Detection of Platelet Antibodies Using a Micro-Enzyme-Linked Immunosorbent Assay (ELISA)

By Charles A. Schiffer and Virginia Young

An enzyme-linked immunosorbent assay (ELISA) for the measurement of circulating platelet antibody and platelet-associated IgG (PAIgG) is described. The test is done in microtiter plates and rapidly provides quantitative and highly reproducible results. Alloantibodies from 28 of 30 multiply transfused patients and isoantibodies from 3 of 4 patients with immune thrombocytopenic purpura (ITP) were detected. PAIgG was elevated in all 4 patients with ITP, and HLA and platelet-specific antigens were reliably detected using HLA typing sera and anti-Pl antibody, respectively. Platelets preserved either by dessication in the wells of the microtiter plates or in liquid suspension in saline at 4°C gave results comparable to values using fresh platelets. Storage periods ranged from 30 days for dessicated platelets to more than 1 year for platelets stored in suspension. The ability to utilize preserved platelets may allow relatively convenient screening of large numbers of potential platelet donors for alloimmunized patients.

A NUMBER OF in vitro assays have recently been developed to detect both circulating antiplatelet antibody and antibody fixed to platelets (so-called "platelet-associated" IgG, PAIgG). Measurement of PAIgG using immunofluorescence,1 inhibition of complement-mediated red blood cell lysis,2 a radioactive125I Coombs test,3 and immunoperoxidase assays,4 among others, has been helpful in the study and treatment of patients with immune thrombocytopenic purpura. This technology also has potential application for the selection of histocompatible donors for alloimmunized patients requiring platelet transfusions. Presently, donor selection is done by HLA typing of lymphocytes because of the recognition that refractoriness in most alloimmunized patients is associated with antibody against HLA antigens.8-10 HLA typing can now be done reliably in many centers, and the donor information can be retained in a permanent form in computer files for long-term use.11,12 Although fully or partially HLA matched transfusions are successful in the majority of patients, as many as 30%-50% of these transfusions fail to produce satisfactory increments,2,9,10,13,14 in some patients because of antibody against non-HLA platelet-specific antigens.2,13,15

Platelet antibody tests have been used for "cross-matching" purposes with variable success.2,3,13,14,16,18 In order to be useful as a crossmatching technique, it is necessary that the assay detect both HLA and platelet-specific antigens and be performed with preserved platelets. This latter requirement is important because it is impractical for donors to come to the blood center repeatedly for platelet crossmatching to be followed by donation if in vitro results are "compatible." The micro enzyme-linked immunosorbent assay (ELISA) to be described was designed with these goals in mind. This assay detects PAIgG by a "sandwich" technique utilizing anti-human IgG conjugated to alkaline phosphatase. Measurement of enzyme activity then provides a quantitative endpoint. Although other ELISA techniques for platelet antibody testing have been reported,19,20 none utilize preserved platelets. One of these ELISA tests has a qualitative endpoint,20 while the other assay is done in test tubes, consumes larger amounts of reagents, and is not easily adapted for large scale use.19

MATERIALS AND METHODS

Platelet Preparation

Platelet-rich plasma was prepared from blood anticoagulated with acid citrate dextrose, formula A (7.5 ml ACD-A plus 42.5 ml blood) by centrifugation at 2000 rpm (1030 g) for 5 min at 22°C in a Sorvall RC-3 centrifuge (Sorvall, Newtown, Conn.). The upper two-thirds of the PRP was aspirated and recentrifuged at 1800 rpm (830 g) for 10 min. The platelet button was washed twice and resuspended in Ringers-citrate-dextrose (RCD) solution (Isolyte E with 5% dextrose, McGaw Lab., Irvine, Calif.). The final volume was adjusted to produce a platelet count of 10⁶/μl. Examination under phase microscopy revealed negligible leukocyte or red blood cell contamination (<1/1000 platelets). Sixty microliters of this platelet preparation (10⁶ platelets) were placed in wells of a Falcon Microtest II microtiter plate (Becton Dickinson, Oxnard, Calif.). The plates were covered with plastic and centrifuged at 1800 rpm (590 g) for 10 min in a Sorvall GLC-1 centrifuge and the supernatants decanted.

