aliquots of diluted microspheres with decreasing serial twofold dilutions (1 in 20 through 1 in 81,920) of positive serum. The specificity of binding of IgG to the IgA-coupled microspheres was tested in inhibition assays with purified polyclonal IgA (Fitzgerald Industries International Inc.) and IgA-deficient serum inhibitors. Aliquots of diluted, purified IgA or diluted, IgA-deficient serum (100 μL in PBS with 1% BSA diluent) were incubated overnight with an equal volume of diluted (1 in 40) positive serum. The incubated mixtures were then tested in a standard assay, and inhibition by purified IgA or IgA-deficient serum was calculated by comparing the fluorescent responses of the incubated mixtures to results for the uninhibited positive serum. Nonspecific binding of IgG protein to the IgA-coupled microspheres was determined by testing sera from healthy adults in standard assays. A limit of inhibition was not chosen as inhibition is dose dependent (see Results section).

Standard Assay Format

Tests for IgG anti-IgA were performed in filter microtiter plates (MABV N1250, Millipore Inc., Billerica, MA) as follows: 100 μL of the positive calibrator, internal control, or test sera diluted 1 in 40 in PBS with 1% BSA was incubated with 100 μL of IgA-coupled microspheres in darkened, covered microtiter plates for 30 minutes at room temperature. After incubation, the microspheres were washed five times with 1% BSA in PBS, then 100 μL of diluted, R-phycocerythrin conjugated, goat anti-human IgG (Jackson ImmunoResearch, West Grove, PA) was added to each well, and the plates were again incubated in the dark for 1 hour at room temperature. Binding of IgG antibodies to the microspheres was measured using a Luminex 100 platform (Luminex Corp.) according to the manufacturer’s instructions.

Results

Specific binding of IgG antibodies to the IgA-coupled microspheres was determined by testing serial twofold dilutions of known positive sera. Results of a typical dose-response analysis for one strongly positive serum are shown in Figure 1. The maximum response for this serum exceeded 20,000 arbitrary FRU at a 1 in 40 dilution (Fig. 2). This particular positive serum was chosen as a calibrator and internal control for all subsequent assays. The serum was used as a calibrator at a 1 in 1280 dilution (100 U, described earlier) and as an internal control at a 1 in 640 dilution (200 U, previously described).

Nonspecific binding of IgG to the IgA-coupled microspheres was determined by testing sera from 133 healthy, non–IgA-deficient adults. The results are shown in Figure 2 for tests performed on serial twofold dilutions of sera from 1 in 20 to 1 in 80. The mean, standard deviation, and maximum response for sera in the normal group displayed in Figure 2 were 984, 819, and 5884 FRU, respectively. All but five sera from these healthy adults had fewer than 3000 FRU at a 1 in 40 dilution. The upper limit of nonspecific binding in the fluorescent microsphere immunoassay (5079 FRU) was calculated as the mean plus five standard deviations for tests of normal sera at a 1 in 40 dilution using the standard assay format. This level of binding corresponded approximately to the response of the positive serum calibrator (4206 FRU), which was assigned an arbitrary value of 100 U. The 100-U value for the assay calibrator obtained by testing this serum at a 1 in 1280 dilution represents a 32-fold greater dilution of the positive serum calibrator than is used routinely to test patients’ specimens. The cutoff for
defining a positive result was established at 200 U (a 1 in 640 dilution of the positive serum, 7165 FRU) to avoid misclassifying any patients’ sera as positive that did not contain IgG anti-IgA. Results between 100 and 200 U were classified as equivocal. In this classification, serum from one healthy adult shown in Figure 2 would be classified as equivocal, and no sera would be classified as positive.

