Utility of adsorption techniques in serological evaluation of warm autoimmune haemolytic anaemia

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Background. Various adsorption techniques are available to remove serum autoantibodies and subsequently detect the underlying alloantibody in previously transfused patients with autoimmune haemolytic anaemia. We planned to establish a suitable adsorption technique in our transfusion service which can remove all autoantibodies and detect underlying alloantibodies rapidly, cheaply and effectively.

Study design and methods. We evaluated 71 direct antiglobulin test-reactive patients with warm AIHA over a period of 20 months. Twenty-three of these 71 patients who had a previous history of blood transfusion or pregnancy and were confirmed carriers of autoantibodies (indirect antiglobulin test-reactive) were considered for the adsorption study. Depending on the adequacy of samples, history of blood transfusion and severity of anaemia either autoadsorption or alloadsorption or both using polyethylene glycol (PEG) or low ionic strength saline (LISS)-papain were performed.

Results. Underlying alloantibodies were detected in 7 of the 23 patients (30.4%) and all these were specific to Rhesus antigens. The mean number of alloadsorptions for complete autoantibody removal using PEG was 1.43 which was significantly lower than the 3.9 using the LISS-papain method (p<0.05). The mean time required by PEG alloadsorption and LISS-papain alloadsorption for autoantibody removal was 93.6 minutes and 177.7 minutes, respectively (p<0.05). Discordant results were not observed in any case and identical alloantibodies were detected by both the techniques.

Conclusion. We found that the PEG method is a rapid, cheap and effective way to remove autoantibodies and detect underlying alloantibodies.

Key words: AIHA, adsorption, autoantibody, alloantibody

Introduction

Red cell-bound autoantibodies detected in various autoimmune disorders often cause in vivo haemolysis. Patients with significant haemolysis and severe anaemia require blood transfusion. Approximately 12-40% of transfused patients develop clinically significant alloantibodies that may induce rapid haemolysis and cause haemolytic transfusion reactions. Detection of these alloantibodies masked by overlying warm autoantibodies at times poses challenge to immunohaematologists. Adsorption techniques, such as autoadsorption and alloadsorption, using reagents such as polyethylene glycol (PEG) or low ionic strength saline (LISS), are widely applied to detect such alloantibodies. Although autoadsorption is considered cheap and safe and avoids altering the antibody level, it is not suitable for use in recently transfused or severely anaemic patients. In such cases, alloadsorption is necessary, despite the technique having the disadvantage of...
adsorbing alloantibodies against high prevalence antigens. Working in a tertiary care hospital with an established haematology department, we regularly encounter patients with autoimmune haemolytic anaemia (AIHA). Most of these patients have a history of blood transfusion elsewhere and are admitted with a severe haemolytic crisis. We, therefore, planned to establish adsorption techniques in our laboratory with the aim of detecting the underlying alloantibodies and selecting the technique most suitable for our transfusion service.

Materials and methods

The study was conducted in the Department of Transfusion Medicine, Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow, India over a period of 20 months from July 2004 to February 2006 after approval from the institutional review board and written consent from the patients.

We evaluated 71 direct antiglobulin test (DAT)-reactive patients with warm AIHA. Sera from all these patients were subjected to antibody screening [indirect antiglobulin test, (IAT)] through gel technology (DiaMed, Cressier s/Morat, Switzerland) using the reagent three-cell panels (DiaMed). For each sample, a positive control, negative control and an auto-control were tested in parallel as described elsewhere. Eight samples reactive with the three-cell panels were further tested for antibody identification using gel cards and the reagent 11-cell panels (DiaMed). Warm autoantibodies were considered to be present only when the test samples reacted optimally at 37°C with the entire 11-cell panels (pan-reactive) and also with the patient’s own red cells (reactive autocontrol). The presence of autoantibodies was also confirmed by parallel testing of eluate obtained by cold acid elution of patient’s DAT-reactive red cells. Twenty-three of these 71 DAT-reactive patients had a previous history of blood transfusion or pregnancy and simultaneously carried autoantibodies in their sera (reactive IAT). These 23 patients were considered for the adsorption study to investigate any clinically significant underlying alloantibody.

Adsorption study

We performed both PEG and LISS-papain adsorption methods using the patient’s own red cells (autoadsorption) and partial patient’s phenotype-matched (Rh, Kidd & Kell) allogeneic ‘O’ group red cells (alloadsorption).

The choice of whether to use one or other or both of the techniques depended on the adequacy of the sample or the patient’s history of blood transfusion and severity of anaemia. Both PEG and LISS-papain adsorption techniques could be performed in only eight of the 23 patients. The quantity of blood sample to be collected was based on the type of adsorption to be performed. For autoadsorption studies, 10 mL of blood were collected in EDTA and 5 mL in a plain vial; for alloadsorption studies, 3 mL of blood were collected in EDTA and 5 mL in a plain vial. Autoadsorption was carried out in 11 patients and both alloadsorption and autoadsorption could be done in only seven patients. Of the other 12 patients for whom only alloadsorption was performed, seven had a recent history of blood transfusion (within the preceding 90 days) and five were severely anaemic.