The wells were then washed once with RCD plus 5% human serum albumin (HSA). After decanting by inversion of the plate, the wells were filled again with RCD-5% HSA and equilibrated uncovered for 30 min at room temperature. This equilibration was intended to coat the sides of the well to prevent nonspecific adsorption of serum proteins to the well during subsequent incubations. The RCD-HSA was then decanted, the wells blotted on filter paper, and the assay done either using fresh platelets or differing types of preserved platelets as described below. Counts were done on the washes and decanted supernatants and there was minimal (<5%) platelet loss.

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during these maneuvers when 10⁶ platelets prepared in ACD-A were used. When greater numbers (between 2 x 10⁶ and 10⁶) of platelets were used, nonhomogeneous, sometimes double layers, of platelets were noted with a considerable loss of platelets from the wells following washing and highly variable results. In contrast, preliminary experiments using EDTA-anticoagulated platelets showed erratic adherence with large numbers lost in the washes even after resuspension in RCD and centrifugation into the wells. Hence, ACD-A, which produced overall consistent results, was used in subsequent experiments.

ELISA Assay

**Indirect Assay**

Fifty microliters of serum were added to each well to be tested and incubated for 60 min at 37°C in a water bath. After decanting, the wells were washed 3 times with RCD-2% HSA and 100 µl of RCD-2% HSA, and 100 µl of an appropriate dilution of goat anti-human IgG conjugated to alkaline phosphatase (Miles Laboratories, Elkhart, Ind.) were added. The conjugate dilution was selected to produce a final absorbance reading (see below) of 0.1-0.2 with known negative sera. The conjugate was diluted in 0.05 M Tris buffer with 1% bovine serum albumin and 0.1% sodium azide. The plate was then kept uncovered at 22-24°C for 45 min. Strict attention must be paid to this temperature as falsely increased final values occurred at higher temperatures. After decanting and washing, 100 µl of p-nitrophenyl phosphate (final concentration 3.8 mM) in diethanolamine buffer (97 ml diethanolamine, 843 ml distilled water, 60 ml 1 N HCl, 100 mg MgCl₂-6H₂O, 0.2 g sodium azide, pH 9.8) was added and the plate was placed in the dark in an air incubator at 37°C for 30 min. The reaction was stopped by the addition of 50 µl of 1 M NaOH to each well. After mixing for 5 min, the reaction product, p-nitrophenolate, was quantitated at 405 nm using an automated microELISA reader (Dynatech MR580, Alexandria, Va.).

**Direct Assay**

Measurement of PAIgG on normal and patients' platelets was done in an identical fashion except that the incubation step with patient serum was eliminated.

Controls included autologous serum when platelets from normal donors were tested and a serum pool from nontransfused males of AB blood type. Experiments were also done to measure the "background" in wells without platelets (serum or buffer alone) and in wells with platelets incubated with buffer alone. Platelets were also tested without the anti-human IgG conjugate but with the p-nitrophenyl phosphate substrate to demonstrate background alkaline phosphatase activity intrinsic to the platelets. This was used as a "blank" against which all other reactions were measured. The "blank" value was always extremely low and quite close to values obtained using an empty well. All experiments were done in duplicate, with the mean values of duplicate wells reported.

**Platelet Preservation**

A number of different methods of preserving platelets for subsequent testing were analyzed. In all these experiments, "preserved" platelets were compared with results using fresh platelets from the same individual and the same serum using the assay conditions described above.

