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VOLUME 16, NUMBER 4, 2000



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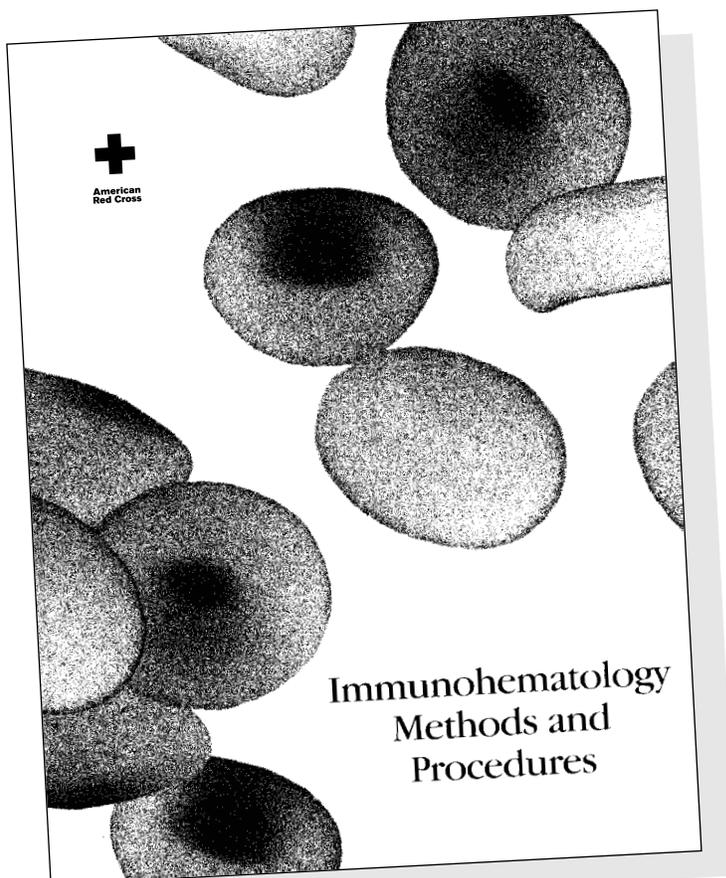
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# Immunohematology

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# New protocols in serologic testing: a review of techniques to meet today's challenges

D.H. RUMSEY AND D.J. CIESIELSKI

## Introduction

In 1945, Coombs et al.<sup>1</sup> described the indirect antiglobulin test (IAT), and Diamond and Abelson<sup>2,3</sup> described albumin, as a potentiator for the detection of "incomplete" or IgG antibodies. It has been 55 years since Coombs's and Diamond's revelations. The classic saline IAT is still viewed as the gold standard. According to the literature, an IAT incubated for 60 minutes at 37°C in saline will provide 99 percent of antibody uptake onto red blood cells (RBCs) from any sample containing an appropriate IgG antibody.<sup>4</sup> If the incubation time is reduced to 45 minutes, still in saline, there will be 95 percent uptake of antibody.<sup>5</sup> All subsequent methods and enhancement media were developed from this reference point. For example, low-ionic-strength solutions (LISS) were developed and used to speed up the rate of antigen-antibody association so that the optimal range of antibody uptake (95 to 99%) could be achieved using 15- to 30-minute incubations. Methods such as a capillary technique and potentiators such as Polybrene<sup>®</sup> have come into and out of favor. Over the years it has become increasingly clear that no single method is perfect. The IAT, in its traditional test-tube format, has, however, proven invaluable in the detection of clinically significant RBC antibodies and is still used by many as the test of choice.

As good as the IAT is, there are many factors that can affect the reproducibility and reliability of this gold standard. Inherent to this standard test-tube system are the undesirable effects that arise from the necessity of the wash step prior to the addition of anti-human globulin. Low-affinity immunoglobulins bound to RBCs may elute off during the wash procedure. Techniques for reading and grading reactivity are difficult to learn, and it may be difficult for staff to maintain competence in low-volume laboratories. The unstable endpoints for the tube method can make reading, grading, and

interpreting tests almost impossible. Alternatives have been developed to address some of these issues. In particular, some of these relatively new methods represent attempts to control the undesirable effects of the washing step, individual reading techniques, the level of expertise needed to grade results accurately, and the unstable endpoint, as well as to optimize pH, ionic strength, the serum-to-cell ratio, and the use of automation techniques. By controlling these variables, ease of testing, better reproducibility, and better reliability of test results can occur.<sup>6</sup>

To maintain the quality of testing, documentation, and overall patient safety required by regulatory agencies, it has been crucial to develop new methods. The alternative methods currently licensed for use in the United States are commonly called gel tests, i.e., RBC-affinity column technology, and solid-phase technology. The purpose of this review is to summarize the principles of the different methods, their advantages and disadvantages, and the automated equipment available.

## Principles of the Tests

### *The gel test*

The gel test (ID-MTS; Ortho-Clinical Diagnostics, Raritan, NJ) was released in Europe in 1988 and was available in the United States in 1995. The technology was developed by Lapierre et al.<sup>7</sup> and is manufactured in the United States by Micro Typing Systems, Inc. The basic principle of the gel test is that, instead of a test tube, the serum and cell reaction takes place in a microtube consisting of a reaction chamber that narrows to become a column about 15 mm long and 4 mm wide. Each column contains about 35  $\mu$ L of a dextran acrylamide gel prepared in a buffer solution such as LISS or saline. The gels may also contain other

elements: preservatives such as sodium azide, sedimenting agents such as bovine serum albumin, and, in some cases, specific reagents such as anti-IgG or other RBC-specific antisera (ABO and D). When gels are to contain specific reagents, the reagents are added to the gel during preparation by the manufacturer before the microtube is filled. Thus, the reagent is dispersed throughout the length of the gel column. The gel column is about 75 percent packed gel and 25 percent liquid. Six of these microtubes are embedded in a plastic card to allow ease of handling, testing, reading, and disposal.<sup>8</sup>

The manufacturer's insert outlines the following procedure to perform an IAT. Using a gel microtube that contains IgG, 25  $\mu\text{L}$  of serum or plasma and 50  $\mu\text{L}$  of LISS-suspended RBCs at an 0.8 percent concentration (pipetted separately) is added to the reaction chamber of the microtube. The microtube is incubated at 37°C for 15 minutes, then spun for 10 minutes in a dedicated centrifuge at approximately 70  $\times g$ . During centrifugation, RBCs in the reaction chamber are pulled into the gel column. The serum mixture along with unbound serum proteins lack the weight necessary to be pulled from the reaction chamber and therefore do not enter the column where they could potentially neutralize the anti-IgG in the gel. Sensitized RBCs will pass through the upper part of the gel column and agglutinate in the presence of anti-IgG. Agglutinated RBCs are too large to pass through the gel matrix, so they are trapped at various places within the gel, depending on size of the agglutinates, while unagglutinated RBCs slip easily through the gel and pellet at the bottom of the microtube.

After centrifugation, positive reactions are indicated by RBC agglutinates trapped anywhere in the column of the gel. Positive reactions can be graded from 0 to 4+. A 4+ reaction is indicated by a solid band of RBCs on top of the gel. A 3+ reaction displays agglutinated RBCs in the upper half of the gel column. A 2+ reaction is characterized by RBC agglutinates dispersed throughout the length of the column. A 1+ reaction is indicated by RBC agglutinates mainly in the lower half of the gel column with some unagglutinated RBCs pelleted at the bottom. Negative reactions display a pellet of RBCs on the bottom of the microtube and no agglutinates within the matrix of the gel column. Mixed-field reactions can also be observed in the gel test. These reactions are more commonly encountered during RBC typing procedures rather than during serum or plasma testing methods, but in either case they are easy to recognize. Antigen-

positive RBCs, in this case, are completely agglutinated by the specific antisera present and they lie at the top of the gel, whereas the remainder of the RBCs that are antigen negative do not agglutinate and pellet at the bottom.

#### *RBC-affinity column technology*

The other column technology test (ReACT<sup>®</sup>; Gamma Biologicals, Houston, TX) was released for the United States market in 1997.<sup>9,10</sup> Recently, Gamma Biologicals has become a subsidiary of Immucor, Inc. (Atlanta, GA). The ReACT test detects IgG-sensitized RBCs in an immunologically active matrix. In the ReACT test system, six or eight microcolumns approximately 12 mm in length and 3 mm in diameter are molded together in a plastic strip. Each microcolumn consists of a wider test chamber that narrows to a microtube. The microtubes are filled with an immunologically reactive agarose gel, which is physically separated from the contents of the test chamber by a viscous serum-cell barrier. The immunoreactive matrix is composed of sepharose gel particles to which protein G is covalently bound and sephacryl particles to which protein A is covalently bound. Protein G and protein A are bacterial proteins from *Streptococcus* group G or C and *Staphylococcus* group A, respectively, that bind specifically to the Fc portion of IgG molecules. Protein G has a high affinity for all subclasses of IgG molecules, whereas protein A will not detect IgG3. The blend of sepharose-protein G and sephacryl-protein A results in a layer of protein G at the top of the gel column. This layering effect helps clearly distinguish and visualize positive reactions.

The manufacturer's insert outlines the following procedure to perform an IAT. One drop or 50  $\mu\text{L}$  of 0.8 percent LISS-suspended RBCs and one drop or 50  $\mu\text{L}$  of serum or plasma are added to the test chamber. The entire test strip then is incubated at 37°C for 15 minutes and then centrifuged using a programmed cycle for approximately 3 minutes. During centrifugation, the RBCs in the reaction chamber are pulled through the viscous barrier into the agarose gel to which protein G and A are bound. The serum mixture remains in the test chamber because it cannot pass through the viscous barrier. This avoids the neutralization of protein A and G by unbound serum immunoglobulins. Any RBCs sensitized with IgG will adhere near the top of the column to the immunoreactive agarose gel. If there is no IgG bound to the RBCs, they migrate through the gel and are deposited at the bottom of the microtube.

Positive reactions are therefore visualized by a RBC band of any size left near the top of the agarose gel column. Positive reactions may be graded as strong positive, positive, weak positive, and negative or by the 4+ to 0 grading system, depending on the size of the RBC band at the top of the gel. Negative reactions result in the absence of a band of RBCs at the top of the gel column and deposited RBCs at the bottom of the microtube. Negative tests can be quality controlled by the addition of IgG-coated "check cells," if desired.

#### *Solid phase technology*

The RBC solid-phase adherence assay is commonly referred to as "solid-phase" (Capture-R<sup>®</sup>; Immucor, Inc.). The test has been in use since the early 1980s in Canada<sup>11,12</sup> and in the United States since shortly thereafter.<sup>13,14</sup> The solid-phase platform is a 96-well microtiter plate that can be used as a whole or broken down to individual 8-well strips. The plates are stored in sealed foil pouches with a moisture indicator. If the indicator has turned pink, the plate has been exposed to too much humidity and should be discarded. The commercial product can be purchased in several formats. The wells can be coated with the RBC membrane lysate from either pooled, two-cell, or four-cell antibody screening cells selected for antigen combinations in the same manner as the liquid product or with membranes that compose a 13-cell panel. One can also purchase plates that are coated only with the proprietary adhesive and prepare a custom antibody screen or select-cell panel using liquid or frozen intact RBCs. The reagent that replaces anti-IgG in this method is the Indicator RBCs (Immucor) that are labeled with IgG directed against human IgG. All the Capture-R<sup>®</sup> kit products include a LISS additive with a protein indicator that changes the reagent's color from purple to blue when serum or plasma is added to a well. Positive and negative controls that are used to validate each test run are also included in the kit.

To follow the manufacturer's directions to perform an IAT, add two drops of the LISS additive to each well being tested. One positive and one negative control well must be included with each batch tested. The sample of choice is EDTA plasma, but serum can be used. One drop (50  $\mu$ L) of patient sample is added to each test well. One drop of control is added to the appropriate well and the plate frame (containing 1 to 12 strips) is put on a plate mixer or is lightly tapped at the corners until the blue color of the LISS and sample mixture is visible in all the wells. The plate frame is

incubated at 37°C for 15 minutes and then the mixture is washed out of the wells. This can be done with a squeeze bottle (3 washes and blot), a hand-held strip washer, or an automated plate washer with predetermined programs. In all cases, the plate is blotted dry onto absorbent material and two drops of indicator cells are added to each well. The plate or strips are centrifuged for 3 minutes at 350  $\times$  g in a centrifuge and read. A positive reaction is indicated by a smooth coating of indicator cells over the round surface of the bottom of a well, which shows that the indicator cells have bound to antibody. A negative reaction is a button of cells at the bottom of the well. Grading can be performed using the classic 0 to 4+ scale. ABO and Rh testing can be performed in strips as well, but these tests still use a direct agglutination method, and a special coating on the bottom of the well is not required.

#### **Advantages and Disadvantages**

Although each of the three methods look very different from each other and from tube testing, they have all been designed to meet the same testing needs. They all have moved from glass to plastic as a safer choice of material and they all have standardized endpoints in preparation for automation. Within each method there are advantages and disadvantages due to their unique design.

#### *The gel test*

Advantages of the gel test include small sample size, decreased variation in volume delivery, greater uniformity between repeat tests, no cell washing step, and decreased technique dependence. The gel test is also easy to learn, since there are relatively few steps in the procedure and it provides a clear, easy-to-read, stable endpoint. When the microtubes are covered and refrigerated, the gel cards can be read with accuracy for at least 24 hours after testing. Sensitivity and specificity of gel testing have been found to be comparable to the tube LISS-IAT.

