Original Article

Identification of Clinically Significant Alloantibodies in Thai Patients with Autoimmune Hemolytic Anemia

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Abstract:

Objective: To identify clinically significant alloantibodies in Thai patients with autoimmune hemolytic anemia (AIHA) by acid elution technique, standard antibody identification technique and ZZAP allogeneic adsorption technique. Materials and Methods: Fifty patients with AIHA who had positive direct antiglobulin test (DAT) and positive antibody screening were included. Acid elution kit was used to identify IgG antibodies bound on patient’s RBCs. Standard antibody identification and allogeneic adsorption with ZZAP-treated RBCs were used to identify alloantibodies in patient’s plasma. Results: A total of 20 alloantibodies were found in 16 (32%) of 50 samples but not found in eluate. The alloantibodies included 6 (30%) anti-S, 5 (25%) anti-Mi, 4 (20%) anti-E, 1 (5%) anti-c, 1 (5%) anti-Jk, 1 (5%) anti-Jk, 1 (5%) anti-Di and 1 (5%) anti-N. Fifteen (30%) samples had clinically significant alloantibodies (IgG) with a total of 19 specificities. Four samples showed 4 clinically significant alloantibodies which could be identified by allogeneic adsorption techniques but not by standard antibody identification. Conclusion: Patients with AIHA are at significant risk of red cells alloimmunization. Further testing with adsorption technique should be performed to identify alloantibodies for patients who had history of transfusion before transfusion of RBCs.

Keywords: • AIHA • Alloantibody • Identification • ZZAP reagent • Allogeneic adsorption


Introduction

Autoimmune hemolytic anemia (AIHA) is characterized by the production of antibodies directed against self red blood cells (RBCs), so-called “autoantibodies”, leading to enhance clearance through Fc receptor-mediated phagocytosis.¹ Transfusion support in AIHA is complicated by the difficulty of compatibility test and by the shortened survival of the transfused RBCs.²⁻³ However, if transfusion is required, the finding of compatible donor unit is difficult and problematic in the immunohematology laboratory due to the presence of panagglutination in the patient’s plasma and may mask the presence of clinically significant alloantibodies which developed by previous transfusion or pregnancy.²⁵ Several reports indicated that approximately 32% of patients with AIHA have underlying alloantibodies.²⁻³ The need for a method to identify these potentially clinically significant alloantibodies prior selecting appropriate RBCs for any transfusion is essential.²⁴

Antibody identification in patients with AIHA is challenged for the blood banker in the compatibility process to provide compatible unit blood for transfusion.
therapy. Moreover, the literature is still under reported in Thailand. Thus, the aim of this study was to identify clinically significant alloantibodies in Thai patients with AIHA by acid elution technique to detect IgG-alloantibodies bound into RBCs and ZZAP allogeneic adsorption technique in addition to standard antibody identification technique.

Materials and Methods

Patients

Fifty EDTA blood samples were obtained from patients with AIHA who had positive DAT and antibody screening in the Outpatient Clinic of Hematology at Siriraj Hospital during June to December 2012. The study was approved by the ethics committee of Siriraj Hospital, Mahidol University, Thailand.

