A Specific Assay for Anti-HLA Antibodies: Application to Platelet Donor Selection

By Frederick E. Millard, Patricia Tani, and Robert McMillan

Alloimmunization to donor class I HLA antigens represents a major obstacle to successful platelet transfusion therapy. It is desirable to distinguish alloimmunization from nonimmunologic causes of poor platelet survival to assess the need for HLA-matched, single-donor platelets. We describe a new in vitro assay for anti-HLA antibodies and report its application to the problem of platelet cross-matching. In contrast to previously described crossmatch techniques, the immunobead assay is specific for anti-HLA antibodies. The assay was used to evaluate 51 single-donor platelet transfusions given to seven patients from 35 different donors. Recipient plasma was assayed for antibodies directed against HLA antigens present on donor platelets. A one-hour posttransfusion corrected count increment of \( \geq 7,500 \) was considered a successful outcome. Twenty-nine of 33 (87.9\%) transfusion episodes associated with a negative immunobead assay had successful outcomes. The four unsuccessful transfusions were associated with potential nonimmunologic causes of poor platelet survival. Only two of 18 (11.1\%) episodes associated with a positive assay had successful outcomes. Only one unsuccessful transfusion episode was associated with a negative immunobead assay and a positive radiolabeled antiglobulin test result, which suggested that isolated alloantibodies to antigens other than class I HLA antigens are not a common cause of platelet refractoriness. Platelets stored in suspension at 4°C or frozen in liquid nitrogen were found suitable for crossmatch testing.

SINCE ITS BEGINNINGS over three quarters of a century ago,1 platelet transfusion therapy has played an increasingly important role in the supportive care of patients with bone marrow failure. A significant proportion of recipients become refractory to repeated transfusions from random donors due to alloantibodies to foreign class I HLA antigens present on the platelet surface.2,3 Yankee et al first demonstrated that HLA-matched platelets from single donors frequently give good increments in this situation.4,5 Unfortunately, HLA-matched donors are available for only a minority of alloimmunized patients, and the response to partially matched donors is less predictable.6-8 The recognition of cross-reacting groups and the differential expression of certain HLA antigens (eg, B12) on platelets has facilitated educated guessing during donor selection6-11; however, a means of selecting appropriate donors without resorting to trial and error would be advantageous. A variety of approaches to platelet crossmatching have been investigated, but none has proved sufficiently convenient and reliable to become generally accepted.12-20 In this report, we describe and characterize a new method of identifying anti-HLA alloantibodies. The method was used to evaluate 51 transfusion episodes in seven patients. A platelet radioactive antiglobulin test (RAGT) was performed simultaneously and the results compared with the immunobead results.

MATERIALS AND METHODS

Patient and Donor Population

Fifty-one transfusion episodes involving seven patients with bone marrow failure were studied (Table 1). All were refractory to random donor platelets before entry into the study. Transfusion episodes associated with fever, infection, or splenomegaly were not routinely excluded if good increments from any donor were seen within 24 hours. No patient demonstrated evidence of disseminated intravascular coagulation during the study period. Patient no. 1 was dropped from the study when he became refractory to all donors a few days before his death, associated with adult respiratory distress syndrome (ARDS) and mechanical ventilation. Patient no. 2 was dropped from the study when he became persistently febrile and refractory to all donors. He was subsequently determined to have a disseminated fungal infection. Donors were selected from family members when possible (n = 16), otherwise from paid community volunteers (n = 35). HLA matching was as follows: A, 9; B/B2X, 5; C, 19; D, 8; unknown, related, 7; unknown, unrelated, 3.9

Assessment of Transfusion Outcome

Each transfusion episode was evaluated by computing the one-hour corrected count increment (CCI) from the following standard formula: (one-hour postcount – pretransfusion count) \( \times \) body surface area in square meters/number of platelets transfused/100 = 1-hour CCI. When available, a later CCI (8 to 24 hours) was also calculated. A successful outcome was defined as a one-hour CCI of \( \geq 7,500 \). In one instance, a one-hour CCI of 7,289 with a six-hour CCI of 4,252 was considered “successful” when a transfusion from an HLA-matched sibling given less than 24 hours later gave a one-hour CCI of 8,391 and six-hour CCI of 2,670. In two instances where one-hour platelet postcounts were not available, an eight-hour CCI < 0 not associated with overt nonimmunologic causes of poor platelet survival was considered evidence of an unsuccessful transfusion outcome.