There are a few disadvantages. For reference laboratories, batch testing is less of an option because of urgency, timing, and variation in samples. Therefore, the efficiency made possible by batch testing is often lost. Complexity of antibody identification usually requires multiple runs of selected cells. Although an 0.8 percent panel of RBCs is commercially available, any other test cell that needs to be tested must be prepared as an 0.8 percent concentration in the appropriate diluent prior

to use. This is more cumbersome than adding the cells right from the vial into a test tube. The ABO and Rh typing may also have a disadvantage, depending on the laboratory's needs. The RBCs must be incubated in Diluent 1 for 10 minutes prior to centrifugation in the appropriate card. Therefore, it takes a minimum of 20 minutes (10 minutes incubation and 10 minutes centrifugation) to perform an ABO and Rh test. The test-tube method still wins on this one with ease and speed. Although direct antiglobulin tests (DATs) can be done on the gel system, only a DAT using anti-IgG is available. At this time, gel cards containing polyspecific anti-human globulin or anti-C3d are not available. Finally, rouleaux and incompletely clotted samples may cause patterns that resemble positive reactions.

#### *RBC-affinity column technology*

There are advantages to the ReACT system. Compared to the traditional tube testing, the technology is very simple, with few procedural steps. It requires a smaller sample size, does not require a cell-washing step, and goes directly from the incubation step to a 3-minute centrifugation and the final reading. Following interpretation of the centrifuged test, negative tests can be checked for reactivity by the addition of known IgG-sensitized RBCs. The reactions are relatively easy to read and are stable for up to 4 hours when the strip is stored upright. If the test strip is sealed to prevent evaporation and stored upright, the reactivity can be read up to 3 to 5 days after testing is completed. Clinically benign antibodies of the IgM class that can often interfere in other test systems, leading to lengthy and costly investigations, are generally not detected by this method, because Proteins A and G do not react with IgM molecules. The ReACT method has good specificity and sensitivity comparable to a tube LISS-IAT.

The disadvantages to this technology are similar to those found with the gel test. The efficiency made possible by batch testing is not practical in all laboratory settings. Complex antibody identification usually requires multiple runs of selected cells beyond what is available on the commercially prepared 0.8 percent panel. ABO and D typing and polyspecific and C3d-specific DATs are currently not available by this method. With the ReACT system, it is important to remove test strips from the centrifuge within a few minutes following completion of the centrifugation cycle. This is due to the fixed vertical position of the test strips in the centrifuge. If test strips are not

removed from the centrifuge in a timely fashion after centrifugation, the RBC band can dislodge and disperse through the gel, making test interpretation difficult. This difficulty can easily be avoided by removing the test strips from the centrifuge after the 3-minute cycle and storing them upright until they are read. One last concern is the lack, so far, of licensed automated or semi-automated equipment that can process the ReACT test strips.

#### *Solid-phase technology*

The advantages of solid-phase testing are similar to the other two techniques. Compared to tube testing, solid-phase testing requires a smaller sample volume; provides easier handling of large batches, which allows better time management of staff; and is less technique dependent. The most dramatic change, noted by technologists who perform parallel testing, is the greater sensitivity of solid-phase testing compared to the LISS-IAT in tubes.

The disadvantages found with the solid-phase method are quite different from those found with either of the other test methods. There is a learning curve in changing from tube testing to solid-phase testing, which must be completed before the improvement in time management will be achieved. Of interest to the reference laboratories is the fact that this method does not lend itself to RBC membrane modifications such as DTT or AET. Finally, the greater sensitivity, which can be an asset, is also a problem when lack of specificity leads to unnecessary testing. With increased expertise, this concern is manageable.

#### **Instrumentation**

The basic pieces of equipment and materials required for the gel test are the dedicated incubator, centrifuge, and RBC diluents. Accessories are also available to make the testing run smoothly. These include specially designed workstations, automatic pipettes with disposable tips, and an automated reader (Reader M, Micro Typing Systems; Ortho). The Reader M instrument utilizes advanced image analysis to digitally read gel cards within seconds. Results can be archived or printed on customized reports.

To handle a larger number of samples, the gel system can include a fully programmable, random-access liquid-handling station (Megaflex-ID, Tecan; Ortho).<sup>15</sup> This instrument will perform the pipetting activities for all routine blood banking tests for the gel system, and it has a 256-sample capacity. The following tests can be

selected from the instrument's test menu: ABO/D typing, ABO reverse typing, Rh phenotyping, antibody detection, antibody identification, crossmatching, and a DAT using anti-IgG only. The gel system offers positive sample and gel card identification, STAT analyses, and computer interface capabilities, and it can process up to 100 two-cell antibody screening tests per hour.<sup>16</sup> The system is semi-automated in that it performs all the pipetting and reading for the user, but someone still has to transfer the gel cards from the pipetting chamber, to the incubator, to the centrifuge, and then to the reader. To take advantage of the instrument's fast pipetting of large volumes of prepared gel cards, more than one set of workstation equipment (incubator and centrifuge) is necessary.

For its implementation as a new manual system, ReACT has a dedicated centrifuge and incubator designed to hold the strip of microcolumns. ReACT system accessories include a viewbox, control cells, and a centrifuge balance kit. At this time, antibody detection, antibody identification, and IgG autocontrol tests can be performed by the ReACT method in the United States. Crossmatching can be performed as long as the facility validates the method for that purpose prior to implementation. Theoretically, even antigen typing could be performed by this method if antigen-specific IgG antibody is first attached to the RBCs via incubation of the RBCs with specific antisera, prior to centrifugation in the ReACT system.

Like the gel and ReACT systems, solid-phase testing can be performed in a manual setting using a custom-designed incubator, centrifuge, and standard plate washer. Unlike the gel and ReACT systems, there is also a fully automated instrument (ABS2000, Immucor) that is a completely integrated, walk-away system that locks once the run is initiated. Access is denied until testing is complete or the run is cancelled. The ABS2000 is a batch analyzer with a menu that includes ABO/D typing, antibody screening (4-cell screen), type and screen, donor confirmations, and the LISS-IAT crossmatch. It was not designed to be a STAT analyzer. For any situation in which crossmatched products must be provided in less than 60 minutes it would be advisable to use the semi-automated equipment for the solid-phase tests. Sandler et al.<sup>17</sup> concluded that the instrument would be useful in hospitals that tested 6000 to 20,000 samples annually. That covers most small to medium-sized hospital transfusion services. It is a fairly compact instrument that sits on a tabletop and requires no special plumbing. Quality control testing of

all product lot numbers in use must be run every 24 hours or the instrument will discontinue testing. All testing and quality control data can be archived or downloaded to another information system.

## Discussion

In the consideration of a major change in any system, the goals to be achieved act as the starting point and the standard against which the final outcome will be measured. The goals of automation in most cases include increasing workload capacity by increasing the number of tests performed per full-time equivalent, reducing the number of labor-intensive manual procedures performed, improving precision and accuracy, and eliminating subjectivity of results. All three of the protocols reviewed have been designed to address these issues. However, they do this in different ways. To decide which is the most appropriate system for one laboratory, a comparison of the advantages and disadvantages must be done in the context of the laboratory that will be using the chosen system. First it must be determined if the decision will be made according to choice of test method, or to choice of instrumentation.<sup>18</sup> For example, if the ABS2000 is the instrument of choice, then the laboratory must switch to solid-phase testing.<sup>19,20</sup>

If the test method is the priority, it can be useful to read existing literature that compares two or more of the new methods. The two factors that should be considered when contemplating this kind of change in serologic testing are (1) eliminating reading after incubation at 37°C,<sup>21,22</sup> and (2) the factors affecting sensitivity of the DAT and IAT.<sup>23</sup> Over 400 studies have been published comparing gel tests with other methods.<sup>24</sup> The results of these studies are highly variable, depending on the method and reagents used to make the comparison. Most reports seem to conclude that the gel test is at least as sensitive if not slightly more so in the detection of clinically significant antibodies as an LISS-IAT performed in a tube. Some reports have shown it to be particularly good at the detection of Rh system antibodies. Two reports comparing the gel test and the PEG-IAT conflict in their conclusions. One report by Derr et al.<sup>18</sup> claimed the gel test is as sensitive or more so than PEG-IAT; the other report in an abstract by Issitt et al.<sup>25</sup> claimed PEG-IAT detected clinically significant antibody specificities the gel test did not detect. Another study by Reilly et al.<sup>26</sup> compared not only the sensitivity but also the specificity of several IAT methods including LISS and

PEG-IATs. In this study, although PEG had slightly better sensitivity than the other two methods, the gel test had a better balance of both specificity and sensitivity. A study by Weisbach et al.<sup>27</sup> compared the gel test, ReACT, and two other column agglutination systems available in Europe to the LISS-IAT in tubes. They found that the gel test and ReACT had very similar rates of antibody detection (91.6 % and 90.3%, respectively) and also the lowest detection rates of clinically insignificant antibodies (28% and 39%, respectively) of the four column systems. One study, reported by Shirey et al.<sup>28</sup> compared ReACT to LISS-IAT in tubes. ReACT technology was nonreactive with all 130 LISS-IAT negative samples, yielding a 0 percent false positivity. On the other hand, all 84 or 100 percent of the clinically important antibodies were detected by ReACT, as they had been by LISS-IAT.

Long-term cost containment is often cited as one of the reasons to implement automation. Choosing a test method should include consideration of types of antibodies that must be detected by the system versus antibodies that are not considered clinically significant in a laboratory. This frame of reference will shift according to the laboratory involved. For example, transfusion services concentrate on finding compatible blood products, whereas reference laboratories need to identify all antibodies before recommending the safest option for transfusion. The literature contains reports showing how well the techniques in this review detect antibody specificities or how efficiently they miss some specificities, depending on the perspective of the author. For example, Shirey et al.<sup>28</sup> found that five (31%) of the 16 tested samples containing clinically benign specificities, such as anti-Sd<sup>a</sup>, cold auto agglutinins, and Lewis and Knops system antibodies, were nonreactive by ReACT technology, which is an IgG-specific test; Rolih et al.<sup>29</sup> found that antibodies to antigens such as Le<sup>a</sup>, Le<sup>b</sup>, M, N, and P<sub>1</sub> were detected by solid-phase only if there was IgG class antibody present. Rolih et al. were also able to exclude crosslinking of IgM-class antibodies between the monolayer and antigen-positive indicator RBCs as a contributor to the detection of "clinically insignificant" antibodies; in the process, they found that 28 (48%) of 59 insignificant antibodies tested did not react by solid phase. In a cost analysis, Chan et al.<sup>30</sup> reported that the gel test missed 11 (31%) of 36 "cold antibodies" that had been detected by LISS-IAT. Thus, a decrease in detection of unwanted specificities in the range of 30 to 50 percent can be anticipated by

transfusion services before any on-site evaluation is performed.

A comparison of the instrumentation available will involve a work-flow analysis to find the best fit. Each laboratory has its own ways of serving its customers. If the workload requires a large amount of component preparation (platelet pooling) and issuing of components to patients, a system that offers walk-away testing and frees technologists to perform these tasks is preferred. If large amounts of RBCs are transfused and fewer plasma components prepared, a system for faster serologic testing to decrease the number of units released before antibody screening is complete would be the system of choice.

With the decrease in budgets, loss of specialized staff, and increased regulatory requirements, there is no longer the option of choosing the test of record and any accompanying equipment by the old yardstick of popularity with the staff. The educated consumer will prepare a list of requirements, compare all the variables within his or her own laboratory, and choose the system that offers the most improvement over the previous method.

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*Dawn H. Rumsey, ART(CSLT), Technical Editor, Transfusion, American Association of Blood Banks, 8101 Glenbrook Road, Bethesda, MD 20814 (corresponding author); Debra J. Ciesielski, MT(ASCP)SBB, Supervisor, Reference Laboratory, American Red Cross Blood Services, Southern California Region, Los Angeles, CA.*

# Comparison of tube and gel techniques for antibody identification

M.C.Z. NOVARETTI, E. JENS SILVEIRA, E.C. FILHO, P.E. DORLHIAC-LLACER, AND D.A.F. CHAMONE

There are several methods for antibody detection and each technique has advantages and limitations. We compared the performance of the tube (polyethylene glycol-indirect antiglobulin test [PEG-IAT]) and gel test technique for antibody identification. From January to May 1999, we performed antibody screening tests by gel and tube techniques on 10,123 random blood samples submitted to our reference laboratory. Six hundred and twenty-eight (6.2%) reactive samples were tested for antibody specificity by both methods. One hundred and ninety-six were reactive only by gel: 25 anti-D, 33 anti-C, 76 anti-E, 13 anti-c, 5 anti-e, 18 anti-K, 7 anti-Jk<sup>a</sup>, 2 anti-Di<sup>a</sup>, 3 anti-S, 8 combination Rh antibodies (1 with anti-K), and 6 other antibody specificities. Two samples were reactive only by PEG-IAT: 1 anti-K and 1 anti-Di<sup>a</sup>. Four hundred and thirty were positive by the two methods: 156 anti-D, 9 anti-C, 68 anti-E, 15 anti-c, 6 anti-e, 61 anti-K, 12 anti-Jk<sup>a</sup>, 17 anti-Di<sup>a</sup>, 5 anti-S, 73 combination Rh antibodies (2 with anti-K), and 8 other antibody specificities. Based on this study, the gel test is more sensitive ( $p < .01$ ) than the tube test for identifying potentially clinically significant antibodies. *Immunohematology* 2000; 16:138–141.

