Application of the Enzyme Linked Immunospecific Assay (ELISA) for the Detection of Platelet Antibodies

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Many methods have been described to identify platelet antibody, but they are either not very sensitive or too complex for general use. Therefore, we have developed an enzyme immunoassay for the detection of platelet antibodies in serum. The method involves incubating platelets with serum antibody; any attached antibody is shown by the addition of an enzyme (alkaline phosphatase) labeled anti-human IgG, followed by assay of the enzyme reaction with its substrate. The reaction product is indicated by a color change, which is proportional to the antibody concentration. Assay conditions such as the use of paraformaldehyde fixed versus unfixed platelets, conjugate dilutions, and substrate concentration and incubation time were investigated. Positive results were obtained in 16 of 19 sera of patients with various diseases including 2 of 4 patients with idiopathic thrombocytopenic purpura, 2 of 2 with post-transfusion purpura, 2 of 3 with neonatal purpura, and all 9 polytransfused patients. Sensitivity and specificity were 84% and 98%, respectively. Also, enzyme linked immunospecific assay (ELISA) was found to be superior to the lymphocytotoxicity (LCT) and platelet immunofluorescence test (PIIFT) for platelet antibody identification.

M ANY LABORATORY TESTS to detect platelet antibody are available including complement fixation,14C labeled serotonin release,2-3 platelet factor 3 release,4-5 and 51Cr release.6-7 These are useful in the detection of HLA, drug dependent, and isoantibodies. Recent techniques that include Fab-anti-Fab,8-9 complement lysis inhibition,10-14 immunoperoxidase,15 immunofluorescence,16-17 and radioimmunoassay,18-20 and 125I-staphylococal protein A21 have been reported useful for antibody detection in other types of immune thrombocytopenia. However, all of these procedures are difficult to set up and are very time consuming.

The enzyme linked immunospecific assay (ELISA) is a modification of the radioimmunoassay in which an enzyme is substituted for the radiolabel. The advantages are that it is less expensive to perform and requires less sophisticated equipment; a simple photometer is adequate for testing. The stability of the reagents and ease of performance make ELISA especially suitable for determinations that are performed outside large centers.22-23 Therefore, we developed a modification of the assay for the serologic detection of platelet antibody. We report here the details of the assay and the results of our investigation of patients with circulating platelet antibody.

MATERIALS AND METHODS

Conjugate

Two commercial sources of alkaline phosphatase conjugated to anti-human IgG were used (Miles laboratories, Elkhart, Ind., and Orion Diagnostica, Helsinki, Finland).

Platelet Preparations

Platelet-rich plasma (PRP) from 16 healthy, HLA typed donors of blood group O was harvested after differential centrifugation (250 g for 10 min) of fresh blood drawn in EDTA (0.07 ml of 15% EDTA per 7 ml of whole blood). The PRP was centrifuged at 1900 g for 7 min at 20°C, the supernatant was discarded and the platelets resuspended in PBS-EDTA (phosphate-buffered saline: 0.0264 M Na2HPO4, 0.14 M NaCl, 0.009 M Na2EDTA, pH 6.8–7.0). This wash step was repeated twice. When platelets were fixed, 1% paraformaldehyde24 was used as previously described and the fixed platelets were washed three times as before. The platelet count was then adjusted to 7.5 x 10^11/cc mm. Four pools each consisting of an equal number of platelets from four donors were prepared and stored at 4°C until needed for testing. Platelets stored at 4°C without preservative were found to be suitable for testing up to 14 days. Leukocyte contamination of the platelet suspension was less than 0.1% and the erythrocyte contamination was less than 1% based on counts performed by a Coulter Counter Model ZBI.

Sera

Serum samples routinely sent to our laboratory for the determination of platelet antibodies were studied. We selected 10 such samples in which clinical information and/or another technique for platelet antibody detection strongly supported the presence of an antiplatelet antibody. Sera from nine other polytransfused patients who were refractory to random donor platelet transfusions were also referred.

Positive control sera included an anti-PII, an anti HLA A2, and a polyspecific HLA antisemum. A negative “control” serum was made from a pool of sera (negative for red cell and lymphocytotoxic antibody) obtained from five healthy male donors of blood group AB, who had never received a blood transfusion. Sera from 90 healthy nontransfused males were also tested.
Performance of the Assay

The ELISA procedure involved incubation of test sera with target platelets. The cells were washed and the IgG bound to the platelets was determined using alkaline phosphatase conjugated antiglobulin. The degree of IgG binding was considered to be the antiplatelet antibody activity in the test sera. The assay procedure was based on the principles originally developed by Engvall and Perlman with the following modifications: instead of using antigen physically adsorbed to the tubes, we used whole platelets in suspension as the target antigen. Also, shorter incubation times were used than described previously in other ELISA methods.