Murine Monoclonal Antibodies

HB-43 is an IgG\(_{\kappa}\) antibody derived from the 1410K67 cell line, which recognizes the Fc portion of human IgG. HB-95 is an IgG2

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antibody derived from the W6/32 cell line, which recognizes a monomorphic epitope on the heavy chain of human HLA-A, -B, and -C antigens.\textsuperscript{24} The intact heterodimer is required for full expression of the epitope, although HB-95 binds weakly to isolated heavy chains.\textsuperscript{23} Both cell lines were obtained from the American Type Culture Collection (Rockville, MD) and were used to produce ascites in Pristane-primed BALB/c mice from which antibody was purified by protein A affinity chromatography.\textsuperscript{12} Labeling was accomplished by the chloramine-T procedure.\textsuperscript{26}

**Preparation and Storage of Platelets**

Normal platelets. Blood from normal adult volunteers was drawn into EDTA and platelet-rich plasma separated by centrifugation at 400 g for ten minutes. The platelets were pelleted by centrifugation at 1,000 g for fifteen minutes, washed four times with 1.0 mL of 0.05 mol/L citrate buffer (14.7 g sodium citrate, 5.9 g sodium chloride, and 25.0 g dextrose in 1,000 mL distilled H2O, all of which was adjusted to pH 6.2 with 0.05 mol/L citric acid), and resuspended in citrate buffer to approximately 10\textsuperscript{8}/mL. Platelets were used fresh or stored in suspension at 4C for 24 to 48 hours.

Donor platelets. Approximately 3 mL of platelet concentrate was obtained at the end of eachpheresis procedure. This was divided into 1-mL aliquots and centrifuged at 750 g for 90 seconds to remove red cells. The platelets were centrifuged at 3,000 g for five minutes and washed four times. The platelet concentrate could be kept for 24 to 48 hours at 4C before washing without affecting assay results.

Platelet storage. To assess the effect of storage on the immunobead assay, 1.0-mL platelet aliquots (1.3 \times 10\textsuperscript{9}) from two normal donors were stored both in suspension at 4C and by freezing in liquid nitrogen and then tested at intervals of 1, 6, 15, 30, 60, and 120 days against two control plasmas and two plasmas known to contain high-titer anti-HLA antibodies. Platelets to be frozen were suspended in autologous plasma with 5% dimethylsulfoxide in plastic tubes. The RAGT was performed by a modification of the method of LoBuglio et al.\textsuperscript{27} Briefly, 10\textsuperscript{7} washed platelets were sensitized by incubation with 100 mL of test or control plasma for one hour in plastic tubes. The sensitized platelets were washed three times, resuspended in 900 mL of citrate buffer, and solubilized by adding 100 mL of 10% Triton X-100. If the assay was to be completed the same day, the lysate was centrifuged at 3,000 g for five minutes and the supernatant transferred to 15-mL polypropylene tubes. The lysate could be frozen at -20C after solubilization and the assay completed at a later date without affecting the results.

**Immunobead Assay**

The assay is depicted schematically in Fig 1. Aliquots of 10\textsuperscript{7} donor platelets were sensitized by incubation with 100 mL of test or control plasma for one hour in plastic tubes. The sensitized platelets were washed three times, resuspended in 900 mL of citrate buffer, and solubilized by adding 100 mL of 10% Triton X-100. If the assay was to be completed the same day, the lysate was centrifuged at 3,000 g for five minutes and the supernatant transferred to 15-mL polypropylene tubes. The lysate could be frozen at -20C after solubilization and the assay completed at a later date without affecting the results.

