Direct Identification of Unexpected Serum Antibodies against Red Cell Antigens*

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ABSTRACT

A new method to detect the identity of unexpected antibodies in the sera of prospective transfusion recipients is presented. The method is based on a macroscopic hemagglutination inhibition assay. In this assay, incubation of phenotypically defined test red blood cells with patient sera containing non-agglutinating antibodies to one or more specific antigen systems block monoclonal IgM induction of red cell agglutination. Since the specificities of the IgM antibodies are known, the identity of the patient's antibody(ies) can be directly determined. This method, which uses only reagents that are commercially available, is specific and eliminates the use of the indirect antiglobulin test (IAT) and the use of elimination panels.

Introduction

Screening for the presence of unexpected antibodies is a vital component of blood banking procedures. These antibodies react with major red blood cell antigenic determinants that are not of the A, B, O, or D groups and include antibodies to a variety of antigens, including the carbohydrate systems such as Lewis (Le) and P and I systems and the protein systems including the Rh (c,C,e,E), Kell (K, k, kp, kp, Js, Js), Duffy (Fy, Fy), Lutheran (Lu, Lu), M, N, S and s systems and a number of other systems. Unexpected antibodies to any of these antigens, which may be present in the serum of transfusion recipients and/or of donors of fresh frozen plasma, can cause severe and even life-threatening transfusion reactions in recipients of packed red blood cells and fresh frozen plasma, respectively.

Currently, in the United States, there are approximately 50 million transfusions per year. This involves roughly 10 million individuals, all of whom must undergo screening for unexpected antibodies. The number of positive screens for unexpected antibodies that result varies greatly from center to center and has been estimated from as low as 0.3 percent to as high as 38 percent. Thus, as high as 3 million screens are positive for at least one

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* J. Hall, Gamma Corp. personal communication.
unexpected antibody in transfusion recipients. In addition, there are approximately 12 million donors for transfusions, all of whom require screens for antibodies. Approximately 1 percent of the sera of these individuals is positive for unexpected antibodies or an additional 120,000 positive screens per year. Thus, screening for the presence of unexpected antibodies in serum is a major function of transfusion services.

Since most of the unexpected antibodies that induce transfusion reactions are of the IgG class, they do not usually induce agglutination of test cells that contain the corresponding antigenic determinants. Therefore, detection of these antibodies is based on the use of the well-known two-stage indirect antiglobulin test (IAT) that detects the presence of antibodies on the surface of phenotypically defined test red blood cells. Since this method detects only the presence of antibodies on the test cells, determination of the specificity of individual antibodies can only be determined by the use of multiple panels of test red blood cells. The actual antigenic specificities of bound antibodies are then inferred by a process of elimination. The process is manual and highly labor-intensive, and the process of elimination can be error-prone. Newer methods have recently been introduced that reduce the time necessary for performing the IAT and visualizing the results, such as the gel visualization test.

In addition, some antibody specificities may be determined by the use of hemagglutination inhibition by exogenously added antigen. For example, secreted A antigen from the body fluid of a donor or recipient will block red cell agglutination induced by antibodies to A-positive cells. In addition certain reagents, such as dithiotreitol (DTT), are known to destroy antigens, such as the Kell system, on red cell surfaces and, therefore, can be used as an aid in the detection of antibodies to these antigens. However, these methods are not in general use, in part because they are not needed for the A system and other secretor systems; the use of DTT is used in cases where panel elimination results are equivocal. Furthermore, most unexpected antibodies are directed against complex protein antigens which are not available for use in hemagglutination assays, with the exception of the (M,N) system, for which inhibiting peptides from the amino terminus of glycophorin A have been employed.

Recently, monoclonal IgM antibodies have been developed for the purpose of test cell typing. These antibodies cause direct agglutination of red cells with the corresponding antigen system. Thus far, monoclonal agglutinating antibodies have been developed against the A,B,O,D,M,N,C,e,E,e,K,Jk\textsuperscript{a} and Jk\textsuperscript{b} antigens.

