Evaluation of methods for detecting alloantibodies underlying warm autoantibodies

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BACKGROUND: In pretransfusion testing of patients whose sera contain autoantibodies reacting optimally at 37°C, it must be determined whether alloantibodies are also present. Two approaches, testing a 1-in-5 dilution of patients’ sera and the adsorption of sera in the presence of polyethylene glycol (PEG), have been proposed as alternatives to the time-consuming approach of adsorbing sera with ficin- or ZZAP-treated red cells (RBCs). The three approaches were compared.

STUDY DESIGN AND METHODS: Patients’ sera containing warm autoantibodies, with and without alloantibodies, were retested 1) after dilution (1-in-5) and 2) after adsorption with allogeneic RBCs in the presence of PEG. Results were compared to those after adsorption with ZZAP-treated allogeneic RBCs.

RESULTS: Dilution (1-in-5): Twenty-seven of 119 sera (27% with and 22% without alloantibodies) did not react; one example each of alloanti-D, -E, -e, -Fya, and -Jka, and two examples of anti-Jkb were not detected at a dilution of 1 in 5. Alloantibodies were identified in 5 (19%) of 26 1-in-5 diluted sera containing alloantibodies; 87 (73%) of 119 sera still reacted with all cells and would have required further workup. PEG adsorption: Thirty-nine sera were tested after parallel PEG and ZZAP adsorptions. The PEG adsorptions required a total of 55 aliquots of adsorbing cells and 13.75 hours, whereas ZZAP adsorptions required 61 aliquots and 30.5 hours. All alloantibodies (anti-D [3], -C [2], -c [1], -E [4], -K [2], -Fya [1], -Jk* [2], -Jk* [1]) reacted in the adsorbed serum–PEG mixtures at a strength equal to or greater than that in the ZZAP-adsorbed sera.

CONCLUSION: Although the 1-in-5 dilution approach is convenient, only 22 percent of warm autoantibodies without alloantibodies were nonreactive, and 27 percent of alloantibodies of potential clinical significance were not detected. PEG adsorption appears to give similar results to those of ZZAP adsorption, but it has the advantages of eliminating the cost and time of prior treatment of the allogeneic adsorbing cells and of a reduction of at least a 50 percent in adsorption time.

Patients with autoantibodies in their sera that react optimally at 37°C (warm autoantibodies) present a challenge to serologists who must determine if underlying alloantibodies are also present. Warm autoantibody-adsorption procedures are tedious and time-consuming. Laboratories often treat allogeneic adsorbing red cells (RBCs) with enzymes or ZZAP (a combination of dithiothreitol and enzyme) reagent before adsorption to increase the efficiency of the procedure. Recently, two techniques were proposed as alternatives: 1) testing a 1-in-5 dilution of patients’ sera and 2) adsorption in the presence of polyethylene glycol (PEG). The 1-in-5 dilution technique reported by Øyen and Angeles appears to be a modification of a method proposed earlier by Petz and Garratty. Petz and Garratty proposed testing the patient’s serum, at a dilution at which the autoantibody reactivity is weak, against a panel of RBCs; this dilution was selected by titrating the patient’s serum against a pool of two antibody-detection RBCs. This method was proposed for laboratories that do not have the capability to perform adsorptions; however, it was emphasized that only alloantibodies whose titer is higher than the autoantibodies’ titer will be detected.

Liew and Duncan used PEG to enhance adsorption with ficin-treated autologous RBCs. Subsequently, Miller et al. compared adsorptions of ficin-treated RBCs with and without the addition of PEG (though they did not specify whether the RBCs were autologous or allogeneic), and Ylagan et al. compared adsorptions of 10 sera with ficin-treated allogeneic RBCs to adsorptions with untreated RBCs in the presence of PEG. Barron and Brown recently re-

ABBREVIATIONS: AIHA = autoimmune hemolytic anemia; LISS = low-ionic-strength saline (solution); PEG = polyethylene glycol; RBC(s) = red cell(s).
ported their results from comparing adsorptions of 19 sera with ficin-treated allogeneic RBCs to adsorptions with untreated allogeneic RBCs in the presence of PEG. We compared the 1-in-5 dilution technique (dilution technique) and adsorption with untreated allogeneic RBCs in the presence of PEG (PEG adsorption) to our routine adsorption procedure using ZZAP-treated allogeneic RBCs (ZZAP adsorption).8

MATERIALS AND METHODS

A 12-month audit of reference laboratory cases was performed to determine the number of samples that contained warm autoantibodies that reacted in the low-ionic-strength saline solution (LISS) or PEG antiglobulin test and the number of these samples that also contained alloantibodies. Subsequent samples from repeat patients within the 12-month period were noted.