**Platelet Freezing**

Platelets prepared as described above were frozen at a concentration of 10⁶ platelets/µl using 5% dimethylsulfoxide (DMSO) either in small volumes in test tubes or in 200 µl volumes in the wells themselves. Platelets were frozen by placing the tubes (or plates) into a liquid nitrogen freezer at -120°C. After thawing, the platelets frozen in test tubes were washed with RCD, centrifuged into the wells, washed again, and assayed. Platelets frozen in the wells were washed 3 times prior to assay.

**Fixation**

Fixation with 1% paraformaldehyde was carried out for 5 min at room temperature followed by 3 washes with buffer prior to centrifuging the platelets onto the wells. In other experiments platelets already in the wells were fixed with 1% paraformaldehyde.

**Liquid Suspension**

Platelets were washed, suspended in normal saline with 0.01% sodium azide at a count of approximately 10⁶/µl and kept at 4°C. Prior to testing, appropriate dilutions were made with RCD buffer and the platelets were centrifuged into the wells as described above.

**Dessication**

After the wells with the platelets were prepared as described above, the platelets were placed in a dessicator using CaCl₂ as a dessicant at room temperature for 12 hr. The platelets were then covered with plastic wrapping and stored at either room temperature or -70°C until further testing. Prior to testing, the wells were filled with 5% HSA-RCD for 30 min at room temperature.

**Sera Studied**

Sera were tested from normal donors, a patient with posttransfusion purpura with known PI⁺ antibody, alloimmunized polytransfused patients with high levels of lymphocytotoxic antibody, and 4 patients with immune thrombocytopenic purpura (ITP). The patients with ITP had increased numbers of megakaryocytes in the bone marrow, no splenomegaly, and no other identifiable causes of thrombocytopenia. Specific HLA typing sera obtained from the

<table>
<thead>
<tr>
<th>Table 1. &quot;Background&quot; and Normal Sera Absorbance</th>
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<tbody>
<tr>
<td><strong>Experimental Condition</strong></td>
</tr>
<tr>
<td>---------------------------</td>
</tr>
<tr>
<td>RCD/5% HSA alone</td>
</tr>
<tr>
<td>Sera alone</td>
</tr>
<tr>
<td>Platelets + RCD/5% HSA</td>
</tr>
<tr>
<td>Platelets + autologous serum</td>
</tr>
<tr>
<td>Platelets + pooled AB serum</td>
</tr>
<tr>
<td>Platelets + normal sera</td>
</tr>
<tr>
<td>(Combinations of 28 normal sera, 10 normal donors)</td>
</tr>
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NIH Serum Bank were also tested. In addition, direct assays for PAIgG were done on the platelets of the patients with ITP, all of whom were thrombocytopenic (<50,000/μl) with active disease at the time of study.

RESULTS

“Background and Normal Sera

The “background” values and the results obtained with normal sera are shown in Table 1. The back-grounds using either 5% HSA--RCD or serum alone were low, indicating minimal nonspecific absorbance of conjugate or serum to the wells. The absorbance using platelets alone was 0.077 (range 0.031--0.135), indicating either persistence of donor immunoglobulin on the surface of the platelets despite the multiple washings or some release of intracellular IgG, as recently described by Kelton et al.,22 possibly occurring during the course of the platelet preparation. Background levels of retained IgG have also been found using other PAIgG assays.23 An upper limit of 0.150 (+/-2 SD from the mean) was established for the direct assay. No platelets from normal donors exceeded this value. These levels also increased moderately after addition of normal sera, reflecting further nonspecific binding of IgG, possibly to Fc receptors. There was no statistically significant difference between the results obtained with autologous, pooled AB or allogeneic sera from normal donors. Based on these data, an upper limit of normal of 0.300 was chosen (+2 SD from the mean) for the indirect assay. Of the 110 “normal” donor--recipient pairs tested, only 4 had values greater than 0.300, 3 occurring with sera from females who had had prior pregnancies. Only 19 patients’ own platelets obtained at a time when she had recovered from her thrombocytopenic episode and was also nonreactive against the platelets from a P1A¹-negative donor.