Since this immunoassay was approved for routine use in the clinical laboratory, we have performed more than 90 assay runs for IgG anti-IgA on 724 persons during a period of approximately 18 months using two different lots of IgA-coupled microspheres. Interassay reproducibility across all 90 assay runs as measured by the CV of replicate measurements of the internal control was 13.6 percent (data not shown). Tests performed on sera from these 724 persons yielded 46 results greater than 100 U (6.3%). In this group, 38 sera had results greater than 200 U, and 8 sera yielded equivocal results. The medical histories of four patients seen at this institution who had anaphylactic reactions and results greater than 200 U were available for review: all had IgA deficiency as revealed by serum IgA levels of less than 0.5 mg/dL.

Discussion

The fluorescent microsphere immunoassay described in this report compares favorably with several previously reported methods for detecting anti-IgA.12–17 The assay method has excellent analytical sensitivity as revealed by dose-response studies with positive sera and inhibition studies with soluble IgA. In addition, the results show excellent discrimination between nonspecific binding and binding produced by IgG anti-IgA. Only slightly more than 1 percent of sera tested in the clinical laboratory with this
assay yielded equivocal results during more than 18 months of use, and those positive sera, obtained from four patients whose medical histories were available for review, were confirmed as being associated with immediate hypersensitivity transfusion reactions and IgA deficiency.

Overall, the frequency of positive test results among the 724 patient samples tested during 18 months of use was 5.2 percent. In the paper published by Sandler et al., \textsuperscript{11} anti-IgA was detected in 18 percent of sera submitted for testing. How can this discrepancy with our results be explained? The frequency of positive results is determined primarily by the selectivity of physicians in choosing sera for testing. It is worth noting that our institution accepts specimens from laboratories around the world for testing, and it must be assumed that not all requests are supported by strong clinical evidence of prior transfusion reactions or even diminished levels of IgA in blood. Therefore, no direct comparison can be made with the experience reported by Sandler et al.\textsuperscript{11}

The initial methods for detecting anti-IgA described in the medical literature used an immunodiffusion technique that was sensitive to microgram per milliliter concentrations of antibodies, required purified IgA myeloma proteins as reagents, and took several days to generate test results.\textsuperscript{12,20} Subsequent generations of immunoassays, which included hemagglutination assays, modified radioimmunoassays, and sandwich immunoradiometric assay methods, had improved analytical sensitivity and were capable of detecting nanogram per milliliter concentrations of anti-IgA.\textsuperscript{11,15} Despite having improved analytical sensitivity, these methods also used purified IgA myeloma proteins as reagents. The radioassay methods took days to perform and had the added disadvantage of using radionuclides in the assay procedures, which limited the shelf life of test reagents and required monitoring of testing personnel and disposal of radioactive wastes. Hemagglutination assays were more rapid, but had high levels of interassay variability and generated occasional false-negative results attributable to prozone phenomena. Sandwich immunoradiometric assays could be adapted to the use of purified polyclonal IgA as an antigen source, but these methods still required the use and disposal of radionuclides. More recently, enzyme immunoassay, flow cytometric immunoassay, and particle gel immunoassay methods have been used to measure anti-IgA.\textsuperscript{13,14,16} Compared with enzyme immunoassay, the flow cytometric assay is reported to be more reproducible as larger numbers of latex microbeads are used to test each individual serum specimen compared with one or two wells in a typical microtiter enzyme immunoassay. The fluorescent microsphere immunoassay we have developed is similar in design to a flow cytometric immunoassay. Both assays rely on optical measurement of fluorescence generated by the binding of fluorochrome-conjugated anti-IgG to specific antibodies bound to IgA-coupled beads. In the fluorescent microsphere immunoassay, IgA is coupled covalently to fluorescent microspheres. Covalent coupling of IgA to the microsphere immunosorbent reagent contributes to a long shelf life and very low nonspecific binding. We have noted stable binding of calibrator and control sera to the IgA-coupled microspheres for several months of use. In other respects, the fluorescent microsphere and flow cytometric assays appear quite comparable, although it is not possible to compare the analytic sensitivity of these methods without performing tests on the same sera.