Serologic techniques were performed as previously described.9 Reagent RBCs were obtained from various manufacturers (Gamma Biologicals, Houston, TX; Immucor, Norcross, GA; Organon Teknika Corp., Durham, NC; Ortho Diagnostics Systems, Raritan, NJ). LISS was prepared according to the method of Lüb and Messeter.10 The 20-percent PEG was prepared as previously reported11; commercial PEG reagents were obtained from Gamma (Gamma PeG), Organon Teknika (PEG+), and GTI (GTI PER Brookfield, WI). Anti-IgG (American National Red Cross, Washington, DC) was used for all antiglobulin tests.

Dilution technique

Sera containing warm autoantibodies, undiluted and diluted 1-in-5 in pH 7.4 phosphate-buffered saline, were tested against LISS-suspended RBCs.

ZZAP adsorption

ZZAP reagent was prepared from dithiothreitol (Sigma Chemical Co., St. Louis, MO) and ficin (Sigma), and RBCs were treated for 30 minutes as previously described.1 Equal volumes of packed ZZAP-treated allogeneic RBCs and serum were incubated at 37°C for 30 minutes. After centrifugation, 2 drops of the harvested adsorbed serum were added to 2 drops of a 2-percent LISS suspension of RBCs and incubated for 15 minutes at 37°C before the antiglobulin test. Subsequent adsorptions with additional aliquots of ZZAP-treated RBCs were performed as needed.

PEG adsorption

Unlike the 20-percent PEG prepared in our laboratory, the three commercial PEG reagents are in a low-ionic-strength medium and are used at a different PEG:serum ratio (1:1 for commercial reagents rather than 2:1 for 20% PEG) for serologic testing. In our preliminary studies, we found that, if we used 2 volumes of 20-percent PEG for the adsorption, as in serologic tests, the warm autoantibody reactivity remaining in the adsorbed sera appeared to be enhanced. Thus, 1 volume of PEG was used for the study.

Equal volumes of untreated allogeneic RBCs, serum, and PEG were incubated at 37°C for 15 minutes. Four drops of the harvested serum–PEG mixture (to account for the dilution of the serum by the PEG) were added to 1 drop of 3- to 5-percent RBCs and incubated for 15 minutes at 37°C before the antiglobulin test. When subsequent adsorptions were needed to remove antibody, additional PEG was not added.

RESULTS

In one year, our reference laboratory evaluated 694 samples with warm autoantibodies demonstrable by a routine screening procedure using the PEG antiglobulin test; 457 (66%) of these sera reacted in the LISS antiglobulin test and required adsorptions with ZZAP- or ficin-treated RBCs to detect underlying alloantibodies. Forty-seven percent (194/411) of sera containing PEG-reactive warm autoantibodies and 40 percent (105/263) of sera with LISS-reactive warm autoantibodies, not including repeat samples in the 12-month period, contained alloantibodies. Previous studies reported alloantibody detection rates of 15 to 41 percent12,13 after adsorptions with allogeneic RBCs. 27 to 40 percent14-16 after adsorptions with autologous RBCs, and 14 percent after adsorptions with a combination of autologous (most cases) and allogeneic RBCs17; 92 percent of our adsorptions used allogeneic RBCs.

Dilution technique

A total of 119 sera were retested at a 1-in-5 dilution; when tested without dilution, 108 of these showed reactivity of the same strength with all cells tested ("panagglutinin"), while 11 sera demonstrated variable reactivity from cell to cell. Results for the diluted sera are shown in Table 1. Initial adsorptions with ZZAP-treated RBCs showed that underlying alloantibodies were present in 26 of the 119 sera (see Table 2 for specificities). Alloantibodies were not detected in 7 of these 26 sera. Although alloantibodies were identified in five of the 26 sera when tested at a 1-in-5 dilution, all five of these antibodies had shown variable reactivity when the sera were tested without dilution. Thus, the presence of one or more antibodies was already suspected in those 5 sera.