Methods

Direct antiglobulin test (DAT) by standard tube test was performed in 50 samples using a polyspecific antihuman globulin reagent, anti-IgG and anti-C3d (National Blood Centre, Thai Red Cross Society, Bangkok, Thailand) and antibody screening by standard tube test using O1 and O2 screening cells (National Blood Centre, Thai Red Cross Society, Bangkok, Thailand) as described.6 Additional tests using monospecific anti-IgG, anti-IgM, anti-IgA, anti-C3c and anti-C3d (DC-Screening I) card (BioRad, Cressier sur Morat, Switzerland) were subsequently used to classify immunoglobulin classes and the elutions were performed using the DiaCidel Elution Kit (BioRad, Cressier sur Morat, Switzerland). The antibody screening in eluate was performed by using the same reagents and method as in plasma. If antibody screening was positive, panel cells were used for identifying the specificities (National Blood Centre, Thai Red Cross Society, Bangkok, Thailand). Whereas in plasma, if antibody screening was positive, antibody specificity was subsequently identified by using the same panel cells.6 Allogeneic adsorbing cell which had ABO, Rh, Kidd and Diego matched with the patient’s phenotype was used. Whereas, differential allogeneic adsorbing cells selected by group O, R^1_1, R^2_2, and rr which at least one of the three cells being Jk(a-b+), Jk(a+b-) and Di(a-) were used if the patient’s phenotype was unknown. Each adsorbing cell was treated with ZZAP reagent as described.6,8 If antibody was presented in adsorbed plasma samples, antibody specificities were identified.

Statistical analysis

Comparison between rate of alloantibody and transfusion or pregnancy was performed by Odd ratio and a p value of less than 0.05 was considered to be statistically significant. The results were calculated by SPSS version 11.5 (IBM’s Corporate Privacy, New York, USA).

Results

Fifty adult patients with AIHA were 41 (82%) females and 9 (18%) males (F:M ratio of 4.6:1) with a mean age of 50.7 (SD 17.68) years, median age of 50 years (min = 18, max = 86 years). Thirty-one (62%) patients had been diagnosed AIHA with other diseases. In these patients, 16 (51.6%) patients had associated with underlying SLE disorder and 37 (74%) patients had received prednisolone treatment. In females, 22 (53.7%) patients had history of previous pregnancies. The transfusion history was presented in 30 (60%) patients, of which 26 (86.7%) patients received blood transfusions over 3 months and 4 (13.3%) patients received blood transfusions within 3 months. The remaining 20 (40%) patients did not receive blood transfusion.

The strengths of DAT in 50 blood samples were 4+, 3+, 2+, 1+ and w+ in 26 (52%), 14 (28%), 4 (8%), 4 (8%) and 2 (4%) samples, respectively. Following elution test, 43 (86%) samples gave positive results for IAT and all eluates were presented with panreactivity. The remaining 7 (14%) samples gave negative results for IAT. The specificities of alloantibodies were not found in eluate. In unadsorbed plasma samples,
antibody identification results were presented with panreactivity, alloantibody plus panreactivity and only alloantibody detected in 36 (72%), 9 (18%) and 5 (10%) samples, respectively, as shown in Table 1.

The antibody identification results of plasma are shown in Figure 1. By standard antibody identification, alloantibodies were found in 14 (28%) of 50 samples, as shown in Figure 1 and Table 2. Of these 14 samples, 9 samples had alloantibodies with the strength of reactions higher than autoantibodies which included 3 anti-Miα, 3 anti-S, 1 anti-N, 1 anti-Diα and 1 anti-E+c. The remaining 5 samples were found to contain simple specificities without autoantibody, which included 1 anti-Miα, 1 anti-E, 1 anti-S, 1 anti-Jkα and 1 anti-S + E. Whereas, 36 (72%) samples showed only autoantibodies that reacted equally with all panel cells.

A total of 45 samples which had autoantibody

Table 1 Summary of the DAT and characteristics of antibody presented in 50 eluates and unadsorbed plasma

<table>
<thead>
<tr>
<th>DAT strength</th>
<th>Total n (%) (n = 50)</th>
<th>Characteristics of antibody presented in Eluate (%)</th>
<th>Plasma (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Panreactivity (n = 43)</td>
<td>Nonreactivity (n = 7)</td>
</tr>
<tr>
<td>4+</td>
<td>26 (52.0)</td>
<td>25 (58.1) 1 (14.3)</td>
<td>18 (50.0) 7 (77.8) 1 (20.0)</td>
</tr>
<tr>
<td>3+</td>
<td>14 (28.0)</td>
<td>12 (27.9) 2 (28.6)</td>
<td>13 (36.0) 1 (11.1)</td>
</tr>
<tr>
<td>2+</td>
<td>4 (8.0)</td>
<td>2 (4.7) 2 (28.6)</td>
<td>2 (5.6) 0</td>
</tr>
<tr>
<td>1+</td>
<td>4 (8.0)</td>
<td>3 (7.0) 1 (14.3)</td>
<td>2 (5.6) 0</td>
</tr>
<tr>
<td>W+</td>
<td>2 (4.0)</td>
<td>1 (2.3) 1 (14.3)</td>
<td>1 (2.8) 1 (11.1)</td>
</tr>
</tbody>
</table>