**Key Words:** gel test, antibody detection, PEG-IAT, pretransfusion testing

The gel test has been widely used in blood banking in Europe and Latin America since 1991 for ABO and Rh typing, direct antiglobulin tests (DATs), detecting antibodies, red blood cell phenotyping, and other applications.<sup>1-4</sup> In 1994, the Food and Drug Administration approved the gel test for use in the United States. Since then, ABO grouping, indirect antiglobulin tests (IATs), and direct antiglobulin test studies have been reported.<sup>5</sup>

Many substances (albumin, low-ionic-strength saline [LISS], polyethylene glycol [PEG], Polybrene®, etc.) have been used for enhancing the detection of antibodies.<sup>6-9</sup> Some authors suggest that the use of PEG-IAT is equal to or superior to LISS in the detection of potentially clinically significant antibodies. It also has been demonstrated that PEG-IAT reduces detection of insignificant antibodies.<sup>10-12</sup> The purpose of this study was to compare the gel test and the PEG-IAT in

detecting and identifying RBC antibodies in a routine screening procedure.

## Material and Methods

### *Serum samples*

From January to May 1999, antibody screening tests were performed in parallel by the gel test (DiaMed AG, Switzerland) and by the PEG-IAT technique on 10,123 random blood samples submitted to our immunohematology reference laboratory. Blood samples were collected without anticoagulant. The sera were separated from coagulated whole blood by centrifugation and tested within 48 hours of collection.

### *Tube testing*

Antibody screening and identification by PEG-IAT were performed (with some modification at 37°C) according to the American Association of Blood Banks *Technical Manual*,<sup>13</sup> as follows: 20% PEG was prepared in-house by mixing 20g 3350 mw PEG (Sigma Chemicals, St. Louis, MO) with 100mL PBS, pH 7.3. Red blood cell (RBC) reagents for antibody screening and antibody identification were obtained from Gamma Biologicals (Duet Plus™, Panel Two™, Panel 15™; Houston, TX). Two volumes (100μL) of serum and one volume (50μL) of reagent RBCs were mixed, centrifuged immediately, and observed for agglutination and hemolysis. Four volumes (200 μL) of 20% PEG were then added to the tubes, followed by incubation for 15 minutes at 37°C. The tubes were washed × 3 with saline and two volumes of anti-IgG (Biotest S.A., Brazil) were added. Following centrifugation, the tubes were observed for agglutination and hemolysis. Reactions were read macroscopically and graded as previously described.<sup>13</sup> All negative antiglobulin tests were verified with IgG-coated RBCs (Gamma Biologicals).

### Gel test method

The same RBC reagents used in the tube testing were used in the gel test. Anti-IgG cards, Diluent 2, appropriate incubators, and centrifuges were obtained from the manufacturer (DiaMed-ID, Brazil) and the gel tests were performed using the anti-IgG card.

Reagent RBCs were washed  $\times 3$  with saline and suspended in LISS to a final 0.8% suspension, using 1 mL of LISS (Diluent 2) and 10  $\mu\text{L}$  of RBCs. In addition, an autocontrol was prepared using 10  $\mu\text{L}$  of  $\times 3$  washed patient's cells and 1 mL of LISS (Diluent 2). Antibody detection and identification were performed as recommended by the manufacturer's instructions. Briefly, 25  $\mu\text{L}$  of serum and 50  $\mu\text{L}$  of 0.8% reagent RBCs suspended in a LISS diluent were dispensed in a microtube in the anti-IgG card and incubated 15 minutes at 37°C. The cards were centrifuged for 10 minutes at  $895 \pm 25$  rpm in an appropriate centrifuge. Following centrifugation, the cards were observed for agglutination and hemolysis. Positive reactions were graded from 1+ to 4+ according to the manufacturer's guidelines.

In our laboratory, reactions in gel were read by examining the front and the back of the cards. Doubtful reactions were evaluated using a handheld magnification lens. For each antibody identified, the patient's RBCs were tested for the matching antigen by the tube or gel method.

Results by gel were compared with those by PEG-IAT. Discrepant results were evaluated by repeating both methods. Whenever potentially significant antibodies

were missed by gel, additional tests were performed using LISS additives and adsorption-elution methods. In addition, confirmatory tests using at least three negative and three positive RBCs were performed by the same method used for antibody identification. Potentially clinical antibodies were defined based on their specificity according to the expected reactivity and clinical significance as reported elsewhere.<sup>13</sup>

Statistical analysis was performed as described elsewhere.<sup>14</sup>

## Results

### Antibody detection

Parallel antibody detection was performed on 10,123 blood samples, resulting in 628 (6.2%) positive results by PEG-IAT, gel, or both. Four hundred and thirty (4.24%) samples were positive by both methods. One hundred and ninety-six (1.93%) samples were positive only with gel, and 2 (0.02%) only in PEG-IAT (Table 1).

### Antibody identification

Antibody identification was performed on all 628 blood samples yielding a positive antibody screen. Eighty-one blood samples showed more than one potentially clinically significant antibody. Fourteen antibodies (anti-Le<sup>a</sup>, -Le<sup>b</sup>, -P<sub>1</sub>, and cold agglutinins) that are usually considered not clinically relevant with regard to RBC transfusion were detected; six of them were found only in the gel test (Table 1).

One hundred ninety potentially clinically significant antibodies were identified only by the gel test in 190 patients. In contrast, only two potentially clinically significant antibodies (1 anti-K and 1 anti-Di<sup>b</sup>) not detected in gel were identified by PEG-IAT in two patients.

Additional tests performed using LISS additives on the two samples with antibodies missed by gel test confirmed PEG-IAT results. Four samples (0.65%) with weak positive results in the antibody detection test gave negative results in at least one RBC identification panel when the gel cards were observed by reading the front of the cards but were positive when the back of the cards were read. Those four cases were three anti-K and one anti-Di<sup>a</sup>. All four were confirmed reading the front of the gel cards when the blood samples were retested.

No positive reaction in gel was detected only by a magnification lens. Therefore, its use was not helpful in this study.

**Table 1.** Comparative study: PEG-IAT vs. the gel test

Antibody (number)	PEG+ / Gel+	PEG+ / Gel-	PEG- / Gel+
Anti-D (181)	156	0	25
Anti-C (42)	9	0	33
Anti-E (144)	68	0	76
Anti-c (28)	15	0	13
Anti-e (11)	6	0	5
Anti-K (80)	61	1	18
Anti-Jk <sup>a</sup> (19)	12	0	7
Anti-Di <sup>a</sup> (20)	17	1	2
Anti-S (8)	5	0	3
Anti-D + Anti-C (48)	46	0	2
Anti-D + Anti-E (2)	2	0	0
Anti-D + Anti-K (2)	2	0	0
Anti-C + Anti-E (12)	8	0	4
Anti-C + Anti-e (5)	4	0	1
Anti-c + Anti-E (12)	11	0	1
Anti-Le <sup>a</sup> (6)	4	0	2
Anti-Le <sup>b</sup> (2)	2	0	0
Anti-P <sub>1</sub> (2)	2	0	0
Cold agglutinins (4)	0	0	4
Total (628)	430	2	196

Note: The antibodies listed above the line are considered potentially clinically significant.

## Discussion

Based on this study, the sensitivity of the the gel test is superior ( $p < .01$ ) to the conventional tube PEG-IAT in the detection of alloantibodies of potential clinical significance. Gel detected 190 potentially clinically significant antibodies not reactive by PEG-IAT, but failed to detect 2 potentially clinically significant antibodies (1 anti-K and 1 anti-Di<sup>a</sup>) in two patients.

A higher sensitivity for IgM antibodies like anti-Le<sup>a</sup>, -Le<sup>b</sup>, -P<sub>1</sub>, and cold agglutinins might be considered a disadvantage, but there are numerous advantages to the gel system.<sup>1,5</sup> The gel assay incorporates standardized pipeting of reagents and specimens and reading of agglutination reactions. The stable reaction endpoints may be reviewed, photographed, or photocopied at a later time. The required volume of specimen is substantially less, which is advantageous for pediatric testing. Small sample volumes create less hazardous waste, as does the elimination of saline washes in the performance of IATs.<sup>1,15,16</sup>

In contrast to other reports, it is important to note that in our study, all RBC antibodies detected by the gel test remained reactive during antibody identification. This may be because we used only fresh samples, so attenuation of reactivity during freezing, storage, and thawing could be avoided.<sup>17-19</sup> We designed a prospective study with an enormous number of sera tested in parallel in order to obtain a sufficient number of positive results. This large study shows that the gel test is more sensitive when compared to PEG-IAT.

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*Marcia Cristina Zago Novaretti, MD, PhD, Chief of Immunohematology Division, Fundação Pró-Sangue Hemocentro de São Paulo, Hematology/Hemotherapy Department, University of São Paulo, Av. Dr. Enéas de Carvalho Aguiar, 155 - 1º andar - São Paulo, SP - Brazil 05403-000; Eduardo Jens*

*Silveira, Medical Technologist, Chief of Immunohematology Department, Fundação Pró-Sangue Hemocentro de São Paulo; Edio da Costa Filho, MD, Resident Hematology/Hemotherapy Department, University of São Paulo; Pedro Enrique Dorlbiac-Llacer, MD, PhD, Director, Fundação Pró-Sangue Hemocentro de São Paulo; and Dalton de Alencar Fischer Chamone, MD, PhD, Hematology/Hemotherapy Department, University of São Paulo School of Medicine.*

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# Red blood cell diluent composition is important for detection of some anti-E

D.D. YASKANIN, J.L. JAKWAY, AND D.J. CIAVARELLA

Commercially prepared 0.8% reagent red blood cells (RBCs) eliminate the need to manually dilute 3 to 5% RBCs for use in gel cards. Ortho-Clinical Diagnostics investigated twelve anti-E samples detected in MTS Anti-IgG gel cards using Ortho 3% reagent RBCs manually diluted to 0.8% in MTS Diluent 2™ (MTS2) that were not detected with commercially prepared Ortho 0.8% reagent RBCs. In gel tests, using additional examples of E-positive RBCs, 22 of 26 anti-E were reactive when the cells were suspended in MTS2. Only 6 of 28 anti-E were reactive with E-positive Ortho 0.8% reagent RBCs. Five anti-E were tested in gel with five R<sub>2</sub>R<sub>2</sub> RBCs that had been washed and resuspended in four low-ionic-strength diluents. Fifty-eight percent of tests performed in MTS2 were positive compared to 13 to 41 percent for the other diluents. Anti-E detection also varied from 6 to 56 percent based on the donor of the RBCs. Seven anti-E were characterized by their reactivity in tube techniques and were reactive using PEG and/or ficin-treated RBCs only. As a comparison, 25 archived examples of anti-E were detected using RBCs suspended in MTS2 and Ortho 0.8% reagent RBCs. These data show that some anti-E are not detected by Ortho reagent RBCs in MTS Anti-IgG gel cards. However, these anti-E have characteristics of antibodies of questionable clinical significance. *Immunohematology* 2000; 16:142-146.

**Key Words:** gel columns, anti-E, low-ionic-strength diluents

## Introduction

Gel card technology for antibody screening and identification offers the advantages of small sample volume requirement, a more standardized testing method, and a relatively stable red blood cell (RBC) agglutination endpoint compared to tube techniques. However, gel card technology has the disadvantage of requiring the use of a low concentration of reagent RBCs suspended in a low-ionic-strength (LIS) diluent. Traditional LIS diluents are not suitable for long-term storage of RBCs due to their hypotonic nature and the time-consuming daily preparation of reagent RBCs for gel card testing. Ortho-Clinical Diagnostics (Raritan, NJ) developed a new product line, Ortho 0.8% Reagent Red Cells, which combines the low-ionic strength desired

for indirect antiglobulin testing with longer RBC storage.

Within several months of the introduction of 0.8% reagent RBCs, Ortho-Clinical Diagnostics received 12 anti-E samples from customers for resolution of differences in MTS Anti-IgG card test results using 0.8% reagent RBCs and Ortho 3% reagent RBCs. The latter were stored in an isotonic solution and resuspended to 0.8% with an LIS diluent, MTS Diluent 2™ (MTS2; Micro Typing Systems, Pompano Beach, FL) on the day of use. This report summarizes the investigation and characterization of these anti-E.

## Materials and Methods

### *Anti-E samples*

Twelve anti-E samples were obtained from patients as referrals to Ortho-Clinical Diagnostics. For comparison to the referral samples, 25 samples were retrieved from frozen storage. Twenty-three of these antibodies were identified between 1973 and 1979, before the development of gel column technology, and two were source plasmas purchased from a commercial supplier (Serologicals, Inc., Birmingham, AL).

### *LIS diluents*

MTS2™, Ortho Antibody Enhancement Solution (OAES), and 0.8% red cell diluent were obtained from Ortho-Clinical Diagnostics. MTS2™ is a hypotonic buffered saline solution formulated at an appropriate pH and ionic strength to be compatible with dextran-acrylamide gel. Ortho Antibody Enhancement Solution is a phosphate-buffered LIS solution containing glycine. Ortho 0.8% Red Cell Diluent is a LIS diluent to which a purine and a nucleoside have been added. Löw-Messeter LIS diluent was prepared at Ortho-Clinical Diagnostics.<sup>1</sup>

### Polyethylene Glycol

PEG (Gamma PeG™) was obtained from Gamma Biologicals, Houston, TX.

### Reagent RBCs

All reagent RBCs were manufactured by Ortho-Clinical Diagnostics.