ITP

Sera and platelets from four patients with active ITP were tested. Elevated values of PAIgG were detected in all four patients (range 0.538--0.859). Adequate numbers of platelets for testing could be obtained from a patient with a platelet count of 7000/μl. Three of four patients had antiplatelet antibody detected in their serum (values of 0.413, 0.393, 0.408) with a borderline value (0.280) in the other patient. There were too few patients to attempt to correlate PAIgG and serum antibody levels with the patients’ platelet counts.

HLA Antisera

Two sera with anti-HLA-A2 activity, 1 anti-HLA-B7 serum, and 1 anti-HLA-B12 serum were tested against platelets from HLA-typed donors known to have these antigens on their lymphocytes and appropriate negative controls (Table 3). All sera were positive

The effects of ABO incompatibility are summarized in Table 2. There were no statistically significant differences between compatible and incompatible ABO pairings. Three of 4 values in “normals” greater than 0.300 occurred in the O serum/A platelets group, accounting for the elevated mean values of this group. It is unknown whether ABO antibodies could have resulted in increased values in these 3 experiments.

Table 2. Effect of ABO Compatibility

<table>
<thead>
<tr>
<th>Serum ABO</th>
<th>Type O Platelets</th>
<th>Type A Platelets</th>
</tr>
</thead>
<tbody>
<tr>
<td>O</td>
<td>.132 ± .056*</td>
<td>.187 ± .094</td>
</tr>
<tr>
<td></td>
<td>(.033--.237)</td>
<td>(.076--.409)</td>
</tr>
<tr>
<td>n = 21</td>
<td>n = 23</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>.119 ± .068</td>
<td>.142 ± .067</td>
</tr>
<tr>
<td></td>
<td>(.033--.252)</td>
<td>(.062--.325)</td>
</tr>
<tr>
<td>n = 25</td>
<td>n = 37</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>.163 ± .03</td>
<td>.105</td>
</tr>
<tr>
<td></td>
<td>(.130--.187)</td>
<td></td>
</tr>
<tr>
<td>n = 3</td>
<td>n = 1</td>
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</table>

*Mean absorbance ± SD (ranges).

Table 3. HLA Antisera

<table>
<thead>
<tr>
<th>HLA Antiserum</th>
<th>Positive*</th>
<th>Autologous Control</th>
<th>Negative Control*</th>
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<tbody>
<tr>
<td>No. 1</td>
<td>A2</td>
<td>.904†</td>
<td>.18</td>
</tr>
<tr>
<td></td>
<td>B7</td>
<td>.18</td>
<td></td>
</tr>
<tr>
<td>No. 2</td>
<td>A2</td>
<td>.504</td>
<td>.18</td>
</tr>
<tr>
<td></td>
<td>B7</td>
<td>.591</td>
<td>.21</td>
</tr>
<tr>
<td></td>
<td>B12</td>
<td>.720</td>
<td>.18</td>
</tr>
<tr>
<td></td>
<td></td>
<td>.987</td>
<td>.17</td>
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*Platelets were tested from HLA-typed donors known to be positive or negative for HLA-A2, B7, or B12
†Absorbance values.
SERA FROM ALLOIMMUNIZED PATIENTS

Fig. 1. Sera from 30 alloimmunized patients were tested against platelets from 3 individuals. All sera were positive (absorbance > 0.300) except for sera from 3 individuals (+, 0, +), which gave borderline results perhaps because of HLA similarities between these patients and the individuals tested.

against platelets from donors possessing these antigens and negative against appropriate controls.