As it happens, the development of such directly agglutinating antibodies has another unexpected use: the direct detection of unexpected antibodies in serum by a hemagglutination inhibition assay. The basis of the method is summarized in figure 1. As shown, the monoclonal antibody directly agglutinates the test cells containing the antigenic determinant against which the IgM is directed. This result gives a reference amount of agglutination. The same cells are incubated with non-agglutinating antibodies from the patient's antiserum that coats the antigenic sites present on test cells. At this point, the antibody-coated cells are incubated with the agglutinating antibody. Since now many of the antigenic sites are blocked by the patient's antiserum, agglutination induced by the IgM monoclonal antibody is much weaker than in the control reaction. The result is a reduction in the agglutination score owing to the competition of the patient's non-agglutinating antibodies with the agglutinating IgM. Since the IgM monoclonal antibody has a known specificity, reduction in the agglutination score could only have been caused by the presence of non-agglutinating serum antibodies to the same red cell antigen.

In this paper, the results of studies on several antigen systems are presented in which this assay is shown to be capable of detecting the presence of non-agglutinating serum antibodies to a number of different antigenic determinants on test red cells and illustrating...
the possible feasibility of this assay in directly identifying the antigen systems against which unexpected antibodies are directed. The studies presented here focus on unexpected antibodies that induce macroscopic agglutination using the indirect antiglobulin test (IAT).

**Materials and Methods**

**Materials**

All phenotypically defined panel cells employed were purchased from the Gamma
DIRECT IDENTIFICATION OF UNEXPECTED SERUM ANTIBODIES

Corp.* (Houston, TX). Monoclonal IgM antibodies with defined specificities for the Jk\textsuperscript{a} and Jk\textsuperscript{b} antigens were obtained from the Ortho Corp.; K (Kell) antigen, and the Rh group antigens, c, and E from the Gamma Corp.* Non-agglutinating human antisera directed against the Jk\textsuperscript{a} and Jk\textsuperscript{b} antigen determinants were obtained from the Gamma Corp.* Anti-c and anti-E antisera were obtained from two patients whose sera were found by the usual screening procedures based on the IAT to contain antibodies to these two antigenic determinants, respectively. The test cells for the indirect antiglobulin test and the Coomb's agglutinating anti-human immunoglobulin reagent were obtained from the Ortho Corp.†

METHODS

\textit{Indirect Antiglobulin Test (IAT)}

All antisera tested in the experiments to be described were subjected to an IAT in the standard manner as described.\textsuperscript{1} Briefly, a three percent suspension of test panel red blood cells (approximately 100 \textmu l) was incubated with 100 \textmu l of human antisera at 37°C for 30 min. To the incubation mixture, a volume of 100 \textmu l of Coomb's antiglobulin was added, and the mixture was incubated for another 30 min after which the mixture was subjected to centrifugation, and agglutination was scored. The usual controls were performed to confirm the reliability of the antiglobulin reagent.

\textit{Agglutination Inhibition Assays}

In these assays, a three percent suspension of phenotypically defined test red blood cells (approximately 100 \textmu l) were incubated with 100 \textmu l of human antiserum for 30 min at 37°C. The mixture was cooled to room temperature. At this point, a volume of 100 \textmu l of monoclonal IgM antibody to the same antigen system as that for the antiserum was added and incubated for 5 min at room temperature. The mixture was subjected to centrifugation, and agglutination was scored. Reference reactions for these reactions were performed in which saline replaced the human antiserum and in which the IgM monoclonal antibody was added directly to the test cells. These control reactions served to give a baseline reference agglutination score.

In addition, other control reactions were performed in which, after human antiserum was incubated with the test red blood cells, an IgM monoclonal antibody, with a specificity different from that of the human antiserum, was added to the cell suspension as described in the preceding paragraph, and agglutination was then scored.

\textit{Agglutination Score}

All red cell agglutinations were scored on a scale of 0 to 4 in the standard manner.\textsuperscript{1} Agglutination scores of 4 represent clumped red cells that formed sediments that were not disrupted on shaking; 3 represented red cell sediments that fragmented only minimally on shaking; 2 represented sediments that could be disrupted on shaking; 1 represented sediments that broke into many fragments on gentle shaking; and 0 represented completely disruptable sediments that formed on gentle shaking. All agglutination experiments were performed in duplicate, and at least two different phenotypically defined red cells containing a specific antigen were employed in each assay for agglutination inhibition. The specific phenotypes for each of the test cells employed are identified for each reaction described in the Results and Discussion section.