Immunobeads were prepared by incubating 1/4-in polystyrene beads (#0023804, Pierce Chemical Co, Rockford, IL) with anti-human IgG (HB-43, 10 \mu g/bead) in 0.5 mL per bead of 0.01 mol/L NaHCO\textsubscript{3} buffer (pH 8.5) for one hour on a rotator. After four washes with 1.0 mL phosphate-buffered saline (PBS) Tween, they were incubated for an additional hour with 2% bovine serum albumin (1 mL/bead) to block residual binding sites. After four more washes, one bead was added to each tube containing platelet lysate and incubated with constant agitation for one hour. During this incubation, IgG alloantibodies that had been bound to the platelet before solubilization attach via the Fc portion to the HB-43 bound to the bead. The class I HLA antigen remains bound to the Fab portion of the alloantibody after solubilization and is thus bound indirectly to the bead. The beads were washed four more times in PBS-Tween, and incubated for one hour with 400,000 cpm of \textsuperscript{125}I-labeled monoclonal anti-HLA antibody (HB-95) in 1 mL PBS-Tween. After four final washes, the beads were dried on a paper towel and counted in a Beckman gamma counter (Beckman Instruments, Inc, Fullerton, CA). All incubations were carried out at room temperature.

The test of interest was run in triplicate along with duplicates of one positive and two negative controls. One technician could run 24 simultaneous assays in approximately six hours.

**RAGT**

The RAGT was performed by a modification of the method of LoBuglio et al.\textsuperscript{27} Briefly, 10\textsuperscript{7} washed platelets were sensitized by incubation with 100 mL of plasma for one hour at room temperature and then washed three times in citrate buffer. A 3 \times 10\textsuperscript{7}-aliquot was incubated for 30 minutes with 300,000 cpm of \textsuperscript{125}I-labeled antihuman IgG (HB-43 and the anti-HLA, HB-95).

**Table 1. Patient Characteristics**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age/Sex</th>
<th>Diagnosis</th>
<th>Evaluable</th>
<th>Number of Different Donors</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>42/M</td>
<td>AA, allo-BMT</td>
<td>22</td>
<td>14</td>
</tr>
<tr>
<td>2</td>
<td>32/M</td>
<td>ALL, auto-BMT</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>21/M</td>
<td>AML</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>36/F</td>
<td>AML</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>41/M</td>
<td>AML</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>71/M</td>
<td>AML</td>
<td>17</td>
<td>11</td>
</tr>
<tr>
<td>7</td>
<td>57/M</td>
<td>Secondary MDA→ AML</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

Totals 51 35

*Abbreviations:* AA, aplastic anemia; allo-BMT, allogeneic bone marrow transplantation; auto-BMT, autologous bone marrow transplantation; AML, acute myelogenous leukemia; MDS, myelodysplastic syndrome.

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**Fig 1.** Schematic representation of the antigen-antibody "sandwich" that remains fixed to the polystyrene bead at the end of the assay. The anti-IgG is HB-43 and the anti-HLA, HB-95.
man IgG (HB-43). Triplicate 50-mL aliquots containing $6 \times 10^8$ platelets were layered over 200 mL of 30% sucrose in 400-μL plastic microcentrifuge tubes with tapered tips and centrifuged at 11,000 g for five minutes. After quick-freezing at −70°C, the tips containing the platelet button were removed and counted in a Beckman gamma counter.

Statistics

The overall predictive values of the immunobead and RAGT assays were compared with the binomial proportions test.

RESULTS

Characterization of the Immunobead Assay

Triplicate assays using 12 negative control plasmas against the same normal platelets gave a mean percent variation of 8.7% ± 0.0% (1 SD). Absolute values of negative controls generally fell in the range of 80 to 300 cpm. A single ± variation of 8.7% against the same normal platelets gave a mean percent variation of 6.3% ± 6.6%. Assays run using citrate buffer in place of plasma gave results comparable to negative controls.

To assess the possibility that the assay might recognize antiplatelet antibodies directed against non-HLA antigens, plasmas from 18 patients with the clinical syndrome of idiopathic thrombocytopenic purpura were tested against normal platelets. Seven of these had plasma autoantibodies to the glycoprotein (GP) IIb/IIIa complex, two had autoantibodies to glycoprotein Ib, and the remainder gave negative results for both these autoantibodies. Three plasmas gave positive results in the immunobead assay, two with GP IIb/IIIa autoantibodies and one with a strong antiplatelet autoantibody without demonstrable specificity. All three patients had a history of pregnancy and/or transfusion. Of the negatives, eight had a history of pregnancy or transfusion, two did not, and in five cases these data were not available.