Ortho 3% reagent RBCs (Selectogen®, Surgiscreen®, and Resolve™ Panels A and C) are stored in an isotonic phosphate-citrate buffered diluent to which a purine, a steroid, and nucleotides have been added to maintain reactivity, retard hemolysis, or both during the dating period. For routine MTS IgG card testing, 3% RBCs were resuspended to 0.8% in MTS2™ (0.8% MTS2™) by adding 10 µL of packed RBCs to 1 mL of diluent (or proportional volumes as needed). In some experiments, 3% RBCs were resuspended to 0.8% in other LIS diluents by the same technique.

Ortho 0.8% reagent RBCs (0.8% Selectogen®, 0.8% Surgiscreen®, and Resolve™ Panel A) are manufactured and stored in an LIS RBC diluent (0.8% Red Cell Diluent) and are intended for use in gel or glass bead column technology only. For routine MTS gel cards, 0.8% RBCs were used directly from the vials. In some experiments, aliquots of 0.8% RBCs were centrifuged, and the LIS RBC diluent was removed and replaced with an equal volume of MTS2, Ortho Antibody Enhancement Solution, or Löw-Messeter LIS diluent.

Ortho 3% ficin-treated RBCs (Resolve™ Panel C) were used in tube testing directly from the vials.

### MTS Anti-IgG Card™ testing

Fifty µL of a 0.8% RBC suspension was added to a test column. Twenty-five µL of antibody was then added to the column. The cards were incubated for 15 to 30 minutes at 37°C and centrifuged in an ID-MTS centrifuge™.

### Tube testing

One drop of Ortho 3% reagent RBCs (ficin untreated) was added to two drops of anti-E in a glass test tube. The tubes were centrifuged and read immediately. Two drops of Ortho Antibody Enhancement Solution or PEG were then added and the tests were incubated at 37°C for 15 to 30 minutes. For tests containing Ortho Antibody Enhancement Solution, the tubes were centrifuged and read after incubation and at the antihuman globulin (AHG) phase. For tubes containing PEG, results were observed at 37°C without centrifugation. Centrifugation after incubation is not recommended in tests

with PEG because it can cause falsely positive reactions. However, the appearance of agglutination or aggregation in uncentrifuged tubes at 37°C was recorded as an investigative tool to understand customer complaints. Tests containing PEG were then completed at the AHG phase. For tests with ficin-treated cells, no additive was included with the RBCs and anti-E.

### Reaction grading

Agglutination in MTS Anti-IgG gel cards was graded according to the following scale: 0 = no agglutination, all cells form a button at the bottom of the column; w = weak positive, agglutinated cells extend slightly above cell button into column; 1 = agglutinated cells in the lower half of the column; 1.5 = agglutinated cells extend through more than half of the column but not the entire column length; 2 = agglutinated cells throughout the length of the column; 3 = most agglutinated cells in upper half of the column; 4 = agglutinated cells form a band across the top of the column.

Agglutination in tube tests was graded as follows: 0 = no agglutination; 1 = many small clumps with reddish background; 2 = many medium clumps with pink background; 3 = many large clumps with clear background; 4 = one clump with clear background.

## Results

### Comparison of anti-E vs. 0.8% RBCs in MTS2™ diluent and 0.8% Ortho reagent RBCs

Initial investigation of the twelve anti-E samples consisted of testing the samples with one to four examples of RBCs washed and suspended to 0.8% in MTS2™ and one to four examples of cells manufactured as 0.8% Ortho reagent RBCs. A total of 11 blood donors were represented in the examples of 0.8% MTS2

**Table 1.** Anti-E reactivity with R<sub>2</sub>R<sub>2</sub> red blood cells (RBCs)

Sample	RBCs in MTS2 diluent		Ortho reagent RBCs (0.8%)	
	# Positive	# Tested	# Positive	# Tested
0685	2	2	0	2
1053	2	2	2	2
1206	1	1	0	2
1302	2	2	0	1
1435	2	2	0	3
1488	NT*	NT	1	1
CC1	3	3	0	4
CC2	2	3	1	3
CC3	1	3	0	2
2511	1	1	1	1
0492	1	1	0	1
2128	0	1	0	1
Totals	17	21	5	23

\* NT = Not tested

reagent RBCs; 11 different blood donors comprised the examples of 0.8% Ortho reagent R<sub>2</sub>R<sub>2</sub> RBCs. The results for tests with R<sub>2</sub>R<sub>2</sub> RBCs are presented in Table 1. Anti-E reacted in 17 of 21 tests (81%) with 0.8% MTS2™ RBCs, whereas only 5 of 23 (22%) of 0.8% Ortho reagent RBCs were positive with the anti-E samples. In reactions with E-positive RBCs of other phenotypes, four anti-E (1302, 1435, 1488, and 2511) were positive with a single example of 0.8% MTS2™ r'r RBCs, but three of four anti-E that were tested with 0.8% Ortho reagent r'r RBCs were negative (samples 1206, 2511, 0492, and 2128). Only one sample (2511) was tested with R<sub>2</sub>r RBCs; the R<sub>2</sub>r cells prepared in MTS2 were positive whereas the RBCs manufactured as 0.8% Ortho reagent RBCs were negative. Overall, including all E-positive phenotypes, there were 22 positive results in MTS Anti-IgG cards out of 26 total tests when the anti-E samples were tested with cells suspended to 0.8% in MTS2. In comparison, only 6 of 28 total tests were positive when the same samples were tested with E-positive RBCs manufactured as 0.8% Ortho reagent RBCs.

#### Effects of diluent and reagent RBC donor

To assess the impact of the RBC diluent on the reactivity of these anti-E samples, R<sub>2</sub>R<sub>2</sub> cells were selected from Ortho 3% reagent red cells (two examples) and 0.8% reagent red cells (three examples).

**Table 2.** Reactions of anti-E with different R<sub>2</sub>R<sub>2</sub> red blood cells (RBCs) and diluents

Anti-E Sample	Diluent	3% Cells		0.8% Cells		
		3SS671	RC222-3	8S101-2	8S102-2	8SS102-2
0685	MTS2	w*	0	w	0	0
	RCD	0	0	0	0	0
	OAES	1	NT <sup>†</sup>	0	0	0
	LM	0	NT	0	0	0
1435	MTS2	2	2	w	1.5	1
	RCD	w	1.5	0	0	0
	OAES	2	2	0	w	0
	LM	w	2	0	0	0
CC1	MTS2	2	2	1.5	w	0
	RCD	0	2	0	0	0
	OAES	0	1.5	0	w	0
	LM	0	1.5	0	0	0
CC2	MTS2	1	0	0	w	0
	RCD	0	0	0	0	0
	OAES	1	w	0	0	0
	LM	0	0	0	0	0
CC3	MTS2	0	0	NT	w	0
	RCD	0	0	NT	0	0
	OAES	0	w	NT	0	NT
	LM	0	0	NT	0	NT

\* Weak positive, less than 1+

<sup>†</sup> Not tested

MTS2™ = Ortho LIS solution

RCD = Ortho 0.8% reagent RBC diluent

OAES = Ortho Antibody Enhancement Solution

LM = Löw-Messeter LIS diluent

Aliquots of each RBC reagent cell were resuspended to an 0.8% cell concentration in MTS2™, 0.8% Red Blood Cell Diluent, Ortho Antibody Enhancement Solution, and Löw-Messeter LIS solutions. The cell suspensions were tested in ID-MTS Anti-IgG cards with five anti-E, which had sufficient volume for this experiment. The results are presented in Table 2. When reactivity was compared by diluent, tests performed in MTS2™ resulted in the highest percentage of positives (14 positives of 24 tests, or 58%), followed by Ortho Antibody Enhancement Solution (9/22 tests, or 41%), Löw-Messeter (3/22 tests, or 14%), and 0.8% Red Blood Cell Diluent (3/24 tests, or 13%). Anti-E detection also varied by RBC source. The anti-E were most reactive with RBC number RC222-3, which was positive in 10 tests out of a total of 18 (56%). Cell 3SS671 showed 9 positives in 20 tests (45%), cell 8S102-2 was positive in 6 of 20 tests (30%), 8S101-2 was positive in 3 of 16 tests (19%), and 8SS102-2 was positive only once in 18 tests (6%; Table 2).

#### Characterization of anti-E reactivity by tube tests

Seven anti-E samples were examined by conventional tube techniques to compare the characteristics of their reactivity in tube tests with the results of MTS Anti-IgG card testing. None of the samples were reactive when tested in the presence of Ortho's LIS additive (Table 3). Six of seven samples were tested using PEG as the additive; five samples were positive at the AHG phase of testing and one showed agglutination-aggregation with PEG at 37°C only. When the anti-E were tested with ficin-treated RBCs, three samples were positive at immediate spin, six of seven were reactive at 37°C, and only one sample remained reactive at the AHG phase.

#### Comparison with archived anti-E

To determine whether examples of anti-E routinely react more strongly with RBCs suspended in MTS2™ compared to cells manufactured as 0.8% Ortho reagent RBCs, 25 examples of anti-E were retrieved from frozen storage and tested with Ortho 3% R<sub>2</sub>R<sub>2</sub> RBCs diluted to 0.8% in MTS2 and with 0.8% Ortho reagent RBCs. Most (23 of 25) of these samples were archived before the development of gel column agglutination technology; therefore, sample selection was not biased in favor of any diluent used in gel column technology. All 25 samples were detected by both sets of reagent RBCs at approximately equal strength (data not shown). This suggests that only selected anti-E samples show differential reactivity in MTS Anti-IgG cards.

## Discussion

Commercially prepared 0.8% reagent RBCs eliminate the need to manually dilute 3 to 5% reagent RBCs for use in gel cards. In a population of 805 random and 277 antibody-reactive samples, Ortho 0.8% reagent RBCs were shown to perform comparably to Ortho 3% reagent RBCs resuspended in MTS2™.<sup>2</sup> It was therefore of some concern that 12 anti-E samples were identified that reacted differently when tested in MTS Anti-IgG gel cards with Ortho 0.8% reagent RBCs versus Ortho 3% reagent RBCs. A study of 25 archived anti-E samples suggested that the 12 referred anti-E were a subset of antibodies that reacted differentially between the two RBC sources. Several hypotheses were considered to address these discrepancies. First, variation in the E antigen expression of different RBC donors was the most obvious explanation. The donors used to manufacture 3% and 0.8% reagent RBCs are different. It is impossible to directly compare antibody reactivity between Ortho 3% reagent RBCs and Ortho 0.8% reagent RBCs, regardless of the test method used. The bias for some anti-E to react with one RBC sample versus another was clear when five antibodies were tested with five different RBCs and reacted from 6 to 56 percent of the time, depending on the donor cell. Variations in the E antigen have been recently described, which may explain the preference of some antibodies for particular cells.<sup>3,5</sup> As the understanding of E antigen structure and expression becomes better characterized, it may be useful to include examination of the E antigen structure in future investigations.

Diluent composition and use also were considered in the explanation of the differences in anti-E detection. In experiments in which the same RBCs were washed and resuspended in different LIS diluents, the antibodies in this study reacted most consistently with RBCs suspended in MTS2. However, it is important to note that for routine use in gel card technology, 3% RBCs are stored in an isotonic solution and resuspended in an LIS diluent on the day of use. In contrast, 0.8% RBCs are manufactured and stored in an LIS diluent and are not washed or resuspended prior to use. The effects of long-term storage on RBC membranes in different diluents as well as diluent composition may therefore play a role in the variable detection of anti-E.

Finally, there are reports that describe anti-E antibodies in individuals that appear to be unrelated to RBC exposure; i.e., by transfusion, by pregnancy, or otherwise immunized by RBCs.<sup>6-10</sup> It is unknown if any of the antibodies in this study could be so characterized.

**Table 3.** Results of anti-E tube testing using Ortho Antibody Enhancement Solution (OAES), PEG, and ficin

Anti-E sample	OAES	PEG	Ficin		
	AHG*	AHG	IS†	37°C	AHG
1435	0	1	2	0	0
1488	0	2	1	2	1
CC1	0	1	0	2	0
CC2	0	2	1	1	0
2511	0	NT‡	0	1	0
0492	0	1	NT	1	0
2128	0	0§	NT	1	0

\* antihuman globulin test

† immediate spin

‡ not tested

§ 0 at AHG; 1+ at 37°C

Further samples from the 12 patients with anti-E used in this study were unavailable. Similarly, we did not have access to the patients' histories. We were, however, able to determine that seven anti-E reacted only with PEG, ficin-treated R<sub>2</sub>R<sub>2</sub> cells, or both when examined by tube methods. These findings are important in light of previous reports that PEG or enzyme-only antibodies are not clinically significant and that their detection is undesirable.<sup>10,11</sup>

The 12 examples of anti-E described in this report were confirmed to be less frequently detected with Ortho 0.8% reagent RBCs in comparison to Ortho 3% reagent RBCs resuspended in MTS2. However, the observation that seven of the antibodies subjected to further investigation reacted under conditions not well correlated with clinical significance show that failure to detect anti-E with Ortho 0.8% reagent RBCs may save the time and cost associated with antibody identification and prevent unnecessary use of antigen phenotyped blood.