As noted earlier, the fluorescent microsphere immunoassay for IgG anti-IgA we developed has proved reliable for more than 18 months of routine use in the clinical laboratory. Compared with the immunoradiometric assay we used for several years, the fluorescent microsphere immunoassay has improved our practice by markedly reducing the time required to evaluate patients who might be at risk of anaphylactic transfusion reactions or might require blood products free of IgA. The results of the microsphere immunoassay are available within 4 hours, whereas those of the immunoradiometric assay require 48 hours to be available. This has the potential to decrease utilization of the rare IgA-deficient plasma product inventory maintained at our institution.

References


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Kandelaria M. Rumilla, MD, MN, Jeffrey L. Winters, MD (corresponding author), Jessica M. Peterman, BS, Eric A. Jedynak, BS, and Henry A. Homburger, MD, Department of Laboratory Medicine and Pathology, Division of Transfusion Medicine, Mayo Clinic, 200 First St. SW, Rochester, MN 55905.
Charles Salmon, MD (1925—2009)

Charles Salmon was born in Trédaniel (Côtes-d’Armor) on February 13, 1925. With his doctorate in medicine (1951), he occupied a position of first assistant at the Institut Pasteur de Lille, and he created a laboratory for blood groups at the Departmental Center of Blood Transfusion (CDTS) at Saint-Antoine Hospital in Paris, the same place where Professor Jean Dausset discovered the first antibody against white blood cells that would define the HLA system.

Charles Salmon devoted his entire life to blood groups and their major applications in blood transfusion; his works are located at the crossroads of several disciplines, including hematology, transplantation, immunogenetics, and human genetics. He obtained his doctoral dissertation-ès-sciences in 1960 by developing a thermodynamic study of antigen-antibody reactions, which revealed for the first time the relationship between physicochemical properties of natural antibodies in the ABO system and the nature of red cell antigens. These original studies enabled him to describe physicochemical changes between antibodies and antigens in various normal and pathologic conditions, such as acute leukemia.

In 1965, Charles Salmon became eligible for the aggregation of medicine, allowing him to become a professor and teach at a university. He created a research unit (INSERM U76) on blood groups in 1967 that was a multidisciplinary research team of physicians and scientists assembled to study blood groups in their many facets: serology; immunology; genetics; biochemistry; and, later, molecular biology. With his team, he described the existence of the “dangerous universal donor,” proposed a classification of autoantibodies responsible for autoimmune hemolytic disease and their specificity and etiology, and defined a hierarchy of immunogenic antigens responsible for posttransfusion immunizations. He made many other original observations, especially with Professors Bernard Dreyfus and Henri Rochant, such as variations in the expression of blood groups in the setting of the leukemic process, indicating a genetic dysfunction before the description of oncogenes. In human genetics, Charles Salmon used the tremendous discriminating power of blood groups to demonstrate, with Professors Raymond Turpin, Jerome Lejeune, and Jean de Grouchy, the existence of a “monozygotic heterokaryote,” showing that twins are not necessarily of the same karyotype. Charles Salmon also had a real fascination with polymorphisms of blood groups, and of many other markers detectable in the blood. With his wife, Denise, he made substantial contributions to demonstrating the usefulness of such markers for studies of parentage and paternity.

The expertise of Charles Salmon and his scientific reputation led to his appointment as university professor, and he created a master’s degree in immunology and human biology, together with Professors Jean Dausset, Guy Voisin, and Maxime Seligman. In subsequent years he created a university-specific blood transfusion degree, the DUTS (Diplôme Universitaire de Transfusion Sangüine), then a doctorate based on the acquisition of a DEA (Diplôme d’Etudes Approfondies) of immunobiotechnology, immunogenetics, and transfusion. From the 1980s, Charles Salmon also invested heavily in vocational training, in which he pioneered audiovisual courses. Charles Salmon was an exceptional teacher. His outstanding teaching skills helped train many physicians and scientists in immunohematology in France and abroad, in both the academic and training fields.