Table 2 The specificities of alloantibodies in 16 patients with AIHA

<table>
<thead>
<tr>
<th>Cases</th>
<th>Sex</th>
<th>Age (years)</th>
<th>History</th>
<th>Antibody(ies) in Eluate</th>
<th>Unadsorbed plasma</th>
<th>Adsorbed plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Female</td>
<td>53</td>
<td>Yes</td>
<td>Yes</td>
<td>AutoAb</td>
<td>Miα</td>
</tr>
<tr>
<td>2</td>
<td>Female</td>
<td>76</td>
<td>Yes</td>
<td>Yes</td>
<td>AutoAb</td>
<td>Miα + warm autoAb</td>
</tr>
<tr>
<td>3</td>
<td>Female</td>
<td>39</td>
<td>Yes</td>
<td>Yes</td>
<td>AutoAb</td>
<td>S + warm autoAb</td>
</tr>
<tr>
<td>4</td>
<td>Female</td>
<td>51</td>
<td>Yes</td>
<td>No</td>
<td>AutoAb</td>
<td>S + E</td>
</tr>
<tr>
<td>5</td>
<td>Female</td>
<td>38</td>
<td>Yes</td>
<td>Yes</td>
<td>AutoAb</td>
<td>S + warm autoAb</td>
</tr>
<tr>
<td>6</td>
<td>Female</td>
<td>57</td>
<td>Yes</td>
<td>No</td>
<td>AutoAb</td>
<td>Jkα</td>
</tr>
<tr>
<td>7</td>
<td>Male</td>
<td>21</td>
<td>Yes</td>
<td>No</td>
<td>AutoAb</td>
<td>Miα + warm autoAb</td>
</tr>
<tr>
<td>8</td>
<td>Female</td>
<td>60</td>
<td>No</td>
<td>No</td>
<td>AutoAb</td>
<td>S (IgM antibody)</td>
</tr>
<tr>
<td>9</td>
<td>Female</td>
<td>55</td>
<td>Yes</td>
<td>Yes</td>
<td>AutoAb</td>
<td>N + warm autoAb</td>
</tr>
<tr>
<td>10</td>
<td>Female</td>
<td>54</td>
<td>No</td>
<td>Yes</td>
<td>AutoAb</td>
<td>Diα + mixed-type autoAb</td>
</tr>
<tr>
<td>11</td>
<td>Female</td>
<td>18</td>
<td>Yes</td>
<td>No</td>
<td>AutoAb</td>
<td>Warm autoAb</td>
</tr>
<tr>
<td>12</td>
<td>Female</td>
<td>36</td>
<td>Yes</td>
<td>No</td>
<td>AutoAb</td>
<td>E + c + warm autoAb</td>
</tr>
<tr>
<td>13</td>
<td>Female</td>
<td>44</td>
<td>Yes</td>
<td>No</td>
<td>AutoAb</td>
<td>Warm autoAb</td>
</tr>
<tr>
<td>14</td>
<td>Female</td>
<td>34</td>
<td>Yes</td>
<td>Yes</td>
<td>AutoAb</td>
<td>Miα + IH</td>
</tr>
<tr>
<td>15</td>
<td>Female</td>
<td>32</td>
<td>Yes</td>
<td>Yes</td>
<td>AutoAb</td>
<td>E</td>
</tr>
<tr>
<td>16</td>
<td>Female</td>
<td>50</td>
<td>No</td>
<td>Yes</td>
<td>AutoAb</td>
<td>S + warm autoAb</td>
</tr>
</tbody>
</table>