In disposable polystyrene tubes (12 x 75 mm) that had been coated with Bovine Serum Albumin (BSA) overnight at 4°C, 0.2 ml of platelet suspension was incubated with an equal volume of test serum for 30 min at 37°C. The cells were then centrifuged at 1900 g for 7 min at room temperature and the supernatant serum discarded. Platelets were resuspended following centrifugation with buffer by gentle pipeting with a pipet tip attached to a 200 μl adjustable pipetter. This method of resuspension resulted in a homogeneous platelet suspension with no visual aggregates present. The platelets were washed three times with PBS-EDTA containing 1% BSA. After the final wash, the platelet button was resuspended in 0.2 ml of buffer and an equal volume of anti-human IgG conjugated to alkaline phosphatase was added. The reaction mixture was incubated at room temperature for 45 min, after which the platelets were washed twice. To the final platelet button, 0.2 ml of freshly prepared substrate solution (p-nitrophenyl phosphate, 1 mg/ml, pH 9.8) was added and the mixture incubated at room temperature for 15 min. The reaction was stopped by adding 0.1 ml of 1 M NaOH. The reaction product, p-nitrophenolate from p-nitrophenyl phosphate, was quantitated in a spectrophotometer (Gilford 300 N) at 405 nm, to obtain optical density (OD) reading.

In each assay, a negative and positive control serum was tested. All tests were performed in duplicate.

Evaluation of ELISA

The OD for the test serum was calculated by subtracting the OD of negative control serum from that obtained with the test serum. An OD > 2 SD above the mean of the negative controls, which was based on the OD values of 50 healthy normal donors, was considered positive.

Sera were also screened for antibodies by the standard NIH lymphocyte cytotoxicity technique (LCT) and by the platelet indirect immunofluorescence test (PIIFT) as described by Van dem Borne. To insure uniformity in the results among the three tests, the lymphocytes and/or platelets from the same individuals were used for the LCT, PIIFT, and ELISA.

RESULTS

During the development of the technique, various conditions of the assay were studied to optimize the difference between the negative and positive reference sera.

Platelet Preparation

If the platelet preparation was contaminated with more than 1% red blood cells, the OD difference between the positive and negative control sera was less than expected. This was due to the high OD value obtained with the negative reference serum. Removal of contaminating red cells from a platelet preparation by treatment with NH₄Cl for 5 min, followed by three washes, decreased the OD of the positive reference serum. For example, when platelets were not treated with NH₄Cl the positive reference serum OD was 0.614 as compared to the negative reference serum OD of 0.089. On the other hand, when the same platelets were treated with NH₄Cl, the OD values were 0.068 and 0.053, respectively. It therefore appeared that NH₄Cl treatment of platelets somehow inhibited platelet antibody binding. Minimal red blood cell and leukocyte contamination was best achieved by careful aspiration of the top ⅔ of the PRP following centrifugation of anticoagulated whole blood, and by another slow centrifugation of PRP (250 g for 10 min) to sediment red blood cells. Occasionally, the second centrifugation step was repeated during the platelet wash procedure.

In this study, normal sera and patient sera were studied using pools of paraformaldehyde fixed platelets but subsequently we have used unfixed platelets with equal success. Paraformaldehyde fixation resulted in the loss of about 50% of the platelets. Moreover, by not fixing the suspension we could reduce the procedure time about one hour.

Nonspecific Binding

The OD value obtained with platelets incubated with normal serum represented nonspecific conversion of the substrate. The presence of platelets made the greatest contribution to nonspecificity; 60% of the nonspecific OD was due to normally bound platelet IgG that was routinely present despite three wash cycles in the preparation of the platelet suspension. Approximately 33% of the nonspecific binding was attributed to adsorption of IgG by platelets upon incubation with normal serum. About 2% of the nonspecificity was due to adsorption of serum IgG to the test tube, while about 5% represented adsorption of conjugate to the test tube. Nonspecific conversion of substrate by platelets was minimized by washing the platelets 3 times before and 2 times after incubation with serum. Reduced serum and conjugate adsorption to the test tube was achieved by albumin coating of the test tubes prior testing and by including 1% BSA in the wash buffer.