<table>
<thead>
<tr>
<th>Antibody(ies) identified</th>
<th>All cells reactive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonreactive</td>
<td>Antibody(ies)</td>
</tr>
<tr>
<td>Warm autoantibodies only</td>
<td>20 (22%)</td>
</tr>
<tr>
<td>Warm autoantibodies + alloantibodies</td>
<td>7 (27%)</td>
</tr>
</tbody>
</table>

TABLE 1. Results after 1-in-5 dilution of 119 sera
TABLE 2. Alloantibody specificities underlying warm autoantibodies tested by the 1-in-5 dilution technique*

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Not detected</th>
<th>Identified</th>
<th>Suggested</th>
<th>Masked</th>
</tr>
</thead>
<tbody>
<tr>
<td>D</td>
<td>D</td>
<td>D</td>
<td>C</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>E (2)</td>
<td>D+C (2)</td>
<td>E (3)</td>
<td></td>
</tr>
<tr>
<td>e</td>
<td>K</td>
<td>e</td>
<td>C+e</td>
<td></td>
</tr>
<tr>
<td>Fy*</td>
<td>Jk&lt;sup&gt;e&lt;/sup&gt;</td>
<td></td>
<td></td>
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<tr>
<td>Jk&lt;sup&gt;e&lt;/sup&gt; (2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Jk&lt;sup&gt;b&lt;/sup&gt; (2)</td>
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</tbody>
</table>

* Alloantibody specificities were initially determined after adsorptions with ZZAP-treated allogeneic RBCs.

Warm autoantibodies continued to mask the presence of alloantibodies in the 1-in-5 dilutions of the remaining 14 sera, although, in 4 sera, the presence of anti-D, D+C, and -e was suggested (i.e., reactivity with antigen-negative RBCs was weaker than that with the respective antigen-positive RBCs). Most of the sera, with or without underlying alloantibodies that were negative when tested at a 1-in-5 dilution, reacted only 1+ (a few reacted 2+) when tested without dilution; the majority of the sera that still reacted after dilution at 1 in 5 were reactive 2+ to 4+ when tested without dilution.

PEG adsorption

Parallel PEG and ZZAP allogeneic adsorptions were performed on 28 sera, 8 of which contained alloantibodies. For the 28 sera, a total of 36 RBC aliquots and 9 hours were required for the PEG adsorptions, compared to 37 aliquots and 18.5 hours for the ZZAP adsorptions. For 23 of the 28 sera, an equal number of PEG and ZZAP adsorptions were required; 3 sera needed one less PEG adsorption, and 2 sera needed one more PEG adsorption. All alloantibodies detected (Table 3) reacted in the serum-PEG mixture at a strength equal to or greater than that in the ZZAP-adsorbed serum, presumably because of enhancement by the PEG that remained in the adsorbed serum. One previously identified, very weak anti-E did not react after either PEG or ZZAP adsorption.

4°C adsorption with PEG for removal of cold autoantibody

A mixture of a strong autoanti-I and alloanti-E, after three 15-minute adsorptions with PEG at 4°C or 37°C, reacted 1+ and 4+, respectively, with E- RBCs at room temperature. At antiglobulin test, the 4°C adsorbed serum did not react with E- RBCs and reacted 1½+ with E+ RBCs; the 37°C adsorbed serum agglutinated the E- RBCs (1+) and E+ RBCs (3+) before the addition of anti-IgG and reacted ½+ and 1½+, respectively, after the addition of anti-IgG. Thus, the 4°C PEG adsorption was more efficient than the 37°C PEG adsorption in removing the cold antibody, and the anti-E was still demonstrable.

In a comparison of PEG and ZZAP adsorptions at 4°C (15 min each), three PEG adsorptions and five ZZAP adsorptions were required to remove a strong autoanti-I (4°C ti-
ter, 2048). Thus, PEG was more efficient than ZZAP in removing this high-titer cold antibody.

Is weak antibody activity lost through adsorption with PEG?

We observed that, after one or two adsorptions in the presence of PEG (20% [prepared in our laboratory] or commercial reagent), plasma and sera became clear, both in comparison to unadsorbed plasma and sera and to ZZAP-adsorbed plasma and sera. In addition, it was noted that a precipitate formed in serum–PEG mixtures after storage at 4°C. Several experiments attempted to determine if these phenomena represented loss of specific antibody reactivity after adsorption with PEG.