Seven positive and seven negative controls were run with serum and compared with results using plasma. No significant difference was noted between negative controls. The absolute value of serum-positive controls averaged 60% of plasma values. No positives were missed by using serum.

Interpretation of Assay Results

The mean test result was divided by the mean of the negative controls to give a test/control ratio (TCR). Experiments with 12 normals gave a mean normal TCR of 1.00 ± 0.14 (1 SD). A TCR of <1.43 was initially defined as negative and included 3 SD. In practice, TCRs between 1.43 and 3.0 were associated with successful transfusion outcomes in five of six cases. This was interpreted as evidence that the immunobead assay can detect levels of alloantibody below that necessary to significantly affect platelet survival. That this was not due to random variation is supported by the observation that all nine A match transfusions were associated with TCRs <1.43. For clinical purposes, therefore, a TCR of ≤3.0 was considered negative and predictive of a successful transfusion outcome.

A TCR of ≤2.0 was empirically defined as negative for the RAGT by similar retrospective data analysis.

Correlation With Transfusion Outcome

Each evaluable transfusion episode was categorized according to whether the immunobead assay was positive (TCR >3.0) or negative (TCR ≤3.0) and the outcome successful (one-hour CCI, ≥7,500) or unsuccessful (one-hour CCI, <7,500). The data for both the immunobead assay and the RAGT are summarized in Table 2. The immunobead assay was positive in 18 episodes; 16 of 18 (88.9%) had unsuccessful outcomes. In 33 cases the assay was negative; 29 of 33 (87.9%) had successful outcomes. The mean one-hour CCI was 2,199 for assay-positive episodes and 13,072 for assay-negative episodes.

Twenty-one episodes were RAGT-positive; 15 of 21 (71.4%) had an unsuccessful outcome. Of the 30 RAGT-negative episodes, 25 of 30 (83.3%) had a successful outcome. The mean one-hour CCI was 3,897 for RAGT-positive episodes and 11,118 for RAGT-negative episodes.

Thus, the immunobead assay correctly predicted the outcome of 45 of 51 (88.2%) transfusion episodes, whereas the RAGT was correct in 40 of 51 (78.4%) episodes. The difference was not statistically significant ($P = .14$).

In a single instance (involving patient 1), a patient-donor combination initially associated with a positive immunobead assay and an unsuccessful outcome gave a negative assay and a successful outcome 1 month later. The initial transfusion was given just before preparation for bone marrow transplantation with high-dose cyclophosphamide and total nodal irradiation, which suggests that the alloantibody-producing lymphocytes were ablated, after which preformed antibody disappeared.

Figure 2 displays a plot of TCR v one-hour CCI that demonstrates the definition between positive and negative results.

Misclassified Transfusion Episodes

Of the six episodes misclassified by the immunobead assay, only one was correctly classified by the RAGT. Six of the 11 episodes misclassified by the RAGT were correctly classified by the immunobead assay. In five instances, both tests gave misleading results.

In the first immunobead assay–positive, successful outcome episode (patient no. 1), the TCR was 5.3 and the one-hour CCI, 16,897. The six-hour platelet postcount had fallen to below the pretransfusion level. The second case

Table 2. Correlation of Immunobead and RAGT Results With Transfusion Outcome

<table>
<thead>
<tr>
<th>Assay-Positive</th>
<th>Assay-Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCI &lt; 7,500</td>
<td>CCI &gt; 7,500</td>
</tr>
<tr>
<td>Immunobead</td>
<td></td>
</tr>
<tr>
<td>16/18 (88.9%)</td>
<td>2/18 (11.1%)</td>
</tr>
<tr>
<td>RAGT</td>
<td></td>
</tr>
<tr>
<td>15/21 (71.4%)</td>
<td>6/21 (28.6%)</td>
</tr>
</tbody>
</table>
Feasibility of Using Stored Platelets

The mean positive control/negative control ratios for both platelet donors at each time interval are displayed in Table 3.