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*Dania D. Yaskanin, PhD, MT(ASCP)SBB (corresponding author), Senior Scientist, Immunohematology Assay Research and Development, Ortho-Clinical Diagnostics, 1001 US Highway 202, Raritan, New Jersey 08869; Janice L. Jakway, MA, MT(ASCP)SBB, and David J. Ciavarella, MD, Ortho-Clinical Diagnostics, Raritan, New Jersey.*

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# Quantitation of red cell-bound immunoglobulins and complement in lymphoma patients

M. PODBEREZIN, A. LEVINA, L. ROMANOVA, O. MARGOLIN, O. NASIBOV, AND A. V. PIVNIK

Quantitative ELISA may be useful for determining the amount of red blood cell (RBC)-associated immunoglobulins (Igs) in patients with autoimmune hemolytic anemia (AIHA). In idiopathic AIHA, there is about 20 times more RBC-associated IgG and complement than in normal persons. In patients with low-grade lymphomas (particularly, B-CLL and splenic marginal zone lymphoma) autoimmune hemolysis is a component of their anemia. In high-grade malignant lymphomas (i.e., diffuse large B-cell lymphoma and peripheral T-cell lymphoma), as well as in Hodgkin's disease, autoimmune hemolysis contributes little, if any, anemia. The quantitative ELISA for RBC-associated IgG and complement is useful for following the effects of treatment in patients with immune hemolysis. *Immunohematology* 2000; 16:147–153.

**Key Words:** autoimmune hemolytic anemia (AIHA), enzyme-linked immunosorbent assay (ELISA), red blood cell (RBC), immunoglobulins (Ig), complement

Autoimmune hemolytic anemia (AIHA) which may be primary or secondary, represents approximately 8% of all cases of anemia.<sup>1</sup> Approximately 50 to 60 percent of all patients with secondary AIHA have autoimmune hemolysis.<sup>1</sup> The most frequent diseases associated with AIHA are low-grade chronic lymphoproliferative disorders (LPD), various autoimmune diseases (connective tissue diseases, ulcerative colitis, Hashimoto thyroiditis, pernicious anemia, autoimmune hepatitis, etc.),<sup>1-4</sup> and solid tumors.<sup>5</sup>

Among all lymphoproliferative disorders, B-cell chronic lymphocytic leukemia (B-CLL) is the most common disorder associated with secondary AIHA, representing 11 percent of all cases.<sup>6</sup> Some researchers consider autoantibodies in B-CLL to be tumor-derived and thus clonal in origin.<sup>7</sup> Others propose a reactive etiology of AIHA (i.e., autoantibody production by polyclonal, nontumor lymphocytes).<sup>8-11</sup>

There are relatively few case reports associating AIHA with Hodgkin's disease (HD).<sup>12-14</sup> Kuhbock et al.<sup>15</sup> found features of overt hemolysis in about 2.5 percent of patients with red blood cell (RBC) autoantibodies.

In some patients with HD, AIHA was a presenting feature; in others, HD was diagnosed several years after autoimmune hemolysis. AIHA is more common in advanced stages of HD and steroid therapy is not effective. AIHA presenting in remission of HD may signal impending relapse, but, because the number of such patients is relatively small, there are no recommendations that autoimmune hemolysis serve as a prognostic marker in HD. Progression of disease and overall survival in AIHA-complicated HD patients is similar in HD patients with or without hemolysis.<sup>16</sup>

Warm type AIHA is more common than cold types, but a small proportion of patients with LPD have cold-type AIHA.<sup>17,18</sup>

The most important factors determining the severity of AIHA are the amount and class of RBC-associated immunoglobulin (Igs), as well as the involvement of complement.<sup>19-21</sup>

IgG1 and IgG3 predominate in AIHA. The presence of IgA, IgM, or both, in addition to IgG, may enhance the severity of anemia.<sup>22</sup> There are few reports of IgA as the only immunoglobulin in AIHA.<sup>23,24</sup> Opsonization of RBCs with complement seems to have an additive role in eliminating senescent cells and in the severity of the hemolytic process.<sup>25,26</sup> The mechanisms of RBC destruction (i.e., elimination of senescent RBCs) in physiologic conditions are very similar to those in AIHA. Autoantibodies to the so-called "senescent cell antigen" (modified band 3 protein) are the principal stimuli for RBC removal.<sup>27,28</sup>

One study suggests that defective control of IgG autoreactivity by autologous IgM is an underlying mechanism for hemolysis in warm AIHA.<sup>29</sup>

The direct antiglobulin test (DAT) is the most widely used assay for the diagnosis of AIHA. However, the DAT cannot detect less than 200 molecules of IgG per RBC.

Thus, approximately 10 percent of all cases of AIHA are DAT (Coombs)-negative.<sup>30</sup>

Research efforts aimed at the development of more sensitive assays for the detection of red cell-bound proteins include the Polybrene® test, RBC incubation with proteolytic enzymes, aggregate-hemagglutination test, and flow cytometry.<sup>31,32</sup> Flow cytometry is used increasingly to measure RBC-bound Ig.<sup>33</sup> This technique has the advantage of using more than two fluorescent labels, enabling researchers to analyze different immunoproteins simultaneously. Recently, gel column technology has been used, which allows separation of plasma from RBCs in the sample and makes RBC washing unnecessary, but still detects RBC-bound Ig.<sup>34</sup>

The enzyme-linked immunosorbent assay (ELISA) is sensitive, relatively straightforward, and allows quantification of RBC-bound Ig and complement.<sup>35-37</sup> We have used this method since 1995 and pilot results are promising.<sup>38</sup>

In the following report, we evaluate ELISA for the diagnosis of idiopathic and symptomatic AIHA in acute attacks and long-term follow-up. Also, we assess the contribution of autoimmune hemolysis in anemia in lymphoma patients.

## Materials and Methods

A total of 79 patients were enrolled in the study (41 females and 28 males). Ages ranged from 15 to 76 years (mean = 36). The patients were divided into two groups:

(A) Idiopathic AIHA ( $n = 11$ ); warm autoantibodies (10); cold autoagglutinins (1).

(B) Secondary AIHA ( $n = 68$ ); non-Hodgkins lymphoma (43), including diffuse large B-cell lymphoma (11), follicular lymphoma (1), peripheral T-cell lymphoma (5), chronic lymphocytic leukemia (8) including B-CLL (6) and T-CLL (2), splenic marginal zone lymphoma (5), hairy cell leukemia (2), Waldenstrom macroglobulinemia (2), mantle-cell lymphoma (1), and symptomatic pure red cell aplasia (8); and Hodgkin's disease (25).

All patients had physical examinations according to standard protocol, including specific hematological and immunological tests.

The main method used in this study was a quantitative ELISA for RBC-associated Ig and complement.<sup>38</sup> This test evaluates Ig and complement components fixed to the RBCs using enzyme-conjugated monospecific antibodies. We used peroxidase instead of alkaline phosphatase for this assay due

to ready availability of the former and the absence of different results using either enzyme. Commercial monospecific IgG, IgA, IgM, C1q, and C3 antisera were used in the assay (Biolab, Moscow, Russia). The correlation between the color intensity and RBC-associated Igs, C1q, and C3 amount was calculated. The ELISA test was performed as follows: 0.5 mL of donor group O, I-positive, Rh(D)+ washed RBCs was incubated with serial dilutions of anti-D followed by repeated washing (x 3). Phosphate-buffered solution (PBS; 0.2mL) was added to 0.2 mL of peroxidase-labelled antisera and 0.1 mL of patient and donor RBCs, and incubated for 30 minutes. Acetate- and phosphate-buffered orthofenilenediamine with hydrogen peroxide (0.05% solution) was used as a substrate. Then, 0.2 mL of PBS and 0.2 mL of substrate were added to the mixture and centrifuged after a 30-minute incubation. The supernatant layer was transferred to the plate wells prefilled with 0.1 mL of 0.1% hydrogen peroxide solution. The reaction was stopped with 0.05 mL of 50% sulphuric acid. Optical density of solutions in each well was measured by a spectrophotometer. To calculate the amount of RBC-associated Ig, we took into account the Ig concentration in the anti-D serum, molecular weight of the various Ig classes, the amount of reagents, and the Avogadro coefficient. Curves relating the color intensity to RBC-associated Igs, C1q, and C3 were generated. The method assumes that all anti-D is bound to the RBCs.<sup>39</sup> The method is similar to that used by others.<sup>39,40</sup>

After measuring RBC concentration in samples tested, we calculated the extent of RBC sensitization molecules of IgG, IgA, IgM, C1q, and C3 per RBC.

We tested 52 healthy donors as control samples.

## Results

### Normal controls

We established the following normal ranges for the values of RBC-bound Igs: IgG < 150 mol/ 1 RBC, IgA < 20 mol/ 1 RBC, IgM < 5 mol/ 1 RBC, C1q 10  $\mu$ g/ 1 RBC, and C3 40 to 60  $\mu$ g/ 1 RBC.

### Idiopathic AIHA

All 11 patients with idiopathic AIHA had normochromic anemia with increased reticulocyte counts, indirect hyperbilirubinemia, and bone marrow erythroid hyperplasia. Splenens were enlarged in six patients (54%). DATs were positive in eight patients (72%). ELISA results were strongly positive in all 11.

**Table 1.** Hematologic results and RBC-associated Igs, and complement in patients with idiopathic AIHA

Time period	Hemoglobin g/dL	Reticulocytes %	IgG mol/RBC (n < 150)†	IgA mol/RBC (n < 20)	IgM mol/RBC (n < 5)	C1q µg/RBC (n < 20)	C3 µg/RBC (n = 40-60)
Before treatment (11)*	6.6 ± 0.84	10.3 ± 4.0	2276 ± 593	157 ± 60	65 ± 19	19.8 ± 10.5	127.5 ± 97
After steroid treatment (11)*	9.76 ± 0.65	7.69 ± 3.3	602 ± 221	150 ± 45	60 ± 43	7.0 ± 2.7	16.5 ± 12.0
After splenectomy (4)*	10.5 ± 1.45	8.75 ± 4.25	1286 ± 545	131 ± 69	10 ± 7	Not done	Not done

\* Number of patients

† Normal values

Laboratory results of idiopathic AIHA patients are summarized in Table 1.

We observed a three- to tenfold increase of all Ig classes on the RBCs in idiopathic AIHA. Decreases in the number of RBC-bound autoantibodies correlated with the clinical and hematological responses to steroid therapy. In steroid treatment failures, the level of RBC-associated Ig remained relatively increased.

Splenectomy was performed in four patients who failed steroid therapy. Despite apparent clinical and hematological remission, RBC-associated Ig was increased for several months in two patients. In the remaining two patients, RBC sensitization decreased to normal concentration in a few days. These results correspond to the findings of DAT positivity observed in splenectomized AIHA patients by others.

We compared the results of DATs and ELISAs for the 11 patients (Fig.1). The seven DAT-positive patients all bound more than 1000 Ig molecules per RBC. Only one DAT-negative patient bound more than 1000 Ig molecules.

*Secondary anemia in LPD*

Clinical and laboratory findings were similar in patients with low-grade LPD and idiopathic AIHA. DAT results were positive in three of six (50%) CLL patients and four of five (80%) patients with splenic marginal zone lymphoma (SMZL).

CLL patients were treated with prednisone (60 to 80 mg/day). Following steroid treatment failure, fludarabine was administered 50 mg/day in two patients for five courses. The first-choice method of treatment in SMZL was splenectomy. In three of four splenectomized patients, relapse occurred on an average of 8 months after splenectomy. In two of those three relapsed patients, fludarabine was efficacious.

Patients with large B-cell lymphomas and peripheral T-cell lymphomas were treated by various standard protocols (CHOP, ProMACE-CytaBOM, DHAP, or MACOP-B), alone or in combination with radiation therapy. Increases in Hb concentration and decreases in reticulocyte counts were observed only in patients with

CLL and SMZL. Cytotoxic treatment of high-grade B-cell lymphomas was associated with moderate decreases in Hb concentrations and slight increases in reticulocyte counts. In patients with T-cell lymphomas and low-grade non-Hodgkin's lymphoma (NHL), associated with secondary pure red cell aplasia (PRCA), chemotherapy had no effect on Hb and reticulocyte counts.

Levels of RBC sensitization in different LPDs, complicated by anemia, are represented in Table 2. No differences in pre- and posttreatment RBC-associated Ig levels in PRCA-complicated LPDs were observed.

Low-grade LPDs were associated with a seven- to tenfold increase of Ig molecules on RBCs. In those patients, RBC-associated complement, particularly C1q, was increased before treatment. Treatment was associated with measurable decreases of RBC-associated Ig and full normalization of complement concentration.

To evaluate the contribution of autoantibody-mediated hemolysis in different types of low-grade LPD, patients with CLL, SMZL, and hairy-cell leukemia (HCL) were analyzed separately (Table 3).

The highest RBC-associated Ig levels were observed in patients with CLL and SMZL; less sensitization was observed in HCL cases. In CLL, the major complement component on RBCs was C3. In SMZL, it was C1q. There was a mild increase of RBC-associated Ig in

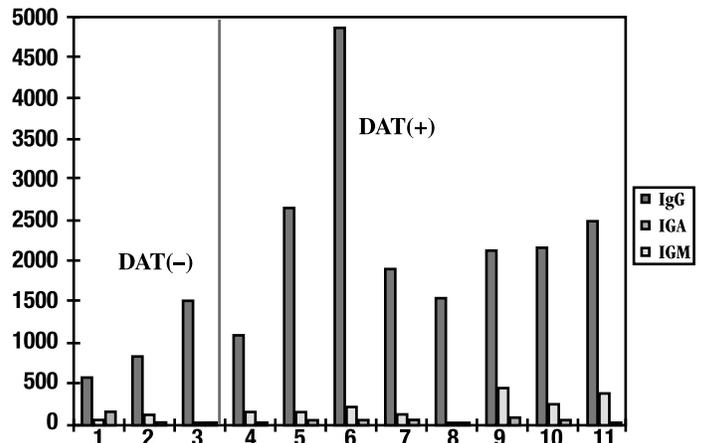


Fig. 1. RBC-associated Ig level in 11 patients with AIHA compared with DAT results. Patients' numbers are shown on the X axis; the Y axis represents the number of Ig molecules per RBC.