ND : Not done; * : New alloantibody
and autoantibody plus alloantibody were included for antibody identification by allogeneic adsorption. The antibody specificity results are shown in Figure 1 and Table 2. Two new samples (4.4%) were found to contain alloantibodies including 1 anti-Mi\(^a\) and 1 anti-Jk\(^b\) and two samples (4.4%) revealed new and previously identified alloantibodies including 1 anti-Mi\(^a\) + anti-E and 1 anti-Di\(^a\) + anti-S as shown in Table 2. Whereas, 7 samples (15.6%) were found only previously identified alloantibodies including 3 anti-S, 2 anti-Mi\(^a\), 1 anti-N and 1 anti-E + anti-c. The remaining 34 samples (75.6%) were found not contain alloantibody.

For overall 50 plasma samples, the alloantibodies were detected in 16 samples (32%). Among these 16 samples, single and multiple alloantibodies were found in 12 (75%) and 4 (25%) samples, respectively. Furthermore, 15 of 50 samples (30%) of patients who have been previously transfused and/or pregnant had IgG alloantibodies which were considered to be clinically significant alloantibodies. There was significant association (p = 0.03) between the history of transfusion and red cell alloimmunization which are shown in Table 3. However, there was no significant association between pregnancy and red cell alloimmunization (p = 0.54).
### Discussion

When DiaCidel acid elution kit was included to elute the positive DAT in the present study, no alloantibody was detected in all eluates even though could be detected in plasma (Table 2). In Thailand, only one study reporting 95 patients with a positive DAT that they found alloantibody specificities in 21(22.1%) eluates of all samples.\(^9\) But only 6 samples were presented autoantibodies in their serum. Similar findings were reported in positive DAT patients.\(^10-11\) In other studies, they found that the most common specificities of autoantibodies were antibodies in Rh system (often, anti-e).\(^12-15\) Since, the samples in this study were included from patients with several types of AIHA which were warm, cold and mixed-type AIHA. In some cases, if the elution test was performed in the presence of complement alone, the eluate gave negative with reagent RBCs.\(^2,4,14\) In addition, 4 samples obtained from AIHA patients with a history of blood transfusion within 3 months, no alloantibodies detected in eluates. However, elution test is essential and should be performed in patients with AIHA who have positive DAT with anti-IgG and who have been recently transfused.\(^2,4\) In addition, dilution study and adsorption of eluate could be considered for further identification in these patients because alloantibodies were masked by autoantibodies.\(^9\)

The previous studies of alloantibodies in Thai patients with AIHA is poorly reported. Especially those who had presented with panreactivity in the plasma. In this study, autoantibody and alloantibody were found in 50 unadsorbed plasma which gave positive reactions in antibody screening test. In addition, simple specificities were apparent in some cases, however, these findings were considered to be alloantibodies and confirmed by negative antigen typing results with monoclonal standard antisera. It has been described that if the autoantibody has been adsorbed by the patient’s RBC in vivo, free autoantibody could not be detected in the plasma.\(^2\) However, 5 patients have been continuously managed by corticosteroid treatment before the study. Therefore, low titer autoantibodies due to this drug may be occurred, then autoantibodies could not be detected in their plasma.\(^1,5\) Furthermore, a higher rate (45%) of alloantibodies were identified by this technique which were similar to the previous report (58%) and most alloantibodies had a higher reactive titer than autoantibodies.\(^8\) Further testing with allogeneic adsorption were included to identify alloantibody which may be masked by autoantibodies in plasma of 45 samples. Interestingly, alloantibodies were identified in 4 (8.9%) of 45 samples after allogeneic adsorption.