Conjugate Studies

The optimal conjugate dilution was determined by block titration for each new batch of conjugate. One example of such a titration is illustrated in Table 1.
Platelets used in this experiment were not treated with paraformaldehyde. A fixed number of platelets (1.5 x 10^8) was tested with a positive and a negative reference serum in a series of dilutions of the serum and the stock conjugate. The conjugate dilution that yielded a high OD with a positive serum (e.g., 1.0 or greater) and a low OD with a negative serum (e.g., 0.2 or less) after 15 min of substrate incubation time was selected for subsequent testing. The largest difference between the positive reference serum OD and the negative reference serum OD was recorded at the highest concentration of conjugate tested (Table 1), but it overloaded the spectrophotometer. Also, at low conjugate dilution, the negative reference serum OD was greater than 0.2. Consequently, we selected a dilution of 1:100 and 1:200 for conjugate A and B, respectively (Table 1) since it permitted the measurement of OD without introducing a dilution step. Also, by using the highest possible dilution of the conjugate we could reduce the cost of this reagent per assay. Moreover, we were even able to visually interpret the results at these conjugate dilutions. Using diluted conjugate, normal sera imparted no visually detectable color in contrast to positive sera. On the other hand, at low conjugate dilution (e.g., 1:50), both positive and negative sera manifested a strong yellow color that could not be differentiated visually. Visual assessment of the test results was felt to be important since it may facilitate the application of the present technique.

**Substrate Studies**

Substrate incubation times from 0 to 60 min were investigated. Increasing OD values were noted as the substrate incubation time increased. However, the difference between the positive and negative reference serum OD (expressed in terms of a ratio) decreased when the incubation time was extended beyond 30 min. At incubation times of 15 and 30 min, the ratio was stable. However, the shorter incubation time (15 min) was chosen to reduce the overall procedure time.

We also investigated different substrate concentrations, from 1 mg/ml to 4 mg/ml (Table 2). Higher substrate concentrations resulted in overloading of spectrophotometer and therefore the lowest concentration (1 mg/ml) was chosen that also lead to reagent economy. Substrate concentration was not the limiting factor for the development of color, since with increasing incubation time and with increasing antibody concentrations, greater OD values were obtained. Substrate incubation at room temperature and at 37°C gave similar results.

**Specificity**

To demonstrate the immunospecificity of the ELISA reaction, we studied the absence of antigen or platelet antibody in the assay. As shown in Fig. 1, a much higher OD was obtained when platelets were incubated with antiplatelet serum as compared to the OD obtained upon incubation with normal serum. Also, the OD was low when an antibody was incubated with platelets lacking the corresponding antigen. The mean (range) OD value of 49 normal sera with four pools of fixed platelets were 0.005 (0-0.03), 0.006 (0-0.04), 0.013 (0-0.05), and 0.013 (0-0.05). With one additional normal serum, an OD greater than the mean ± 2 SD of normal was obtained with each pool and was considered a false positive reaction. This serum was also tested for red cell antibody and for platelet antibody by LCT and PIIFT and was found to be nonreactive. We also studied 24 normal sera from single unit rather than pooled platelet preparations (not fixed with paraformaldehyde) and the mean OD ± SD was 0.134 ± 0.037. It was apparent from these experiments that normal control OD values were higher with unfixed platelets compared to fixed preparation but correspondingly higher OD values were also found with positive sera. Therefore, overall results remained unaffected.

The reproducibility of ELISA is shown in Table 3.
Patient Sera

Nineteen sera from patients were assayed by all three techniques. The clinical diagnosis and results of all three techniques are listed in Table 4 and the reactivity of each platelet pool by ELISA is shown in Table 5. The sensitivity of ELISA was superior (84%) to that of LCT (26%) and PIIFT (68%). The specificity of each technique was similar: ELISA (98%), LCT (100%), and PIIFT (94%) (Table 6).

Idiopathic thrombocytopenic purpura (ITP) patients. Sera from three patients with chronic ITP and serum (J.W.) from the mother of a patient with neonatal ITP were negative by LCT; one serum (M.B.) was positive with PIIFT and two (S.H. and M.B.) were positive with ELISA.

Post-transfusion purpura patients. Serum from both patients with post-transfusion purpura gave strong positive results with all four platelet pools by PIIFT and ELISA. Negative reactions were seen by LCT. One sample gave negative results when tested with platelets from five P1A\(^A\) negative donors. The other serum was not tested with P1A\(^A\) negative platelets because of an insufficient amount available for testing.