**Alloimmunized Patients**

Sera from 30 alloimmunized patients, all of whom were refractory to random donor platelets, were tested against platelets from 3 normal individuals. All sera were positive against at least 70% of a panel of 80–100 lymphocytes and all demonstrated cytotoxicity against multiple HLA antigen specificities (done by P. Terasaki, UCLA, Los Angeles, Calif.). As shown in Fig. 1, sera from 28 of the patients were positive against 2 of the donors, with 27 sera reactive against the other donor. Sera from 3 patients with either negative or marginally positive against all 3 donors. Two of these patients had HLA types similar to the platelet donors

with only single antigen mismatches which may in part explain the lower values in these pairings.

Dilution experiments were done using HLA and P1A1 antisera and sera from alloimmunized patients. A representative experiment is shown in Fig. 2, which demonstrates a progressive fall in absorbance with increasing dilution of serum. No prozone effect was noted with the sera tested.

**Preserved Platelets**

**Frozen Platelets**

Absorbance values were always greater when frozen-thawed platelets (frozen 1–7 days) were tested as compared to fresh controls. Two to four fold increases were seen in many experiments using either autologous or antibody containing sera. Results were poor whether platelets were frozen in the wells themselves or in test tubes and subsequently placed in the plates after thawing.

<table>
<thead>
<tr>
<th>Table 4. Dessicated Platelets</th>
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<tbody>
<tr>
<td>Normal Sera</td>
</tr>
<tr>
<td>---------</td>
</tr>
<tr>
<td>n = 100</td>
</tr>
<tr>
<td>Fresh controls</td>
</tr>
<tr>
<td>− 70°C</td>
</tr>
<tr>
<td>n = 91</td>
</tr>
<tr>
<td>Fresh controls</td>
</tr>
<tr>
<td>Room temperature</td>
</tr>
</tbody>
</table>

*Mean absorbance ± SD (ranges).

The same platelet-serum pairs were tested using fresh platelets or dessicated platelets stored at −70°C or room temperature.

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Paraformaldehyde Fixation

Inconsistent results were obtained using fixed platelets in experiments using 20 sera and 2 platelet donors, both with increases and decreases in absorbance compared to fresh controls. In general, decreases in absorbance were noted using fixed platelets and antibody “positive” sera, and in some experiments these decreases were substantial (>0.500). Less profound discrepancies were seen with normal sera, although both increases and decreases in absorbance were noted. Variable results were seen using platelets fixed either in solution or in the wells.

Dessicated Platelets

Dessicated platelets from 12 individuals were tested against multiple normal or “positive” sera after storage from 7–30 days at either room temperature or -70°C. The duration of storage did not affect the results, and the data in Table 4 represent a summary of all the experiments. There was no statistically significant difference between the results using fresh platelets and platelets stored at either temperature. There was a slight overall fall in absorbance values compared to fresh platelets, although increases were seen in some experiments. This suggests that significant leakage of immunoglobulin did not occur using this method of storage. More consistent results (Fig. 3) were obtained after storage at -70°C with only 19% (27/142) of values either increasing or decreasing more than 0.100 from the fresh controls compared to 41% (59/143) of values with room temperature storage (p = 0.00003). This is also reflected in the higher correlation coefficient (r = 0.91) for -70°C storage compared to r = 0.83 for room temperature storage when paired fresh and dessicated values were compared by linear regression analysis. Most (16/27) of the discrepancies of greater than 0.100 were noted with positive sera (i.e., with high initial absorbance values). Duplicate values from platelets stored at -70°C agreed closely (mean difference 0.039, range 0–0.183).

Liquid Suspension

Platelets from 3 normals were stored in liquid suspension in normal saline with 0.01% sodium azide for more than 1 yr (maximum 417 days). Results using fresh platelets from those donors with both normal sera and sera from alloimmunized patients were compared with the stored platelets (Table 5). Stored platelets tested without serum gave low “background” results (mean 0.073, n = 3), similar to results using fresh platelets (Table 1). There was no significant difference between the fresh and stored platelets with either normal or “positive” patient sera (Table 5). The correlation coefficient for the paired fresh and liquid suspension stored platelets was 0.88. In 9 of 35 paired experiments, there was a difference of more than 0.100 between fresh and stored platelets. Only 2 of the 9 discrepancies were greater than 0.200 and 7/9 occurred with “positive” antisera.