Antigen phenotyping and selection of allogeneic red cells for adsorption

Minor antigen phenotyping (C, c, E, e, K, k, Jkα, Jkβ, Fya and Fyb) could be performed in 11 patients on their eluted red cells. Allogeneic ‘O’ group red cells for adsorption were selected either on the basis of the patient’s antigen phenotype or in patients with a recent history of blood transfusion; group ‘O’ red cells of the phenotype R1R1, R2R2 and rr, one of these cells being K- and Jk(a+b+) and the other Jk(a+b−), were selected.

The PEG method for autoadsorption and alloadsorption

We performed the PEG tests as described previously. Briefly, a 20% PEG solution was prepared in-house by dissolving 3350 MW, 20 g PEG (Sigma-Aldrich, New Delhi, India) in 100 mL phosphate-buffered saline maintaining a pH of 7.3. Equal volumes of patient’s serum, PEG solution and autologous/allogeneic packed red blood cells were mixed properly, incubated at 37°C for 15 minutes and centrifuged for 5 minutes at 1000g. The absorbed serum-PEG mixture was then harvested for further analysis.

LISS-papain method for autoadsorption and alloadsorption

Briefly, equal volumes of patient’s serum,
commercially available LISS (DiaMed) and 1% papain (DiaMed) treated autologous/allogeneic packed red blood cells were mixed properly, incubated at 37°C for 20 minutes and centrifuged for 5 minutes at 1,000g. The absorbed serum-LISS mixture was then harvested for further analysis\(^6\).

In all the procedures, samples containing equal volumes of serum and PEG/LISS were tested in parallel with only serum without PEG/LISS in order to observe whether dilution of serum by PEG/LISS had any effect. No dilutional effect was observed as the strength of the IAT was the same in both.

**Detection of alloantibodies on harvested mixtures**

The harvested serum recovered by any of the techniques was subjected to IAT using gel cards and red cell panels. Sera still found to contain autoantibodies were further subjected to adsorption with fresh aliquots of autologous/allogeneic red cells and the procedure repeated until the reagent red cells showed either a specific reaction pattern or absence of any reaction. Sera showing a specific reaction pattern were then tested for alloantibody identification whereas absence of any reaction after complete adsorption indicated the presence of only autoantibodies.

**Statistical analysis**

Wilcoxon’s signed rank test was applied using SPSS software (version 9.0, Apache software foundation, USA) to compare the results of the two methods of adsorption.

**Results**

Of the 71 DAT reactive patients, 32 carried autoantibodies in their sera (IAT pan-reactive). Twenty-three of these IAT-reactive patients had a past history of blood transfusion or pregnancy and were considered for the adsorption study. Monospecific DAT evaluation showed that the red cells of 15 of these 23 patients were coated with only immunoglobulin G (IgG) type of autoantibody and that the other eight patients carried both IgG and complement fractions (C3c/ C3d) on their red cells. Primary warm AIHA was diagnosed in 13 patients and another ten patients suffered form secondary AIHA due to systemic lupus erythematosus (SLE) (n=4), rheumatoid arthritis (n=3), autoimmune hepatitis (n=1), and rapidly proliferating glomerulonephritis (n=2). Seventeen (73.9%) of the 23 patients were females, predominantly of the elderly age group (≥40 years). Underlying alloantibodies were detected in seven patients (30.4%): the sera of four patients carried anti-E, two patients had anti-C only and one pregnant AIHA patient carried both anti-D and anti-C. All these patients were transfused the corresponding antigen-negative blood without any adverse effect.

Table I describes the various adsorption techniques used. The mean number of alloadsorptions for complete autoantibody removal using PEG was 1.43 which was significantly lower than the 3.9 needed with the LISS-papain method (p<0.05). The mean time required by PEG alloadsorption and LISS-papain alloadsorption for autoantibody removal was 93.6 minutes and 177.7 minutes, respectively and this difference was statistically significant (p<0.05). Inadequate sample size restricted the comparative statistical analysis of PEG autoadsorption and LISS-papain autoadsorption; however, the former technique was rapid (mean 34.4 minutes vs 103.3 minutes).