A weak anti-Jk\textsuperscript{a} (1+ by LISS antiglobulin test) was used for sham adsorptions with three aliquots of Jk(a–) RBCs in the presence of 20-percent PEG or Gamma PEG. The anti-Jk\textsuperscript{a} was 1+ reactive with Jk(a+b–) RBCs after each of the three adsorptions with either of the PEG reagents.

The same weak anti-Jk\textsuperscript{a} diluted with an equal volume of a warm autoantibody and adsorbed twice with PEG- or ZZAP-treated Jk(a–) RBCs reacted 1+ with Jk(a+b–) RBCs in both adsorbed sera after removal of the autoantibody. An anti-E reacted 1/2+ in the serum–PEG mixture after each of two sham adsorptions with E– RBCs in the presence of 20-percent PEG; before the adsorption, the anti-E reacted 1/2+ with and without the addition of an equivalent volume (2 drops) of PEG. The anti-E–PEG mixture from the second sham adsorption still reacted (1/2+) after storage at 4°C overnight (and centrifugation to remove the precipitate). An equal volume of PEG was added to master dilutions of an anti-E. Aliquots of the anti-E–PEG mixtures were tested immediately and after 24 hours' storage at 4°C. The stored dilutions were centrifuged to remove the precipitate that had formed. The anti-E titer was 8 before and after 4°C storage.

**DISCUSSION**

The alloimmunization rate (40%) we observed in our patients with LISS-reactive warm autoantibodies is higher than that in previous reports, especially those in which adsorptions were performed primarily with allogeneic as opposed to autologous RBCs.\cite{12-16} Wallhermfechtel et al.\cite{12} and Issitt et al.\cite{13} who performed adsorptions with allogeneic RBCs, reported detection of alloantibodies in 15 percent (19/125) and 41 percent (14/34) of sera, respectively. In the studies of adsorptions with autologous RBCs, Issitt et al.,\cite{13} James et al.,\cite{14} Laine and Beattie,\cite{15} and Morel et al.\cite{16} detected alloantibodies in 27 percent (11/41), 32 percent (13/41), 38 percent (41/109), and 40 percent (8/20) of sera, respectively. Sokol et al.\cite{17} detected alloantibodies in 14 percent (294/2149) of their patients, but that series included samples from patients with warm- and cold-reactive autoantibodies and did not distinguish how many patients with warm autoantibodies had alloantibodies. In addition, Sokol et al.\cite{17} state that they issued K–, Rh phenotype-matched blood to decrease the incidence of alloimmunization. Issitt et al.\cite{13} found that 69 and 19 percent of the suspected alloantibodies detected after adsorptions with autologous and allogeneic RBCs, respectively, had autoantibodies that mimicked alloantibodies. Although adsorptions with autologous RBCs were not separated out in our audit, our data primarily (92%) represent adsorptions with allogeneic RBCs. Adsorptions with autologous RBCs were not the focus of this investigation. Although most serologists would treat sensitized autologous RBCs (e.g., with enzyme, ZZAP, or chloroquine) to remove immunoglobulins before adsorption, Combs and Issitt, as cited in Issitt and Anstee,\cite{18} used untreated autologous RBCs for PEG adsorptions without loss of efficiency.

We should emphasize that we studied more patients than did other investigators (i.e., 263 vs. 20–138),\cite{12-16} excluding Sokol et al.,\cite{17} for the reason stated above. The patient population that is referred to our reference laboratory is from a broad geographic base but may be biased toward patients requiring transfusion and/or patients with stronger antibodies. Another major factor is the number of transfusions the patients have received previously. James et al.,\cite{14} and Wallhermfechtel et al.\cite{12} found that 75 percent (3/4) and 32 percent (6/19), respectively, of patients who had received >5 transfusions had alloantibodies present. Such a high rate of alloimmunization in patients with warm autoantibodies underscores the need for efficient techniques to evaluate all sera from patients with autoimmune hemolytic anemia (AIHA).\cite{19}