The most frequently identified alloantibodies by published reports in Caucasian patients with AIHA were antibodies of Rh, Kell, Kidd and Duffy systems, with a high rate of anti-E and anti-K.\(^8,12,13,15-18\) Whereas, in Asian-Mongoloid, few studies in patients with AIHA were reported. The previous reports in Chinese and Singapore showed the most frequent alloantibodies of Rh, MNS systems (often, anti-E and anti-Mi\(^a\)).\(^13,19\) Similarly, the present study showed that the most frequent alloantibodies were antibodies of MNS and Rh systems. Thus, if transfusion may be required, adsorption technique is also necessary to exclude alloantibody. However, when the adsorption

<table>
<thead>
<tr>
<th>History</th>
<th>Alloantibody (n=16)</th>
<th>No alloantibody (n=34)</th>
<th>p-value</th>
<th>Odds ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transfusion</td>
<td>13 (81.3%)</td>
<td>17 (50.0%)</td>
<td>0.03</td>
<td>4.33</td>
</tr>
<tr>
<td>Pregnancy</td>
<td>9 (60.0%)</td>
<td>13 (50.0%)</td>
<td>0.54</td>
<td>1.50</td>
</tr>
</tbody>
</table>

Table 3: The association between alloantibody and history of transfusion or pregnancy.
technique could not be performed, prophylactic antigen-matched donor blood will ensure the safety of blood transfusion.\textsuperscript{2,15,20}

In our experience, further testing by allogeneic adsorption with ZZAP-treated RBCs was an effective similar to other studies,\textsuperscript{2-7,12,21} since these techniques could remove free autoantibody from patient’s plasma in 41 of 45 samples (91%), whereas 4 samples, the reaction of free autoantibody were remained after adsorption (Figure 1). The reason is that proteolytic enzyme in the ZZAP mixture could denature some blood group antigens (i.e. M, N, S, s, Fy\textsuperscript{a}, Fy\textsuperscript{b}) whereas all Kell system antigens were denatured by DTT in ZZAP reagent.\textsuperscript{2} Even four adsorptions were used for this study, they could not completely remove free autoantibody in some cases. Alternative technique is to perform adsorption with untreated adsorbing cells.\textsuperscript{2} Although allogeneic adsorption with ZZAP treatment may identify antibody of the Kell system but this antibody is rare in Thais.\textsuperscript{22-23} The allogeneic adsorbing cells with enzyme treatment should be selected only for the antigens of Rh (C, c, E and e) and Kidd (Jk\textsuperscript{a} and Jk\textsuperscript{b}) systems, so that clinically significant alloantibody of common blood group systems could be identified. In addition, in patients with AIHA, antigen typing with monoclonal antiserum reagents before initial transfusion especially Rh and Kidd systems should be performed. The beneficial of this procedure is to select allogeneic adsorbing cells which have phenotype-matched with patient’s RBC. Moreover, if possible, donor units with phenotype-matched with the patient’s phenotype may be selected to reduce the risk of alloimmunization.\textsuperscript{2,15,20}

In the present study, 40 patients with AIHA had a history of blood transfusion and/or pregnancy. These groups had high rates of alloimmunization, similar to other studies.\textsuperscript{8,12-13} Moreover, the significant association was observed between blood transfusion and alloimmunization whereas no significant association was observed between pregnancy and alloimmunization. Therefore, the patients with AIHA who had a history of previous blood transfusion are at a high risk of producing clinically significant alloantibodies. Thus, a history of blood transfusion is an important point and further adsorption technique may be required before transfusion of incompatible units.

In summary, the present study showed a high rate of alloantibody in AIHA, especially in patients who presented with panreactivity in plasma and had a history of transfusion. Further testing with adsorption technique should be performed before transfusion.