<table>
<thead>
<tr>
<th>Storage Method</th>
<th>Day of Storage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frozen</td>
<td>1   6  15  30  60  120</td>
</tr>
<tr>
<td>4°C</td>
<td>53.3* 26.7 23.9 25.7 21.0 23.2</td>
</tr>
<tr>
<td>Liquid N₂</td>
<td>32.6 20.7 23.9 25.3 10.5 23.2</td>
</tr>
</tbody>
</table>

Table 3. Immunobead Assay Results Using Platelets Stored Frozen in Liquid Nitrogen and in Suspension at 4°C

*Data are presented as mean positive control/negative control ratios.

DISCUSSION

The immunobead assay was originally developed to detect autoantibodies against platelet membrane glycoproteins. We have adapted the assay to the study of alloantibodies to class I HLA antigens and applied it to the problem of platelet crossmatching. The results indicate the assay has sufficient predictive value to warrant wider-scale application.

The potential contributions of a reliable crossmatch technique to the conservation of blood bank resources and improved patient care are considerable. The incidence of alloimmunization in patients receiving repeated transfusions is estimated to be at least 50%, with only a small fraction having an HLA-matched related donor available. One large center drawing on an HLA-typed pool of over 2,400 donors found an average of only 1.3 A-matched donors per patient, with 25% of patients having no A or B matches. Most physicians treating alloimmunized patients have considerably smaller donor pools available. The use of partially mismatched platelets greatly enhances the usefulness of small donor pools but requires a reliable crossmatch technique for optimal utilization.

Ideally, prospective donors would have platelets stored when they enter the donor pool. When a patient with a poor response to random donor platelets is encountered, his or her plasma could be assayed against a small panel of normal platelets chosen to represent the common HLA antigens. If negative, it could be assumed that a problem other than alloimmunization exists and the expense of single-donor platelets avoided. If positive, HLA-matched platelets would be indicated. In the majority of cases where these are not available, the best matches could be pulled from the donor pool and their stored platelets tested, selecting those who test negative. Although our storage study extended only 120 days, other studies suggest that much longer storage periods are feasible.

One potential drawback to the immunobead assay is its HLA specificity. Many authors have suggested that alloantibodies to platelet-specific antigens account for some percentage of transfusion failures, usually based on poor response to HLA-identical platelets and/or the lack of demonstrable lymphocytotoxic antibody. However, alloantibodies to platelet-specific antigens have been difficult to demonstrate conclusively in this setting, and their true significance remains uncertain. Herman et al used Western blots to study seven patients refractory to HLA-matched platelets and were unable to demonstrate antibody to platelet-specific antigen on the GP IIb/IIa molecule. They concluded that antibodies to platelet-specific antigens are not commonly present. Our finding of only one in 51 instances of poor transfusion outcome where the RAGT was positive and the immunobead assay negative tends to support this. It seems more likely that unrecognized nonimmunologic factors account for many if not all of these cases. However, it would

Fig 2. Plot of TCR v one-hour CCI for 51 transfusion episodes. A relatively sharp definition between positive and negative assays is seen.

(patient no. 6) had a TCR of 253.7 (the second highest recorded during the study), a one-hour CCI of 10,806, and a 16-hour CCI of 1,965. When the same donor was used again 4 weeks later, the TCR was 183 and the eight-hour CCI, 0. Both patients had unsuccessful outcomes with other donors associated with a positive immunobead assay.

The four assay-negative, unsuccessful outcome cases were all associated with potential non–alloantibody-related causes of poor platelet survival. The first (patient no. 2) was associated with fever and occurred five days before the patient became refractory to all donors and was dropped from the study (see the section Patient and Donor Population). The other three involved patient no. 1: the first occurred when platelets were given simultaneously with a granulocyte transfusion–associated febrile reaction, and the other two occurred after the patient had been intubated for ARDS but before he became refractory to all donors.
IMMUNOBEAD ASSAY FOR ANTI-HLA ANTIBODIES

seem prudent at present to use the RAGT or a similar non-HLA-specific assay to evaluate the presence of antibody to other platelet-associated antigens in cases where the HLA-specific immunobead assay is negative in the face of poor platelet survival.

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