**Table 2.** RBC sensitization in patients with chronic lymphoproliferative disorders (LPD) and anemia measured by quantitative ELISA method (average values are represented by *n*)

Diagnosis	Test time	IgG mol/RBC ( <i>n</i> < 150)	IgA mol/RBC ( <i>n</i> < 20)	IgM mol/RBC ( <i>n</i> < 5)	C1q $\mu$ g/RBC ( <i>n</i> < 20)	C3 $\mu$ g/RBC ( <i>n</i> = 40–60)
Large B-cell lymphoma (19)*	Before treatment (12)*	438	102	3	15	28.4
	After treatment (7)*	1042	77	5	1.08	4.98
Peripheral T-cell lymphoma (10)*	Before treatment (5)*	86	29	5	6.8	1.375
	After treatment (5)*	806	76	69	3.2	182.35
LPD complicated by symptomatic PRCA† (16)*	Before treatment (8)*	293	29	17	8.75	81
	After treatment (8)*	346	42	11	0.28	65.6
Low-grade LPD (36)*	Before treatment (18)*	1482	71	45	67.2	57
	After treatment (18)*	372	15	26	4.08	8.7

\* Number of tested samples

† Pure red cell aplasia

patients with large B-cell lymphoma without detectable complement (Table 2). After chemotherapy, there was a twofold increase in the amount of RBC-associated IgG and no significant change in the amount of IgA and IgM on the RBC membranes. Significant increases in RBC-associated Ig and complement levels (especially C3) were observed in peripheral T-cell lymphomas.

We studied 20 HD patients before treatment and divided them into two groups according to their Hb level: 14 with normal Hb levels and 6 with Hb concentrations less than 11.0 g/dL. Only very subtle changes in RBC sensitization were observed between these groups of patients (data not shown). However, we included in the study five HD patients who developed profound anemia during MOPP-ABVD therapy. These patients were analyzed separately. Both Hb concentrations and RBC counts were normal before treatment. All five had high levels of RBC-associated Ig and complement with moderate reticulocytosis (2 to 4%; Table 4).

## Discussion

The ELISA is a highly sensitive quantitative method for measuring RBC-associated Ig and complement. The minimal amount of Ig detectable by conventional antiglobulin tests is as follows (approximately): IgG, 1000; IgA, 100; and IgM, 40 molecules per single RBC (Fig. 1). The ELISA was positive in all patients with idiopathic AIHA, including those with negative DATs,

reflecting the ELISA's greater sensitivity. ELISA results closely paralleled positive or negative outcomes of steroid treatment. An unexpected increase in RBC-associated Ig and complement components was observed in the immediate postplenectomy period. This effect may be due to decreased rate of elimination of sensitized RBCs from the circulation because the site of destruction is absent. The latter event might be explained as the remaining partially compensated hemolytic process. Accordingly, Sokol et al.<sup>1</sup> proposed the term "autoimmune hemolysis" to be more precise than "autoimmune hemolytic anemia." Nevertheless, there is a gradual decrease of RBC autoimmunization during the course of the patient's recovery from AIHA.

Low-grade LPD with anemia was associated with overt laboratory evidence of autoimmune hemolysis (i.e., normocytic, morphology, high reticulocyte count, and indirect hyperbilirubinemia) and RBC sensitization by Igs and complement, illustrating the importance of the diagnostic category of patients with the autoimmune hemolysis. This relationship was particularly evident in CLL and SMZL. The nature of autoantibodies in CLL patients has been described recently. Some researchers propose a clonal origin for these autoantibodies;<sup>7</sup> others reject this hypothesis, stating that RBC-associated Ig in the patients is reactive and produced by different B-lymphocyte clones.<sup>8-11</sup>

There was a correlation between the results of therapy and ELISA results. Recurrences of lymphoma

**Table 3.** RBC-associated Ig and complement in patients with different types of low-grade lymphomas before treatment (average values are shown by *n*)

Type of lymphoma	IgG mol/RBC ( <i>n</i> < 150)	IgA mol/RBC ( <i>n</i> < 20)	IgM mol/RBC ( <i>n</i> < 5)	C1q $\mu$ g/RBC ( <i>n</i> < 20)	C3 $\mu$ g/RBC ( <i>n</i> = 40–60)
CLL (6)*	1942	59	41	47.3	122.2
SMZL (5)*	2651	543	22	227.5	58.7
HCL† (2)*	280 185	32 13	23 16	Not done	Not done

\* Number of patients

† HCL data are shown for both patients

CLL = Chronic lymphocytic leukemia; SMZL = splenic marginal zone lymphoma; HCL = hairy cell leukemia

**Table 4.** Laboratory test results for five Hodgkin's disease patients who developed anemia during MOPP-ABVD treatment (average values are shown by *n*)

Patient	Hemoglobin g/dL	Reticulocytes %	IgG mol/RBC ( <i>n</i> < 150)	IgA mol/RBC ( <i>n</i> < 20)	IgM mol/RBC ( <i>n</i> < 5)	C1q $\mu$ g/RBC ( <i>n</i> < 20)	C3 $\mu$ g/RBC ( <i>n</i> = 40-60)
1	7.2	1.6	1905	157	210	95	152
2	7.7	2.5	4310	483	431	259	330
3	7.0	3.6	2329	206	48	13	2740
4	6.0	3.5	1556	1667	17	7.8	1111
5	6.0	4.1	1875	28	16	150	250

were associated with increased RBC-bound Ig on patients' RBCs, which necessitated fludarabine treatment. In low-grade lymphomas complicated by secondary PRCA, the amount of RBC-associated Ig and complement was within normal ranges and there was no obvious change during treatment. This finding supports the concept that there is no significant contribution of autoimmune hemolysis in the symptomatic anemia of red cell aplasia, although earlier we had shown that standard treatment of AIHA was successful in 90 percent of AIHA-PRCA patients.<sup>41</sup>

There are no data indicating that cytotoxic drugs cause immune hemolysis. Nevertheless, our results suggest that chemotherapy of B- and T-cell lymphomas may result in an increased level of RBC-associated Igs without evident clinical and laboratory signs of AIHA. In a number of HD patients, we observed a severe anemia in the treatment course with very high RBC sensitization by all classes of Ig as well as by complement.

We hypothesize that chemotherapeutic drugs can induce RBC membrane damage with subsequent adsorption of Ig from plasma and accelerated elimination of RBCs from the circulation. This mechanism might be similar to that seen in hereditary hemolytic anemias<sup>42-44</sup> and ineffective erythropoiesis (i.e., megaloblastic anemias, congenital dyserythropoietic anemia, and myelodysplastic syndrome, among others).<sup>45</sup> Therefore, the quantitative ELISA test, which is a sensitive method, may be used in conjunction with other methods to follow patients with symptomatic idiopathic AIHA. The ELISA appears to be more sensitive than the conventional routinely used DAT. The extent of RBC autosensitization appears to correlate with results of therapy, providing a potentially important role for the ELISA during follow-up studies for these patients.

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*M. Podberezin, MD, Postdoctoral Research Fellow, Hematology/Oncology Dept., Children's Hospital Los Angeles, 4650 Sunset Blvd., #57, Los Angeles, CA 90027; A. Levina, PhD, L. Romanova, MD, O. Margolin, MD, O. Nasibov, MD, and A.V. Pivnik, MD, PhD, 125167, Dept. of Hematology and Intensive Care, National Research Center for Hematology, Novozykovsky proezd, 4a, Moscow, Russian Federation.*

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# A quick and simple method for phenotyping IgG-sensitized red blood cells

T.S. SERERAT, D. VEIDT, AND A. DUTCHED

Positive (IgG) direct antiglobulin test (DAT) reactivity ranging from weakly positive to 2+ can be eliminated using a simple "blocking" technique with anti-IgG. This method can be used for antigen typing DAT-positive red blood cells that require the antiglobulin technique. *Immunohematology* 2000;16:154-156.

**Key Words:** blocking technique, positive DAT, phenotyping

Treating IgG autoantibody sensitized red blood cells (RBCs) to render them direct antiglobulin test (DAT) negative prior to antigen typing requiring the antiglobulin technique is usually a complex or time-consuming procedure (gentle heat elution;<sup>1</sup> dissociation of IgG by chloroquine;<sup>2</sup> or acid glycine/EDTA<sup>3,4</sup>). We describe a simple, rapid, and inexpensive method for preparing DAT-negative RBCs that can be performed in any transfusion service or reference laboratory. Although we are not certain how this method works, we believe that "blocking" of the IgG antibody on the sensitized RBCs by anti-IgG is the primary mechanism.<sup>5</sup>

## Material and Methods

Twenty-six blood samples from nontransfused patients whose RBCs had a positive DAT ranging from  $\pm$  weak to 4+ were used in the study. The following antisera were obtained from either Immucor, Inc. (Norcross, GA) or Gamma Biologicals (Houston, TX): anti-S, -s, -Fy<sup>a</sup>, -Fy<sup>b</sup>, -Jk<sup>a</sup>, and -Jk<sup>b</sup>. Anti-IgG (murine monoclonal) was obtained from Gamma Biologicals, and chloroquine and reagents to prepare acid glycine-HCL/EDTA were obtained from Sigma Chemicals (St. Louis, MO).

### *The "IgG blocking" method*

(1) Place one drop of DAT-positive packed RBCs in a 13 x 100 mm test tube. Wash x 4 with normal saline. Discard the last wash.

(2) Add 15 to 20 drops of IgG anti-human globulin (AHG) to the cell button. Mix and resuspend the cell button. RBCs with a weakly positive DAT ( $\pm$  to 1+) may require less AHG.

(3) Allow the suspension to stand at room temperature for 10 minutes. Longer incubation times (15 to 20 minutes) are needed for RBCs with a stronger positive DAT (> 2+).

(4) After incubation, wash the cell suspension x 2 with normal saline. Discard the last wash.

(5) Resuspend the cell button in normal saline (3 to 5% suspension) and repeat the DAT. If the DAT remains positive, spin RBCs to pack and repeat the procedure (steps 2 through 5) until the DAT becomes negative.

(6) The RBCs then can be typed for antigens that require the antiglobulin technique. This technique works well with RBCs that have a weak to moderately positive DAT (weak + to 2+<sup>s</sup>).

To verify the validity of the IgG blocking method, parallel studies using the acid glycine/EDTA and chloroquine diphosphate methods were performed on 26 samples from patients with a positive DAT (IgG) ranging from  $\pm$  to 4+. Fresh EDTA samples were used for initial workups and for phenotyping for the following antigens that required the antiglobulin test: S, s, Fy<sup>a</sup>, Fy<sup>b</sup>, Jk<sup>a</sup>, and Jk<sup>b</sup>. A monoclonal anti-IgG reagent was used for RBC phenotyping as well as for treating the cells in the IgG blocking method.

## Results

Antigen typings for 23 of the 26 samples were in agreement by all three methods (Table 1). One sample, with a 2+<sup>s</sup> DAT (No. 21, Table 1), typed 1+<sup>s</sup> Fy(b+) using the IgG blocking method, but typed Fy(b-) with both acid glycine/EDTA and chloroquine diphosphate methods. Two samples, with 2+<sup>s</sup> and 4+ DATs (Nos. 25

**Table 1.** Comparison of antigen typing of treated direct antiglobulin test (DAT)-negative red blood cells using acid,\* CDP,† and IgG-B‡ methods