DISCUSSION

The microELISA assay for the detection of PAIgG and antiplatelet antibody that we have described is relatively simple to perform and produced highly consistent and reproducible results. The assay detected HLA and platelet-specific antigens as well as PAIgG and circulating antibody in patients with ITP and alloantibodies from polytransfused patients. The advantages of ELISA technology include the stability of the reagents, the avoidance of the biohazard controls necessary when radioisotopes are used, the detection of all subclasses of IgG, as compared to Staph Protein A assays, which will not detect IgG3,23 and the quantitative endpoint of the reaction, particularly as compared to the qualitative, microscopic assessment of immunofluorescence with the PAIgG assays done in many laboratories.1,2 The automated Micro ELISA reader can measure a 96-well plate in approximately 1 min,
thereby rapidly providing final data. It was also usually possible to perform qualitative visual assessments of “positive” or “negative” because of the bright yellow color of the final reaction product compared to controls as was also noted by Horai et al.20

The use of microtiter plates compared to ELISA19,24 and other PAIgG assays done in test tubes considerably simplifies the procedure primarily by making the multiple washes easier to perform, thereby allowing more rapid testing of a larger number of samples. This is of particular importance if the test can be shown to be effective for platelet donor selection in that it would be possible to screen large numbers of donors simultaneously. In addition, the microtiter assay conserves reagents and often scarce antibody supplies. Nonspecific absorption of conjugate or antibody to the plates is minimal (Table I) if the wells are preincubated with a buffer-albumin solution prior to testing. Considering the very low absorption values when platelets were tested with buffer alone, it is unlikely that leakage of platelet alkaline phosphatase25 contributed significantly to the background results. As described in other assays,2 5,22 variable amounts of PAIgG were found on the platelets of normal individuals even after washing. This increased slightly after incubation with autologous or normal sera, possibly due to further nonspecific binding. Nonetheless, there was no overlap in PAIgG between normals and the ITP patients tested, very few “normal” results when sera from alloimmunized patients were tested, and no discrepant results using specific HLA or P1A1 antisera.

Paraformaldehyde fixation and platelet freezing produced variable results. In general, background values were lower using fixed platelets compared to controls, as has also been noted by others.1 The overall results using fixed platelets were less consistent than with dessication, however. Results using frozen platelets were unsatisfactory and usually increased compared to controls. Kelton et al.22 have demonstrated increased levels of intracellular IgG compared to platelet membrane-bound IgG, and it is likely that freezing-induced damage resulted in release of intracellular IgG with overall increased binding of conjugate.

In contrast, platelets preserved for long periods of time either by dessication or in liquid suspension gave reproducible results that correlated well with results obtained using fresh platelets. More uniform results were obtained when dessicated platelets were kept at ~70°C compared to room temperature, although overall results were similar at both temperatures. Because the platelet separation and centrifugation into the wells is relatively straightforward, it should be possible to create large numbers of plates containing platelets from multiple potential donors that can then be stored. “Crossmatches” with potential recipient serum can then be done on large numbers of donors simultaneously in a fashion analogous to lymphocytotoxicity screening when frozen panels are used. Studies are now in progress that will attempt to correlate microELISA crossmatches with transfusion results in alloimmunized patients. Storage of platelets from single individuals in liquid suspension would be more suitable when it is desirable to maintain platelets of a certain antigenicity for screening purposes (e.g., P1A1 negative platelets to serve as negative controls when screening for P1A1 antibody). Our results indicate that platelets can be stored for at least 1 yr under these conditions without significantly altering their antigenicity, suggesting that these antigens are firmly bound to the platelets.

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Detection of platelet antibodies using a micro-enzyme-linked immunosorbent assay (ELISA)

CA Schiffer and V Young