Sera from eight patients were subjected to both

<table>
<thead>
<tr>
<th>Procedure</th>
<th>N. of sera with autoantibodies</th>
<th>Total n. of adsorptions</th>
<th>Total time (min)</th>
<th>Mean time (min)</th>
<th>Mean n. of adsorptions (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LP* alloadsorption</td>
<td>13</td>
<td>51</td>
<td>2310</td>
<td>177.7</td>
<td>3.9 (2-7)</td>
</tr>
<tr>
<td>PEG alloadsorption</td>
<td>14</td>
<td>20</td>
<td>1380</td>
<td>93.6</td>
<td>1.43 (1-2)</td>
</tr>
<tr>
<td>LP autoadsorption</td>
<td>6</td>
<td>18</td>
<td>620</td>
<td>103.3</td>
<td>3 (2-4)</td>
</tr>
<tr>
<td>PEG autoadsorption</td>
<td>8</td>
<td>9</td>
<td>275</td>
<td>34.4</td>
<td>1.12 (1-2)</td>
</tr>
</tbody>
</table>

* LP= LISS-papain
PEG and LISS-papain alloadsorptions (Table II). Fewer adsorptions and a shorter mean time were necessary to remove autoantibodies with the PEG method than with the LISS-papain method (1.37 vs 3.75 and 97.5 minutes vs 175 minutes, respectively) (p<0.05).

**Discussion**

Detection of underlying alloantibodies after removal of autoantibodies from the sera of warm AIHA patients using the conventional ZZAP adsorption technique is not only labour-intensive and time-consuming but also delays urgent blood transfusion in critically ill patients. PEG, LISS and papain are potentiators that enhance antigen antibody reactions and have successfully replaced ZZAP in the performance of various adsorption techniques.

Most patients with AIHA are above 40 years of age and there is a female preponderance. In our series of 23 patients, we encountered 17 females (73.9%), predominantly of an elderly age, all showing strong IAT reactivity (2+ to 4+). The majority of our admitted patients had either been heavily transfused recently or were severely anaemic, thus preventing the use of autoadsorption techniques on them. Sera from only 11 non-transfused and stable patients could be subjected to autoadsorption. This technique has the advantage of being quicker than alloadsorption because the latter requires a search for phenotypically matched allogeneic red cells which is itself time-consuming; moreover, all these patients require elution of their DAT reactive red cells for confirmation of the exact phenotype. Alternatively, in advanced centres, molecular techniques are now being used to phenotype red cell antigens. Adequate autologous red cells and serum could be obtained from only seven patients and both autoadsorption and alloadsorption by LISS and PEG were possible in these cases. Underlying alloantibodies were detected in seven of our 23 patients (30.4%), this detection rate being consistent with the rates in various other studies. Since the detected antibodies were all directed against Rh antigens, Rh phenotype-matched red cell transfusion could be recommended for these patients.

The mean number of alloadsorptions necessary for complete autoantibody removal using PEG was 1.43 which was significantly lower than the mean number of 3.9 using the LISS-papain method (Table II). In a previous study on autoadsorption using PEG, a median of 2 (range, 1-3) adsorptions were necessary to remove all autoantibodies. Similarly, in a subsequent study, Chiaroni et al. had to perform a mean of 2.88 alloadsorptions for autoantibody removal on 50 sera using LISS and papain. The results

**Table II - LISS-papain versus PEG alloadsorptions on the same samples**

<table>
<thead>
<tr>
<th>Samples</th>
<th>LP* alloadsorption</th>
<th>PEG alloadsorption</th>
<th>Alloantibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N. of adsorptions</td>
<td>Time (min)</td>
<td>N. of adsorptions</td>
</tr>
<tr>
<td>1</td>
<td>3</td>
<td>160</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>160</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>140</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>180</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
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<td>200</td>
<td>2</td>
</tr>
<tr>
<td>6</td>
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<tr>
<td>8</td>
<td>4</td>
<td>190</td>
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</tr>
<tr>
<td>Total</td>
<td>30</td>
<td>1400</td>
<td>11</td>
</tr>
<tr>
<td>Mean</td>
<td>3.75</td>
<td>175</td>
<td>1.37</td>
</tr>
</tbody>
</table>

* LP= LISS-papain
of the present study are consistent with the previous findings. The mean time required by PEG adsorption and LISS-papain adsorption for autoantibody removal was 93.6 minutes and 177.7 minutes respectively. Chiaroni et al.6 required only a mean of 57.6 minutes to remove all autoantibodies through LISS-papain adsorption. The longer time required for adsorption in the present study could not be explained and might be attributed to the introduction of a new technique in the department. Larger studies are needed for a comparative statistical analysis of PEG autoadsorption and LISS-papain autoadsorption; however, in the present study it was found that the former technique was faster (mean 34.4 minutes vs 103.3 minutes).

Although the strength of the IAT reaction with the PEG method was weaker than that with the LISS-papain method, both methods could detect underlying alloantibodies. This finding has been documented by other authors as well and has been attributed to PEG-induced immunoglobulin precipitation resulting in a weaker strength of reaction12-14.

Conclusions
With regards to selection of a suitable technique of adsorption in warm AIHA patients requiring urgent transfusion, we recommend the PEG method as it is rapid and detects underlying alloantibodies effectively. The LISS-papain method, given its greater sensitivity compared to PEG, may be used in patients suspected of carrying underlying alloantibodies, such as multiply transfused patients or pregnant women, with negative PEG results.

References