\textbf{Results and Discussion}

\textbf{KIDD SYSTEM}

In the present study, three antigen systems were studied for which non-agglutinating antiserum and monoclonal IgM antibodies were obtained: Kidd, Kell, and Rh systems. Typical results obtained with antisera directed against the Jk\textsuperscript{a} antigen of the Kidd system are shown in table I. In this table, agglutination scores of

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\* Houston, TX.
\† Baretin, NJ.
TABLE I
Effect of Human Anti-Jk\textsuperscript{a} Antiserum\textsuperscript{1} on Monoclonal Anti-Jk\textsuperscript{a} IgM-Induced Agglutination of Jk\textsuperscript{a}-Positive Red Cells

<table>
<thead>
<tr>
<th>RBC Antigen\textsuperscript{2}</th>
<th>Saline</th>
<th>Antiserum Jk\textsuperscript{a}</th>
<th>Jk\textsuperscript{b}</th>
<th>Monoclonal Ab Jk\textsuperscript{a}</th>
<th>Jk\textsuperscript{b}</th>
<th>Agglutination Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Jk\textsuperscript{a}, Jk\textsuperscript{b}</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>4+, 4+</td>
</tr>
<tr>
<td>2. Jk\textsuperscript{a}, Jk\textsuperscript{b}</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>4+, 4+</td>
</tr>
<tr>
<td>3. Jk\textsuperscript{a}, Jk\textsuperscript{b}</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>1+, 1+ (weak)</td>
</tr>
<tr>
<td>4. Jk\textsuperscript{a}, Jk\textsuperscript{b}</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>4+, 4+</td>
</tr>
<tr>
<td>5. Jk\textsuperscript{a}, Jk\textsuperscript{b}</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>4+, 4+</td>
</tr>
</tbody>
</table>

\textsuperscript{1}Human antiserum obtained from Gamma Corp. as described in the Materials and Methods section. Results of duplicate experiments. IAT gave a 2+ score for both anti-Jk\textsuperscript{a} and Jk\textsuperscript{b} antisera.

\textsuperscript{2}Gamma panel cells r' r – 12003, which are Jk\textsuperscript{a}, Jk\textsuperscript{b} positive, were used in this set of experiments.

4+ were obtained either by directly incubating test cells with monoclonal anti Jk\textsuperscript{a} antibody or by diluting this antibody 1:1 with normal saline (first two rows of table I). The antiserum by itself caused no agglutination of the test red cells and gave an IAT score of +2.

When test cells were incubated with human anti-Jk\textsuperscript{a} antiserum and then incubated with anti-Jk\textsuperscript{a} monoclonal antibody, there was a marked reduction in the agglutination score from 4+ to a weak 1+ as shown in the third row of table I. That the blockade of Jk\textsuperscript{a} antigen sites by human antiserum is specific was shown by first incubating phenotypically (Jk\textsuperscript{a},Jk\textsuperscript{b}) cells with human anti-Jk\textsuperscript{b} antiserum and then incubating these cells with anti-Jk\textsuperscript{a} monoclonal IgM. The result, shown in the fourth row of table I, shows that the agglutination score is 4+. This result suggests that Jk\textsuperscript{b} antiserum does not interfere with the Jk\textsuperscript{a}-anti-Jk\textsuperscript{a} agglutination reaction. Similarly, anti-Jk\textsuperscript{a} antiserum does not interfere with the Jk\textsuperscript{b}-anti-Jk\textsuperscript{b} agglutination reaction as shown in the fifth row of table I.

The previous experiments were repeated using three other sets of phenotypically different Jk\textsuperscript{a}-positive red cells with the same results. In table II are summarized the results of these experiments in which anti-Jk\textsuperscript{a} antiserum was incubated with each cell group, and the agglutination induced by monoclonal IgM anti-Jk\textsuperscript{a} was measured as compared with the positive control. As can be seen from this table, major decreases in the agglutination scores were observed for each set of phenotypically distinct red cells. These results indicate that similar decreases in agglutination scores occur with Jk\textsuperscript{a}-positive cells independently of the phenotype of the test cells and confirm the specificity of the agglutinating IgM antibody.