Neonatal purpura patients. Three maternal sera from patients with neonatal purpura were studied.
One sample (S.S.) gave positive reactions by all three techniques; this serum was identified as an anti HLA-B5 cross-reactive with BW35 and B18. Another sample (C.S.) gave positive results only with ELISA and the third (C.G.) was nonreactive in all three techniques.

**Other disorders.** Eight of nine polytransfused patients gave positive results by PIIFT and all of them gave positive reactions by ELISA; only three were positive by LCT. Serum from a nontransfused multiparous woman with lymphoma (J.R.) gave strongly positive results by all methods.

### DISCUSSION

A simple, enzyme labeled anti-human IgG assay for the serologic detection of platelet antibodies has been developed in our laboratory. We selected alkaline phosphatase as the marker enzyme because its substrate (p-nitrophenyl phosphate) is safe and stable.

An alternative enzyme that could have been used is peroxidase. This enzyme is less expensive and can easily be obtained, but its use of this enzyme requires a multistep redox reaction; its substrate, H2O2, is unstable; and one reagent, 5-aminosalicylic acid may present a hazard to laboratory personnel since it is thought to be carcinogenic.

We used platelets pooled from several donors to facilitate screening for platelet antibody. In the present study, we used paraformaldehyde fixed platelets, but in subsequent study washed unfixed platelets gave comparable results and the assay time was shortened, as a consequence, we no longer use fixed platelets.

The low enzyme activity found with platelets in the absence of an appropriate antigen and with negative control serum (Fig. 1) probably represents the normal platelet associated IgG that remains attached to platelets after washing.

Non-specific binding of IgG to the test tube and the presence of alkaline phosphatase normally found in platelets may also contribute to the low background enzyme activity.

In the cases of chronic ITP, we did not find antibody in 2 of the 4 sera tested, however, one of the patients (G.H.) was in clinical remission following steroid therapy. The other patient (J.W.) had chronic ITP for 10 yr., was refractory to cortisone therapy and splenectomy, and delivered two children neonatal ITP. In this patient, negative reactions were seen by all three techniques. In recent reports quantitating platelet associated IgG and C3, it was found that plasma or serum antibody levels are less reliable for assessing the disease activity than the direct platelet IgG determination, and also that platelet-associated C3 may be responsible for the pathogenesis of the thrombocytopenia in these patients. It is possible that the direct assay, as well the use of an anti C3-conjugate could have been useful in such cases.

### Table 6. Sensitivity and Specificity of the Three Techniques for Platelet Antibody Identification

<table>
<thead>
<tr>
<th>Test Result</th>
<th>LCT*</th>
<th>PIIFT†</th>
<th>ELISA</th>
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<tr>
<td>Patients</td>
<td>Controls</td>
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<td>Specificity</td>
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*LCT, Lymphocytotoxicity test.
†PIIFT, Platelet indirect immunofluorescence test.
In one of our cases of neonatal purpura (C.S.), we found a platelet antibody that we have not yet characterized. However, we know it is not anti-P14 since negative results were noted with P14 positive platelets. In the other patient with neonatal purpura (S.S.), we identified an anti HLA-B5 cross reactive with Bw35 and B18 that appears to have caused leuko-thrombocytopenia in the newborn because the lymphocytes from the father and newborn had B5 antigen and the mother’s lymphocytes lacked this antigen. In addition a strong reaction was seen between the maternal serum and paternal lymphocytes, and the titer of the antisera was very high (1:128).

In two patients with post-transfusion purpura, ELISA demonstrated platelet antibody in high titer (1:32), while both sera failed to react in the LCT. One of these antibodies was identified as an anti-P14. The other has not been characterized. Using a direct and indirect radiolabel antoglobulin test, Cines et al. found increased platelet associated IgG and IgG anti-platelet activity in the plasma of a patient with post-transfusion purpura. Our findings support their observation that circulating antibody in post-transfusion purpura is of the IgG type.

All nine polytransfused patients refractory to random donor platelet transfusions were found to have antibody by ELISA and eight of these were also detected by PIIFT. Since only three of them had LCT antibodies in their serum, the remaining six patients may have developed either platelet specific antibodies or HLA antibodies undetected by LCT.

ACKNOWLEDGMENT

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Application of the enzyme linked immunospecific assay (ELISA) for the detection of platelet antibodies

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