As a director of the Institut National de Transfusion Sanguine (INTS) since 1975, he expanded areas of research in transfusion hematology to hemostasis, fundamental immunology, and leukocyte-platelet immunology, as well as virology. This was also the time when Charles Salmon applied biology and cellular engineering and the production of monoclonal antibodies to blood group antigens. The development of knowledge in the biochemistry of several blood group systems, Rh (rhesus) in particular, is largely attributable to the availability of these new techniques. Charles Salmon’s reputation attracted the most complex immuno-
hematology problems across France to his laboratory as well as requests for rare compatible blood units in the most difficult cases. And in 1985, he founded the National Reference Center for Blood Groups (CNRGS), which worked in a network with similar rare blood banks in England, the Netherlands, and the United States.

The scientific work conducted in both the INTS and INSERM U76 laboratories also made use of the rapid development of studies in biochemistry and molecular biology, which opened the door to the understanding of blood groups in molecular terms and an intimate approach to the functioning of genes. Indeed, these studies have allowed the cloning of several blood group genes to elucidate the molecular basis of many blood group systems and clarify the mechanisms of certain diseases of the red cell membrane.

He was a physician, teacher, and researcher of international reputation: the richness of Charles Salmon’s work in immunohematology and genetics resulted in numerous original publications and several books; in 1988 he received the Karl Landsteiner Prize, awarded by the American Association of Blood Banks. Charles Salmon was a member of several scientific societies and served on many committees and boards at the university, on the faculty of medicine, and at INSERM. He was also a Chevalier in the Ordre National de la Legion d’Honneur and Commandeur in l’Ordre National du Mérite.

Charles Salmon was always able to listen, advise, and guide—and sometimes scold—those who had the chance to know him, and they are all deeply grateful for what he has given them and the time he spent with them. He was a demanding master, but also an example of scientific rigor.

For our part, we are proud to express our gratitude, our admiration, and our affection.

Jean-Pierre Cartron
Past Director INSERM Research Unit U76
Scientific Director INTS

Philippe Rouger
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The authors are using royalties generated from the sale of this pocketbook for educational purposes to mentor people in the joys of immunohematology as a career. They will accomplish this in the following ways:

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<th>Phone Contact</th>
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<th>Website</th>
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**Advertisements, cont.**
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
   d. Results
      Presentation of concise and sequential results, referring to pertinent tables and/or figures, if applicable
   e. Discussion
      Implication and limitations of the study, links to other studies; if appropriate, link conclusions to purpose of study as stated in introduction

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

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

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

b. 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).
   b. Place caption for a figure on a separate page (e.g. Fig. 1 Results of . . .), ending with a period. If figure is submitted as a glossy, place first author’s name and figure number on back of each glossy submitted.
   c. When plotting points on a figure, use the following symbols if possible: ○ ● △ ▲ □ ■.

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

III. EDUCATIONAL FORUM

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

B. Preparation of manuscript

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

2. Text
   a. Case should be written as progressive disclosure and may include the following headings, as appropriate
      i. Clinical Case Presentation: Clinical information and differential diagnosis
      ii. Immunohematologic Evaluation and Results: Serology and molecular testing
      iii. Interpretation: Include interpretation of laboratory results, correlating with clinical findings
      iv. Recommended Therapy: Include both transfusion and nontransfusion-based therapies
      v. Discussion: Brief review of literature with unique features of this case
      vi. Reference: Limited to those directly pertinent
      vii. Author information (see II.B.9.)
      viii. Tables (see II.B.7.)

IV. LETTER TO THE EDITOR

A. Preparation

1. Heading (To the Editor)
2. Title (first word capitalized)
3. Text (written in letter [paragraph] format)
4. Author(s) (type flush right; for first author: name, degree, institution, address [including city, state, Zip code and country]; for other authors: name, degree, institution, city and state)
5. References (limited to ten)
6. Table or figure (limited to one)

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