TABLE II
Reduction in Agglutination Score of Different Jk\textsuperscript{a}-Positive Red Cells by Anti-Jk\textsuperscript{a} Monoclonal Antibody in the Presence of Human Anti-Jk\textsuperscript{a} Antiserum

<table>
<thead>
<tr>
<th>Kidd Phenotypes</th>
<th>Agglutination Control</th>
<th>Agglutination with Antiserum\textsuperscript{1}</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. (Jk\textsuperscript{a}+, Jk\textsuperscript{b}+)\textsuperscript{2}</td>
<td>4+</td>
<td>1+</td>
</tr>
<tr>
<td>2. (Jk\textsuperscript{a}+, Jk\textsuperscript{b}+)\textsuperscript{3}</td>
<td>4+</td>
<td>1+</td>
</tr>
<tr>
<td>3. (Jk\textsuperscript{a}+, Jk\textsuperscript{b}−)\textsuperscript{4}</td>
<td>3+</td>
<td>1+</td>
</tr>
<tr>
<td>4. (Jk\textsuperscript{a}+, Jk\textsuperscript{b}+)\textsuperscript{5}</td>
<td>4+</td>
<td>1+</td>
</tr>
</tbody>
</table>

\textsuperscript{1}Human antiserum obtained from Gamma Corp. as described in the Materials and Methods section. IAT gave a 2+ score for both anti-Jk\textsuperscript{a} and Jk\textsuperscript{b} antisera.

\textsuperscript{2}Gamma Corp. panel cells r' r – 12003.

\textsuperscript{3}Gamma Corp. panel cells R1R1 – 6.

\textsuperscript{4}Gamma Corp. panel cells R2R2 – 4950.

\textsuperscript{5}Gamma Corp. panel cells r" r – 11976.
Kell System (K)

This tripartite antigen system complex is similar to the Rh system in that it contains three major antigenic subdeterminants, the major antigen group being K,k.\(^1\) Since the k negative phenotype is present in less than 1 of 500 individuals, unexpected antibodies to this antigen are rare.\(^1\) However, unexpected antibodies to K are more common. Agglutinating monoclonal IgM antibodies to K are available. Similar experiments to those described in the preceding section were therefore performed with K-positive cells. As can be seen in table III, incubation of (K,k) cells with anti-K monoclonal IgM resulted in an agglutination score of 2+ (row 1). Interestingly, this score was enhanced by the addition of saline (row 2) despite the 1:1 dilution of the antibody suggesting that the binding avidity of this IgM is enhanced by increased ionic strength. The antiserum by itself caused no agglutination and gave an IAT score of +1.

When human anti-K antiserum was incubated with the test cells, and the mixture was then incubated with anti-K monoclonal antibody, there was a reduction in the agglutination score from 3+ in saline to 0 to 1+ in the presence of antiserum (row 3 of table III). There was no reduction in the agglutination score when antisera such as anti-Jk\(^a\) antiserum, were preincubated with the K+ red cells (results not shown). Furthermore, incubation of phenotypically K- red cells with anti-K monoclonal antibody resulted in no agglutination, confirming the specificity of the antibody (row 4, table III).

Detection of Serum Antibodies to the Rh c and E Antigens

Maternal antibodies to the C,c,E,e Rh antigens on fetal red blood cells are a common cause of fetal hemolysis.\(^1\) It is critical to determine their presence rapidly. The hemagglutination inhibition assay was used to detect antibodies to the c and E antigens as shown in table IV. Patient antisera containing anti-c and anti-E were employed in these assays. Neither antiserum by itself caused any agglutination of (c+,E+) test red cells and gave IAT agglutination scores of +2. As can be seen in table IV, incubation of test cells with the (c+,E+) phenotype with anti-c serum reduces the agglutination score induced by anti-c monoclonal IgM from control values of 4+ (rows 1 and 2 in table IV) to 1+ (row 3).

Both c and E antigens are closely linked,\(^1\) introducing the possibility that anti-c antibodies might also block E sites non-specifically. Conversely, anti-E antibodies might block c sites non-specifically. In either case, false positive results would be obtained for the presence of anti-E or anti-c antibodies, respectively.