Acknowledgements

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References

Identification of Clinically Significant Alloantibodies in Thai Patients with AIHA


การตรวจหาแอนติบอดีที่มีความสำคัญทางคลินิกในผู้ป่วยไทยที่มีภาวะเม็ดเลือดแดงแตกเท่าจากกลไกอิมมูน

ทินกร ภูนากลม1 ศศิจิต เวชแพศย์1 อิ่ยง ชินธรรมมิตร2 และ วิโรจน์ จงกลวัฒนา1
1ภาควิชาเวชศาสตร์การธนาคารเลือด 2ภาควิชอายุรศาสตร์ คณะแพทยศาสตร์ศิริราชพยาบาล มหาวิทยาลัยมหิดล

บทคัดย่อ

วัตถุประสงค์ เพื่อตรวจสอบแอนติบอดีที่มีความสำคัญทางคลินิกในผู้ป่วยไทยที่มีภาวะเม็ดเลือดแดงแตกเท่าจากกลไกอิมมูนด้วยเทคนิค acid elution เทคนิคมาตรฐาน และเทคนิคการดูดซับแอนติบอดีด้วยเม็ดเลือดแดงของผู้อื่นที่ผ่านการย่อยด้วยน้ำยา ZZAP วัสดุ และวิธีการศึกษา การศึกษานี้ใช้ตัวอย่างเลือดผู้ป่วยที่มีภาวะเม็ดเลือดแดงแตกเท่าจากกลไกอิมมูน จำนวน 50 ราย ที่มีผลการตรวจ direct antiglobulin test และ antibody screen ให้ผลบวก โดยตรวจแอนติบอดีชนิด IgG ที่จับอยู่บนผิวเม็ดเลือดแดงด้วยวิธี acid elution ตรวจแอนติบอดีชนิดในพลาสมาด้วยวิธีมาตรฐานและวิธีดูดซับแอนติบอดีด้วยเม็ดเลือดแดงของผู้อื่นที่ผ่านการย่อยด้วยน้ำยา ZZAP ผลการศึกษา ตรวจพบแอนติบอดีทั้งหมด จำนวน 20 ชนิด ในพลาสมาผู้ป่วยจำนวน 16 ราย (ร้อยละ 32) แต่ไม่พบใน eluate โดยพบแอนติบอดีชนิด S จำนวน 6 ราย (ร้อยละ 30) anti-M จำนวน 5 ราย (ร้อยละ 25) anti-E จำนวน 4 ราย (ร้อยละ 20) anti-c จำนวน 1 ราย (ร้อยละ 5) anti-Jk จำนวน 1 ราย (ร้อยละ 5) anti-Jk จำนวน 1 ราย (ร้อยละ 5) anti-Di จำนวน 1 ราย (ร้อยละ 5) และ anti-N จำนวน 1 ราย (ร้อยละ 5) พบแอนติบอดีชนิดที่มีความสำคัญทางคลินิก (IgG) จำนวน 19 ชนิด ในพลาสมาผู้ป่วย จำนวน 15 ราย (ร้อยละ 30) และยังสามารถตรวจแอนติบอดีชนิดที่มีความสำคัญทางคลินิก (IgG) จำนวน 19 ชนิด ในผิวเม็ดเลือดแดงผู้ป่วย จำนวน 4 ราย (ร้อยละ 8) ซึ่งตรวจไม่พบด้วยวิธีมาตรฐาน สรุป จากการศึกษาพบว่า ผู้ป่วยกลุ่มนี้มีความเสี่ยงที่จะกระตุ้นสร้างแอนติบอดีสูง ดังนั้นควรตรวจให้แน่นอนในการตรวจแอนติบอดีด้วยวิธี การศึกษาพบว่าเทคนิคการดูดซับแอนติบอดีด้วยเม็ดเลือดแดงก่อนใช้เทคนิคการดูดซับแอนติบอดีด้วยน้ำยา ZZAP มากกว่าที่จะกระตุ้นแอนติบอดีด้วยน้ำยา ZZAP

Keywords : AIHA Allantibody Identification ZZAP reagent Allogeneic adsorption