Although the 1-in-5 dilution technique, as described by Øyen and Angeles,\cite{2} would appear to be a convenient approach to screening for underlying alloantibodies, it proved to be inefficient, compared to ZZAP and PEG adsorptions, in our hands. Only 22 percent (20/93) of sera containing warm autoantibodies without alloantibodies were nonreactive after dilution to 1-in-5, in contrast to the 42 percent (50/119) of nonreactive samples reported by Øyen and Angeles.\cite{2} More important, we did not detect 7 (27%) of 26 alloantibodies that were of potential clinical significance. The dilution technique as used by Øyen and Angeles is applied, by them, to study sera that show ≥1+ equivalent reactivity with all cells. Of the seven samples we tested containing alloantibodies that were not detected after dilution, only one (anti-D) showed variable reactivity (1–2+) when tested without dilution; the other six met the criteria set forth by Øyen and Angeles. As discussed by Petz and Garratty,\cite{7} a dilution method might be appropriate if the patient cannot wait for the length of time it takes for adsorptions to be performed. Results from adsorption procedures may take 4 to 8 hours, especially if the sample has to be sent to a reference laboratory. Using the simple dilution ap-
approach is better than transfusing incompatible units, as approximately 35 percent of alloantibodies were detected using this approach. If the dilution technique is used, it may be appropriate to perform adsorption studies retrospectively to detect any alloantibodies that may have been missed by the dilution approach.

Adsorptions with untreated allogeneic RBCs in the presence of PEG appeared to give similar results to adsorptions with ZZAP-treated allogeneic RBCs. We were able to detect the same alloantibody specificities after adsorption with untreated RBCs in the presence of PEG that were found in parallel ZZAP adsorptions. PEG adsorptions at 4°C appeared to be more efficient than those with ZZAP at removing cold autoantibody in one serum tested, but more sera need to be tested to compare this apparent efficiency. Because untreated RBCs do not pack as efficiently as enzyme- or ZZAP-treated RBCs, care must be taken to adequately pack the adsorbing cells before adsorption so that the serum–PEG mixture is not diluted with excess saline. In addition, although we were unable to demonstrate a loss of specific antibody reactivity due to the precipitation of protein that occurs with PEG, the potential still exists; testing of the serum–PEG mixture at the time of adsorption (and not after storage at 4°C) may decrease the likelihood of missing antibodies. In the recent report by Barron and Brown, one anti-K and one anti-Jkβ that were weakly and microscopically reactive, respectively, in LISS antiglobulin test after adsorption with papain-treated allogeneic RBCs, were not detected after adsorption in the presence of PEG. All the methods published to date for adsorption in the presence of PEG, including our own, have varied somewhat in technique or technique comparison (e.g., 30- to 60-min adsorption times, comparison to ficin or ZZAP adsorption, source of PEG).

The primary disadvantage of using PEG rather than ZZAP adsorptions is that there is no visible clue as to the efficiency of the adsorption, such as the agglutination that is seen with ficin- or ZZAP-treated cells that indicates antibodies have adsorbed onto the cells. The second disadvantage is that, because ficin- or dithiothreitol-sensitive antigens will not be destroyed, the interpretation of results is affected, and alloantibodies to antigens of high-frequency, such as anti-Kpβ and anti-LW, will be adsorbed (although it must be remembered that antibodies to other high-frequency antigens can be adsorbed even with ZZAP-treated RBCs). The third disadvantage is that, although PEG appears to be more efficient in adsorbing autoantibodies, warm and/or cold autoantibody reactivity may be enhanced in some cases by the PEG remaining in the adsorbed sera.

The major advantage in using PEG adsorptions rather than ZZAP is that no treatment of allogeneic RBCs is required before PEG adsorption, which saves reagents (e.g., enzyme, ZZAP) and time (30-45 additional minutes for treatment and washing). The second advantage is a ≥50-percent reduction in the time required for adsorptions (15 vs. 30 min) when ZZAP adsorption is performed as recommended in the original method and the Technical Manual, of the American Association of Blood Banks, which results in a shorter turnaround time for serologic resolution. We did not test a shorter adsorption time (e.g., 15 min) with ZZAP-treated RBCs, so the efficacy of the two adsorption methods may be similar. The third advantage is that weak alloantibodies, such as anti-Jkα and anti-Jkβ, may be enhanced by the PEG that remains in the adsorbed sera.

Finally, our large series emphasizes that patients with AIHA become alloimmunized much more commonly than other patients (i.e., 40% vs. 25% of sickle cell disease patients, 5% of thalassemia patients, 5% of multiply transfused patients). If AIHA patients are to receive the same protection as other patients, then we believe efficient procedures for detecting "hidden" alloantibodies, although labor-intensive, must be used in pretransfusion testing, and if American Association of Blood Banks standards are to be satisfied, then these procedures must be repeated every 3 days after transfusion.

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