Case #	DAT before treatment (IgG)	DAT after treatment with			Antigen typing of treated red blood cells (DAT)-using a LISS antihuman globulin test																	
		Acid	CDP	IgG-B	S			s			Fy <sup>a</sup>			Fy <sup>b</sup>			Jk <sup>a</sup>			Jk <sup>b</sup>		
					Acid	CDP	IgG-B	Acid	CDP	IgG-B	Acid	CDP	IgG-B	Acid	CDP	IgG-B	Acid	CDP	IgG-B	Acid	CDP	IgG-B
1	±	0	0	0	2+	2+s	2+s	2+s	2+s	2+	0	0	0	2+	2+s	2+s	3+s	3+s	3+s	2+	2+s	2+
2	±	0	0	0	0	0	0	3+	3+	3+	3+	2+s	3+	2+s	1+s	2+s	4+	3+s	4+	0	0	0
3	+ weak	0	0	0	2+	2+s	2+s	3+	2+s	2+	0	0	0	2+	2+	3+	2+s	3+s	3+s	0	0	0
4	+ weak	0	0	0	2+	2+s	3+	2+	2+s	3+	2+s	2+	2+	2+	2+	2+s	2+s	2+	2+	2+	2+	2+
5	+ weak	0	0	0	2+	2+	2+	0	0	0	0	0	0	2+	2+	2+	2+	2+	2+	0	0	0
6	1+s	0	0	0	3+s	2+s	3+	0	0	0	3+s	3+s	4+	2+s	3+	3+	3+s	3+	3+s	3+	2+s	3+
7	1+s	0	0	0	0	0	0	3+s	2+s	3+	3+	3+	3+	2+	2+	2+s	0	0	0	2+s	2+s	2+
8	1+s	0	0	0	3+s	3+s	3+s	3+s	3+s	3+s	3+s	3+s	3+s	3+	3+s	0	0	0	3+s	3+s	3+s	3+s
9	1+s	0	0	0	0	0	0	2+s	3+	2+s	0	0	0	0	0	0	3+s	2+s	3+s	0	0	0
10	1+	0	0	0	2+	2+	2+	0	0	0	2+	2+	2+	2+	2+	2+	2+	2+	2+	2+	2+	2+
11	1+	0	0	0	3+s	3+s	3+s	3+s	4+	3+s	0	0	0	2+s	2+s	2+s	0	0	0	3+	3+	3+
12	1+w	0	0	0	0	0	0	4+	4+	4+	3+	3+s	4+	2+s	3+	3+	4+	4+	4+	3+s	3+s	3+s
13	1+	0	0	0	0	0	0	3+	3+	3+	3+	3+	2+s	2+s	2+s	2+	4+	3+	3+s	0	0	0
14	2+	0	0	0	2+	2+	2+	2+	2+	2+	2+	2+	2+	2+	2+	2+	2+	2+	2+	0	0	0
15	2+	0	0	0	0	0	0	3+	2+s	2+s	2+	3+s	3+	2+	2+	2+s	0	0	0	2+s	3+	3+
16	2+	0	0	0	0	0	0	3+	2+	2+	0	0	0	2+	2+	2+	0	0	0	2+	2+	2+
17	2+	0	0	0	0	0	0	2+	2+	2+s	2+	2+	2+s	0	0	0	2+	2+s	2+	2+	2+	2+
18	2+	0	0	0	0	0	0	3+	3+	3+	0	0	0	2+	2+s	2+	3+	3+	3+	0	0	0
19	2+s	0	0	0	0	0	0	3+s	3+	3+	2+	2+s	3+	0	0	0	2+s	3+	2+s	2+	2+s	1+s
20	2+s	0	0	0	0	0	0	3+	2+s	3+	3+	2+s	3+	2+	2+	2+	3+s	3+s	3+s	3+	3+	3+
21	2+s	0	0	0	0	0	0	3+	3+s	3+	3+	3+s	3+	0	0	1+s	2+	3+	2+	2+	2+s	2+
22	2+s	0	0	0	2+s	2+s	2+	0	0	0	2+	2+s	2+	2+	2+	2+	3+	3+	3+	3+	3+	2+s
23	2+s	0	0	0	3+	3+	2+s	0	0	0	2+	3+	2+	2+	2+	2+	4+	3+s	4+	0	0	0
24	3+	0	0	0	2+	2+s	2+	3+	2+	3+	2+s	3+	2+s	1+s	1+s	1+s	0	0	0	2+	2+	2+
25	2+s	0	1+s	+ weak	3+s	NT	3+s	3+s	NT	3+s	0	NT	+ weak	2+s	NT	2+s	3+s	NT	3+s	3+s	NT	3+s
26	4+	3+s	0	1+s	NT	2+s	NT	NT	1+s	NT	NT	2+s	NT	NT	2+	NT	NT	3+	NT	NT	3+	NT

\* Acid glycine/EDTA † Chloroquine diphosphate ‡ IgG-blocking technique

and 26, Table 1), remained DAT-positive after the IgG blocking method but were rendered negative with the acid glycine/EDTA method or the chloroquine diphosphate method, respectively.

At the present time, we do not have enough data on RBCs with DATs greater than 3+ to determine if this method would work for those samples.

**Discussion**

This procedure works well with RBCs that have a weak ± to moderately positive (2+) DAT. Additional studies are needed to determine if this technique can be used when acid glycine/EDTA or chloroquine diphosphate methods fail to eliminate strongly positive DAT reactions (3 to 4+).

We cannot explain the discrepant Fy<sup>b</sup> typing results obtained with case 21 (Table 1). However, this may be an example of an individual whose weak Fy<sup>b</sup> antigen expression may be altered by both acid glycine/EDTA

or chloroquine diphosphate treatment. We are currently trying to obtain additional blood samples from this individual to resolve these conflicting results.

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*Thom S. Sererat, BS(MT), SBB(ASCP), Supervisor Special Services Laboratory, American Red Cross Blood Services, Western Lake Erie Region, Toledo, OH 43620; Douglas W. Veidt, MT(ASCP)SBB, Special Services Technologist, American Red Cross Blood Services, Toledo, OH; and Ann M. Dutched, MT(ASCP)BB, Special Services Technologist, American Red Cross Blood Services, Toledo, OH.*

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# Further characterization of transfusion-related acute lung injury: demographics, clinical and laboratory features, and morbidity

M.A. POPOVSKY AND N.R. HALEY

According to Food and Drug Administration data, transfusion-related acute lung injury (TRALI) is the third most frequent cause of transfusion-associated death in the United States and is characterized by an acute respiratory distress syndrome-like clinical picture following transfusion of plasma-containing blood components. It may be underdiagnosed due to unfamiliarity of clinicians with the syndrome. This report describes the largest series to date, 46 cases, occurring between 1992 and 1998. The male-to-female ratio was approximately 1:1. The mean age at diagnosis was 54 years. The most frequent presenting symptom or signs were acute respiratory distress, hypotension, and hypertension. Antibodies to human leukocyte antigens or granulocytes were identified in 61 percent of cases, with 50 percent associated with antibodies in a donor whose blood had been transfused to a patient developing TRALI. Clinical recovery occurred in 87 percent of patients, but TRALI contributed to deaths in 13 percent. Clinicians need to recognize and diagnose this syndrome in order to respond with appropriate interventions. *Immunohematology* 2000;16:157–159.

**Key Words:** TRALI, transfusion reactions, antibodies to leukocyte antigens, antibodies to granulocytes

As recently as the 1990s, most medical textbooks gave scant attention to the respiratory tree as a site of involvement for complications of transfusion.<sup>1,2</sup> Although the massive transfusion literature of the 1950s and 1960s referred to “shock lung,” this was subsequently shown to be a consequence of underlying tissue damage rather than hemotherapy.<sup>3</sup> Other transfusion complications involving the pulmonary system include severe allergic reactions, circulatory overload, and graft-versus-host disease.<sup>4</sup> In 1983, transfusion-related acute lung injury (TRALI) was first recognized as a life-threatening syndrome.<sup>5</sup> Although numerous reports have documented the seriousness of TRALI, fewer than 200 cases are found in the literature.<sup>6</sup> <sup>20</sup> As such, this important problem remains obscure to many transfusing clinicians.

TRALI is characterized by acute respiratory distress, acute pulmonary edema, and hypoxemia, and is frequently accompanied by hypotension and fever.<sup>21</sup> The onset is always within 2 hours of a transfusion of a plasma-containing blood product. Red blood cells, platelet concentrates, pheresis platelets, and frozen plasma have all been implicated.<sup>4,21,22</sup> Recent data from the Food and Drug Administration (FDA) suggest that TRALI is the third most common cause of death from transfusion.<sup>23</sup> This study describes the demographic, clinical, and laboratory features of TRALI.

## Methods

The cases that were included in this study were a result of a review of records of all serious transfusion reactions in an American Red Cross centralized database that stores all reports from each of the 36 Red Cross regional blood centers (38 at the time of the study). Using Red Cross procedures, a serious adverse reaction is defined as—

“any event that suggests significant hazard, contra-indication, side effect, or precaution, and in which medical judgement is required to evaluate recorded information. Included in this category is any occurrence suggesting that a dose received is fatal or life-threatening; results in persistent or significant disability or incapacity; requires or prolongs inpatient hospitalization; or causes a congenital anomaly or birth defect.”<sup>24</sup>

The study period included fiscal years 1992 through 1998. It is a requirement of the Red Cross that all serious reactions must be reported to the organization’s national headquarters. These reports originated at transfusion facilities and were forwarded to regional blood centers. The authors reviewed all cases of

respiratory distress and excluded cases that did not meet previously published criteria for the diagnosis of TRALI.<sup>5</sup> For purposes of this study, TRALI is defined as acute respiratory distress, pulmonary edema, and hypoxemia occurring within 2 hours of transfusion, where other causes of acute respiratory distress have been ruled out (including anaphylaxis and myocardial injury). Tests to identify antibodies to human leukocyte antigens (HLA) and granulocytes were performed in most cases.

## Results

Of the 46 cases meeting the criteria, 24 (53%) were females. The mean age of the recipients was 54 years, with a range of 22 to 82 years. The predominant presenting symptoms or signs were respiratory distress in 35 (76%), hypotension in 7 (15%), and hypertension in 7 (15%) of cases. The respiratory distress was frequently sudden and associated with hypoxemia.

Antibodies to HLA or granulocyte specific antigens were identified in 28 (61%) cases. In 23 (50%) cases, the antibodies were of blood donor origin (of the transfused components), with granulocyte antibodies found in 41 percent and those with HLA-specificity in 28 percent of cases, respectively. In only 11 percent of cases was the antibody identified in the recipients in pretransfusion serum. Of those, 7 percent were HLA-specific, while granulocyte antibodies were found in 4 percent.

The clinical course was marked by recovery of 40 (87%) patients. The implicated products included red cells, platelet products, and fresh frozen plasma. In at least two cases, more than one type of plasma-containing product may have been the cause of the reaction. In six patients (13%), TRALI was judged to be a contributing factor as a cause of death. The mean age of the deceased was 58 years.

## Discussion

In the most recent publication of transfusion-related fatalities reported to the FDA for the period 1990–1998,<sup>23</sup> TRALI ranked only behind hemolysis and bacterial contamination as a cause of death.

Significant questions remain unanswered about this transfusion complication. These include: who is at risk; what is the prevalence of HLA or granulocyte antibodies in the implicated blood components (or recipients); and what is the clinical course? Answers to these questions have been hindered by the lack of large

retrospective or prospective randomized studies. Popovsky and Moore<sup>22</sup> described 36 cases seen at the Mayo Clinic, but since that publication, most papers have referred to single cases.<sup>17-20,22</sup> This study represents the largest group of cases of TRALI reported to date. These data underscore previous observations, the most important being the association of HLA and granulocyte-specific antibodies in the plasma of blood components transfused in the 1 to 2 hours prior to the onset of the transfusion reactions. More than 60 percent of the cases involved passively transfused antibodies. This finding supports the hypothesis that TRALI predominately results from passive transfer of donor antibodies to the recipient.

Given the fact that approximately half of the nation's blood supply is collected by the Red Cross (accounting for more than 10,000,000 blood components per year), the accumulation of TRALI cases over 7 years suggests a lower incidence than that observed elsewhere.<sup>22</sup> This may reflect a lack of awareness of this complication, resulting in under-diagnosis or misdiagnosis.<sup>21</sup>

Some investigators have questioned the etiology of cases in which no antibody has been found and have proposed an alternative pathogenesis.<sup>25</sup> However, a recent report by Kopko et al.<sup>26</sup> in which only Class II HLA antibodies were identified in an implicated donor substantiates the view that complement activating antibodies are important. The elegant experimental rabbit model of Seeger and colleagues provides additional support that passively transfused antibodies are responsible for the clinical and laboratory findings seen in humans.<sup>27</sup> These investigators demonstrated significant pulmonary injury in rabbits only when an admixture of complement, anti-5b granulocyte antibody, and 5b-positive leukocytes were present.

Thus, it is noteworthy that 13 percent of the patients in this study died of TRALI, affirming the clinical importance of this complication. Thus it is important to better understand TRALI. The true incidence of the syndrome and its true mortality awaits a better case-control series.

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*Mark A. Popovsky, MD, Corporate Medical Director and President of Cell Processing, HAEMONETICS CORP., 400 Hood Rd., Braintree, MA 02184; and N. Rebecca Haley, MD, American Red Cross—Biomedical Services, 1616 Ft. Myer Drive, Rosslyn, VA.*

## BOOK REVIEWS

*Blood Bank Regulations: A-Z, 2nd edition.* Kay McCurdy, SBB(ASCP) and Kay Gregory, MS, MT(ASCP)SBB. Bethesda, MD: American Association of Blood Banks (AABB) Press, 1999. Domestic: member \$70, nonmember \$100. International: member \$90, nonmember \$120. Stock number: 992010. To order: call (301) 215-6499, fax (301) 951-7150, or [www.aabb.org/store](http://www.aabb.org/store).

Did Noah Webster change the world when he published the first "American" dictionary? Probably not. But generations of scholars and would-be scholars owe him a large debt of gratitude for minimizing the research one must do to check spelling and definitions. In much the same way, McCurdy and Gregory have created a resource that every serious regulatory affairs office dealing with blood and plasma issues will want on their shelf. The time saved in locating the regulatory basis for even one query will easily repay the small investment for this valuable compendium.

Although the emphasis on American Association of Blood Banks (AABB) and transfusion service issues predominates, even source plasma collectors will find it extremely helpful whenever subject-specific research is needed. However, it is disappointing that American Blood Resources (ABRA), European Plasma Fractionation Association (EPFA), and other international organization documents are not included, but the broad spectrum of references that are included is a major advantage despite the paucity of description of each citation.

The alphabetical arrangement of subjects enables one to turn directly to areas of interest. But most readers would profit from a more detailed index that could be skimmed quickly when the user is uncertain how the target topic is keyed. Topic listings like "FDA" or "Licensing" would benefit from further subcategory breakdown. Several areas, however, do have good crossreferencing to other key words. This reader found the grouping together of several memoranda on a single subject to be more helpful than the traditional chronological lists of FDA memoranda. Helpful appendices include resource lists citing telephone numbers and addresses for obtaining the documents indexed—a rare and extremely useful bonus.