To test this possibility, the (c+,E+) cells were incubated with anti-c antisera. The antibody-coated cells were then mixed with anti-E

**TABLE III**

Effect of Human Anti–K Antiserum\(^1\) on Monoclonal Anti–K IgM–Induced Agglutination of K–Positive Red Cells

<table>
<thead>
<tr>
<th>RBC Antigen(^2)</th>
<th>Saline</th>
<th>Antiserum</th>
<th>Monoclonal AB</th>
<th>Agglutination Score(^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. K+</td>
<td>_</td>
<td>_</td>
<td>+</td>
<td>2+, 2+</td>
</tr>
<tr>
<td>2. K+</td>
<td>+</td>
<td>_</td>
<td>+</td>
<td>3+, 3+</td>
</tr>
<tr>
<td>3. K+</td>
<td>_</td>
<td>+</td>
<td>+</td>
<td>1+, 0</td>
</tr>
<tr>
<td>4. K–</td>
<td>_</td>
<td>_</td>
<td>+</td>
<td>0, 0</td>
</tr>
</tbody>
</table>

\(^1\)Obtained from commercially prepared antiserum, Gamma Corp., found to give score of +1 in IAT.
\(^2\)These cells were (K+,k+) R1R1 –791 Gamma Corp. panel cells.
\(^3\)Results for duplicate experiments are given.
TABLE IV

Effects of Human Anti-c and Anti-E Antisera on Monoclonal Anti-c and Anti-E IgM-Induced Agglutination of (c, E)-Positive Red Cells

<table>
<thead>
<tr>
<th>RBC Antigen2</th>
<th>Saline</th>
<th>Antiserum1</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>c</td>
<td>E</td>
<td>c</td>
<td>E</td>
<td>Agglutination Score</td>
<td></td>
</tr>
<tr>
<td>1. (c+, E+)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2. (c+, E+)</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>3. (c+, E+)</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>4. (c+, E+)</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5. (c+, E+)</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>6. (c+, E+)</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

1 Antisera obtained from two patients whose sera was found to contain unexpected antibodies to c and E, respectively. Results of duplicate experiments. Both antisera gave IAT scores of 2+.

2 Present on R2R2 -4950 Gamma test panel cells.

monoclonal antibody. As can be seen in row 4 of table IV, anti-c sera did not interfere with the E-anti-E agglutination reaction which gave an agglutination score of 4+.

Similarly, anti-E antiserum was found not to inhibit the c-anti-c agglutination reaction as shown in row 5 of table IV. On the other hand, as shown in row 6 of table IV, anti-E antiserum blocks anti-E IgM from agglutinating the test red cells.

Thus, the decrease in agglutination score was caused by a specific block of either c or E sites by the appropriate antibody. Parallel results were found for the Kidd system as described above in which anti-Jka anti-serum did not interfere with the Jkb-anti-Jkb agglutination reaction and vice versa.

ADVANTAGES OF THE METHOD

The positive results obtained in this study apply to three completely different antigen systems for each of which reductions in agglutination scores by human antisera were observed from 3 to 4 down to 1. These results all point to the feasibility of this approach in directly identifying unexpected antibodies in human serum on the macroscopic level.

Advantages of this method are, first, that since it directly detects the presence of unexpected antibodies, it lends itself readily to automation. Secondly, the same test cells can be used to detect the presence of multiple unexpected antibodies because the cells contain multiple antigenic determinants. Thus the necessity for using multiple panel cells is eliminated. Furthermore, the hemagglutination inhibition assay described here utilizes commercially available reagents that are already in use for red cell phenotyping and require no further evaluation as reagents.

Drawbacks to this method are based mainly on the unavailability of agglutinating IgM antibodies to all of the antigenic determinants against which unexpected antibodies can be directed. Thus, for example, no IgM antibodies to the Duffy, Lutheran and S,s systems have yet been developed.

Additionally, the theoretical possibility exists that monoclonal antibodies and the presumably polyclonal antibodies in patient antisera may not bind to the same epitopes on the same antigen. In such a case, false negative results might be obtained. Since most antisera are polyclonal, the likelihood of this occurrence is low. If simultaneous binding of IgM and patient antibodies to different epitopes of the same antigen are found to occur, use of polyclonal agglutinating IgM might be necessary.

It should be noted that the results presented here are all based on scoring of macroscopic agglutination. It may be necessary to perform assays that are based on microscopic agglutination scores.1 Adaptation of the hemagglutin-
nation inhibition assay to microscopic agglutination where the blocking antibodies are present in low titers will be the subject of a future communication.

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References