I believe every facility trying to do business in the blood or plasma arena would find this book invaluable and will eagerly await the 3rd edition. Unfortunately,

changes in standards will soon make this edition obsolete, but fortunately the cost of replacing it is minimal. The lawyers involved in any litigation pertaining to the provision of blood products will also want a copy on their shelf. Let's hope McCurdy and Gregory are already hard at work on the next edition.

*P. Ann Hoppe, MT(ASCP)SBB  
Vice President, Regulatory Affairs  
Serologicals Corporation  
Atlanta, GA*

*Blood Transfusion Therapy: A Physician's Handbook.* 6th ed. American Association of Blood Banks (AABB) Press, 1999. Quantity 1-10: Domestic: member \$9.99, nonmember \$14.99. International: member \$16.99, nonmember \$21.99. Stock number: 99310661 A. To order: call (301) 215-6499, fax (301) 951-7150 or [www.aabb.org/store](http://www.aabb.org/store).

Now in the 6th edition, the American Association of Blood Banks (AABB) *Blood Transfusion Therapy: A Physician's Handbook* remains a useful resource for the practicing physician, the medical student, and ancillary medical staff interested in an overview of transfusion medicine concepts. By design, the authors address major topics in transfusion medicine in a concise manner. Chapters are particularly well referenced, giving the reader an opportunity to seek more in-depth discussion of the topics and access to primary data. This edition has been updated to conform to the 19th edition of *AABB Standards for Blood Banks and Transfusion Services*.

The design is straightforward and accessible. The chapters on blood components and plasma derivatives describe each component and derivative available. There are sections on indications, contraindications, precautions, dose, and administrations. New concepts such as universal leukoreduction solvent detergent-treated plasma, donor-retested plasma, and fibrin sealant have been introduced. In addition, the section on hematopoietic progenitor cells (HPC) in the previous edition has been expanded to a full chapter, reflecting the important developments in collecting and processing these cells. Specifically, this chapter now includes a discussion of different sources of HPC, the use of growth factors, and transfusion therapy during transplantation.

The chapter on transfusion practices gives succinct descriptions of maximum surgical blood ordering; alternatives to allogenic transfusions; transfusion practices in particular situations (e.g., massive transfusion, obstetrics, and pediatrics), therapeutic apheresis, and administration of blood. The chapter on hemostatic disorders gives an overview of hemostasis and then gives a brief discussion of platelet disorders and congenital and acquired disorders of coagulation and fibrinolysis. The chapter on transfusion reactions has a handy table listing the types of reactions along with the signs and symptoms, treatment, and prevention. In addition, there is a concise table on the

treatment of an acute hemolytic transfusion reaction. This will be particularly helpful to the novice.

In these days of weighty specialty textbooks, this authoritative pocket guide serves as a handy resource for the clinician who transfuses patients. In particular, it should be recommended for those who desire quick information about transfusion medicine. Finally, this guide provides an up-to-date "quick read" for those physicians in training in all fields of medicine.

Brenda J. Grossman, MD  
Chief Medical Officer  
Missouri-Illinois Blood Region  
4050 Lindell Boulevard  
St. Louis, MO 63108

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## COMMUNICATIONS

Letters to the Editors

### Re: Gel technology for RhIG dosage

In the paper "A gel technology system to determine postpartum RhIG dosage" (Vol. 16, No.3, 2000; pp. 115-9), the authors (Fernandes JR, et al.) correctly identify a dilution of 20  $\mu$ L of cord blood (packed to a hematocrit of 70 to 75%) into 10 mL of mock maternal blood (packed to a hematocrit of 70 to 75%) as representing an 0.2% fetomaternal hemorrhage. But, somehow, dilutions of 50, 70, and 100  $\mu$ L into 10 mL are given as 0.4%, 0.56%, and 0.8%, respectively. Clearly, they actually correspond to 0.5%, 0.7%, and 1.0% (or 25 mL, 35 mL, and 50 mL of volume based on a maternal volume of 5000 mL). The interpretation for RhIG dosage cutoffs needs to be changed accordingly. It should also be noted that the AABB *Technical Manual*<sup>1</sup> gives 30 mL of fetal whole blood volume as the recommended amount for coverage by 300  $\mu$ L of RhIG as opposed to 20mL as used by the authors.

Stephen Apfalroth, PhD, MD  
Albert Einstein College of Medicine  
Jacobi Medical Center Blood Bank  
1400 Pelham Parkway JH-1E12  
Bronx, NY 10461-1138

1. Vengelen-Tyler V, ed. Technical manual. 13th ed. Bethesda, MD: American Association of Blood Banks, 1999:505.

The above letter was sent to Fernandes JR, et al. The authors offer the following reply.

We thank Dr. Apfalroth for his observations. The issue of inocula percentages is noted. As indicated in our article on page 116, the percentages of 0.2, 0.4, 0.56, and 0.8 refer to the percentages corresponding to 10, 20, 28, and 40 mL of fetal blood in 5000 mL of maternal blood.<sup>1</sup>

While it is correct that the AABB *Technical Manual*<sup>2</sup> states that 300  $\mu$ g of Rh immune globulin (RhIG) is sufficient to counteract the immunizing effects of 30 mL of fetal whole blood, it has been advocated that if one used 300  $\mu$ g of RhIG to cover for only 20 mL of fetal maternal hemorrhage, this would further decrease failures of immune therapy.<sup>3</sup>

In any event, critical to the use of this test is the concomitant use of calibrators to determine when adequacy of RhIG dosage has been met.

Marciana D. Reis, MD, FRCPC  
Chief, Dept. of Clinical Pathology  
Sunnybrook and Womens College  
Health Sciences Centre  
2075 Bayview Avenue  
Toronto, Ontario, Canada M4N 3M5

1. Fernandes JR, Chan R, Coordavia AS, Reis MD, Pinkerton PH. A gel technology system to determine postpartum RhIG dosage. *Immunohematology* 2000;16:115-9.
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Letter From the Editors

**Review: 2000**

This is the last issue that will be edited by Dr. William Sherwood. He has retired from all of his duties with the American Red Cross in Philadelphia as Medical Editor of *Immunobematology*. He has performed that function with great vigor and extraordinary professionalism for 5 years. He also, as many of you know, has a wonderful knowledge of computers and their uses. He was extremely helpful when we made changes in the Web site and worked with the Web manager to make the *Immunobematology* site useful to our readers.

We wish him a happy and healthy retirement and will greatly miss his considerable contributions to the journal.

We thank the authors of the outstanding articles published in 2000. We are especially proud of the "Special Millennium Issue," the first issue of the new century. That issue covered changes in ABO and Rh systems, methodology, and transfusion medicine both in the past and in the present. The 2000 index published in this issue gives you an overview of the scope of material published in all four issues and lists the authors.

We thank our editorial board, especially Dr. William Sherwood, whose names are published in the front of each issue. The board members offer important suggestions for improving the journal and constantly support our efforts. Board members also serve as peer reviewers, as requested.

Last but not least, we thank the following individuals who assisted us in reviewing and selecting papers for publication:

James P. AuBuchon, MD  
Peter Byrne  
Ann Church, MT(ASCP)SBB  
Debra Ciesielski, MT(ASCP)SBB  
Karen M. Cipolone, MT(ASCP)SBB  
Martha Combs, BS  
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Walter H. Dzik, MD  
George Garratty, PhD, FIBMS  
Alfred J. Grindon, MD  
Brenda Grossman, MD  
Andrew Hadley, MD  
Teresa Harris, BA, MT(ASCP)SBB  
Christopher D. Hillyer, MD

Susan H. Hsu, PhD  
Sue Johnson, MT(ASCP)SBB  
W. John Judd, FIBMS, MIBiol  
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Mary Ann Keashen-Schnell, BS  
Christine Lomas-Francis, MSc  
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Marilyn K. Moulds, MT(ASCP)SBB  
Sandra J. Nance, MS, MT(ASCP)SBB  
Lawrence D. Petz, MD  
Steven R. Pierce, SBB(ASCP)  
Joyce Poole, FIBMS  
Dawn M. Rumsey, ART  
Gerald Sandler, MD  
David Stroncek, MD  
Jeffery Trimble, BSc  
Virginia Vengelen-Tyler, MT(ASCP)SBB  
Fumi-ichiro Yamamoto, PhD

The success of the journal is directly related to the quality of the papers and the reviews. Please accept our sincere gratitude and admiration for a year well done.

We also thank both of the companies that sponsored an issue of *Immunobematology* in 2000. Ortho-Clinical Diagnostics has contributed for 10 years and to them we owe a debt of gratitude. Immucor sponsored the June 2000 issue and for that we are most grateful.

January 1, 2000 saw the initiation of the new *Immunobematology* Web site ([redcross.org/immunohematology](http://redcross.org/immunohematology)). All issues published in the past 5 years are now accessible in total! Cover to cover! Viewers use the password (2000) to access the site and can do searches by word or phrase, subscribe on a protected line, read the complete journal, send a letter to the editor, obtain instructions for authors, and much more.

As *Immunobematology* moves into its 17th year of publication, we would like to ask those of you who read and appreciate the journal to think about submitting an article for publication. Without articles that relate to red cell, white cell, and platelet serology, and transfusion, education, computers, and other appropriate subjects, there would be no *Immunobematology*.

Delores Mallory  
Editor-in-Chief

Mary McGinniss  
Managing Editor

## ANNOUNCEMENTS

**Masters (MSc) in Transfusion and Transplantation Science.** Applications are invited from medical or science graduates for the master of science (MSc) degree in transfusion and transplantation sciences at the University of Bristol, England. The course starts in October 2000 and will last 1 year. A part-time option lasting 3 years is also available. Candidates can apply now for October 2000. There also may be opportunities for PhD or MD studies. The syllabus is organized jointly by the Bristol Institute for Transfusion Sciences and the University of Bristol Division of Transplantation Sciences. It includes:

- Scientific principles underlying transfusion and transplantation
- Clinical applications of these principles
- Practical techniques in transfusion and transplantation
- Principles of study design and biostatistics
- An original research project

For further details and application forms, **contact:** Professor Ben Bradley, University of Bristol Division of Transplantation Sciences, Bldg. 11, Zone A, Southmead Health Services, Westbury-on-Trym, Bristol BS10 5ND, UK; Fax: 44-117-9506277; phone: 44-117-9595341; e-mail: ben.bradley@bristol.ac.uk; quote code number: 014.

**Monoclonal anti-Js<sup>b</sup>.** A murine monoclonal anti-Js<sup>b</sup> that reacts by the indirect antiglobulin test using anti-mouse globulin serum is available to anyone who asks. **Contact:** Marion Reid, New York Blood Center, 310 E. 67th Street, New York, NY 10021; e-mail: mreid@nybc.org

**Specialist in Blood Bank Technology Program.** The University of Texas Medical Branch Specialist in Blood Bank Technology Program is accepting applications for the July 2001 class. Training includes theory and clinical practice in transfusion service donor center, HLA management, research, education, and bone marrow transplantation. Students receive approximately \$1800 per month salary and comprehensive benefits. One year of blood bank experience is required with MT(ASCP) or equivalent. Deadline for registration is January 2, 2001. **Contact:** Janet L. Vincent, MS, SBB (ASCP), Education Coordinator, UTMB Blood Bank, 301 University Blvd, Galveston, TX, 77555-0743. Phone: 409-772-4866; e-mail: jvincent@utmb.edu. For additional information, see the Web site at <http://www2.utmb.edu/sbb>. EOE/M/F/H/VAA. UTMB hires only individuals authorized to work in the United States.

## IMPORTANT INFORMATION!

The National Reference Laboratory for Blood Group Serology of the American Red Cross, formerly located in Rockville, Maryland, has transferred all functions to the Musser Blood Center, Penn-Jersey Region of the American Red Cross, in Philadelphia, Pennsylvania. Please use the following address and phone numbers.

Address all mail for the American Rare Donor Program, Reference Laboratory, Quality Control/Blood Components (Factor VIII assays), or *Immunohematology, Journal of Blood Group Serology and Education* to:

**American Red Cross  
P.O. Box 40325  
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**Reference Laboratory:**  
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**Quality Control/Blood Components:**  
(215) 451-4903 (8 am to 5 pm, EST)

**Immunohematology:**  
(215) 451-4902

**Information:**  
(215) 451-4904

**Fax:**  
(215) 451-2538

**E-mail:**  
[immuno@usa.redcross.org](mailto:immuno@usa.redcross.org)

The National Reference Laboratory for Neutrophil Serology remains at the North Central Region of the American Red Cross in St. Paul, Minnesota.

## CLASSIFIED AD

**Reference Laboratory Supervisor:** A large, progressive AABB-accredited reference laboratory located in a metropolitan area, seeks an experienced serologist to be responsible for daily operations, staff training, and compliance. This laboratory performs advanced problem solving, compatibility testing for problem cases, donor screening, routine pretransfusion testing, and platelet studies. The successful candidate will have SBB certification, minimum 3 years' experience in a reference laboratory, and excellent communication and customer service skills. Supervisory, transfusion medicine, and quality assurance experience preferred. Excellent benefits package. **Contact:** send resume to American Red Cross, Southern Region, Human Resources Dept., 1925 Monroe Dr., NE, Atlanta, GA 30324 or fax: (404) 253-5348.

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**St. Paul Regional Blood Services**

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**St. Paul, MN 55107**

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IMMUNOHEMATOLOGY—VOLUME 16, NOS.1, 2, 3, 4, 2000

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# Immunohematology

JOURNAL OF BLOOD GROUP SEROLOGY AND EDUCATION

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