Enzyme Labeled Immunosorbant Assay (ELISA) for Detection of Platelet Antibodies

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ABSTRACT

Although it is widely accepted that patients with immune thrombocytopenia produce platelet antibodies, the demonstration of such antibodies has been difficult and time consuming. A simple and quick enzyme linked immunoassay for platelet antibodies is presented. The platelet associated IgG is coupled with alkaline phosphatase labeled anti-IgG. The resultant complex is determined spectrophotometrically using p-nitrophenyl phosphate as substrate. With this technique, excess of IgG on platelets was detected in 24 out of 33 patients (72 percent) with immune thrombocytopenic purpura and four out of four thrombocytopenic patients with systemic lupus erythematosus. The results of this assay correlate quantitatively with Dixon et al\(^3\) complement lysis inhibition assay (r = 0.82).

Introduction

In 1951, Harrington\(^5\) provided evidence for a humoral factor responsible for thrombocytopenia in patients with idiopathic thrombocytopenic purpura (ITP). In 1965, Shulman\(^15\) demonstrated that the humoral factor was species-specific, could be absorbed by autologous as well as homologous platelets and was present in the 7 S fraction of gamma globulin. Numerous attempts to demonstrate the anti-platelet factor have met with variable success. These include platelet agglutinin test,\(^5,15\) antiglobulin consumption test,\(^15\) and platelet factor 3 immunoinjury test.\(^10\) In 1975, Dixon and co-workers\(^3\) developed a complement mediated red cell lysis inhibition technique for quantitative detection of platelet antibodies. They found all of the tested 17 patients with ITP were positive for platelet antibodies and the quantity of platelet associated IgG (PA IgG) correlated with the severity of the disease. Several new techniques claiming high degree of precision and sensitivity have been developed.
These include immunofluorescence,\textsuperscript{9} Fab-antifab IgG assay,\textsuperscript{12} I\textsubscript{125} Coombs test,\textsuperscript{2} and Fc-I\textsubscript{125} staphylococcal protein test.\textsuperscript{7} In recent years, enzyme linked immunoassays (ELISA) have come in general use. The sensitivity of ELISA is claimed to be equal to or greater than radioimmunoassay.\textsuperscript{14} An ELISA technique is described using alkaline phosphatase labeled anti IgG for detecting platelet antibodies. The technique is simple, sensitive, and reproducible.

**Methods and Material**

Patients and controls in this study are outlined in table I. Antihuman IgG labeled with alkaline phosphatase (Anti IgG*AP) was used.

**ELISA Procedure**

Approximately 30 ml of EDTA blood from a normal donor or patient was spun at 800 \times g for 10 minutes to obtain platelet rich plasma (PRP). The PRP was further centrifuged at 3000 \times g for 10 minutes to obtain a platelet pellet. The pellet was washed three times with at least five ml of EDTA veronal buffered saline (EDTA-VBS).\textsuperscript{\dagger} The pellet was then suspended in EDTA-VBS, and the platelet count was adjusted to approximately 50,000 platelets per cmm. Precisely 0.1 ml of platelet suspension was mixed with 0.1 ml of one in 250 dilution of anti IgG*AP and incubated at 37°C for 30 minutes. The incubation mixture was centrifuged and washed three times with normal saline. After the final centrifugation, the supernatant was discarded and one ml of substrate containing 40 mg per ml of disodium p-nitrophenyl phosphate in 0.1 mol per liter of glycine buffer pH 10.5 was added to the pellet. The pellet was resuspended and incubated for five minutes at 37°C. The reaction was terminated with one ml of five N NaOH. The reaction mixture was centrifuged and the optical density of the supernatant was read at 410 nm. A one in 2500 dilution of anti IgG*AP as a standard. The enzyme activity of the standard was determined by the method of Bowen and McComb\textsuperscript{1} based on the enzymatic rate measurement of hydrolysis of p-nitrophenyl phosphate.\textsuperscript{\S}

\textsuperscript{*} This was obtained from Miles Laboratory, Inc., Elkhart, IN.

\textsuperscript{\dagger} Combine 5.56 g of sodium ethylene diamine tetracetic acid, 5.0 g of sodium barbital, 6.23 g of sodium chloride, and approximately 18 ml of N HC1. Add distilled water to make one liter, pH 7.35.

\textsuperscript{\S} This was done on the DuPont Automatic Clinical Analyzer (ACA).

**TABLE I**

<table>
<thead>
<tr>
<th>Category</th>
<th>Number (n)</th>
<th>Criteria of Selection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>18</td>
<td>Normal CBC and platelet count. No history of a hematologic disorder</td>
</tr>
<tr>
<td>Idiopathic thrombocytopenic purpura (TTP)</td>
<td>33</td>
<td>Clinical diagnosis of TTP. No history of any other hematologic disorder. Platelet count less than 100,000 per cmm. Adequate number of megakaryocytes in bone marrow. Patients who had received steroids, cytotoxic drugs or had splenectomy were excluded from this study.</td>
</tr>
<tr>
<td>Systemic lupus erythematosis (SLE)</td>
<td>4</td>
<td>Clinical diagnosis of SLE, ANA &gt; 1/80. Platelet count less than 100,000 per cmm. Adequate number of megakaryocytes in bone marrow.</td>
</tr>
<tr>
<td>Chronic lymphocytic leukemia (CLL)</td>
<td>3</td>
<td>Clinical diagnosis of CLL. Platelet count less than 100,000 per cmm. Adequate number of megakaryocytes in bone marrow.</td>
</tr>
<tr>
<td>Septicemia</td>
<td>2</td>
<td>Documented gram negative septicemia. Platelet count less than 100,000 per cmm. Adequate number of megakaryocytes in bone marrow.</td>
</tr>
<tr>
<td>Thrombotic thrombocytopenic purpura (TTP)</td>
<td>3</td>
<td>Clinical diagnosis of TTP. Evidence of microangiopathic hemolytic anemia. Platelet count less than 100,000 per cmm. Adequate number of megakaryocytes in bone marrow.</td>
</tr>
</tbody>
</table>
Figure 1. Alkaline phosphatase activity bound to platelet associated antigen by enzyme linked immunosorbant assays (ELISA) technique. ITP = idiopathic thrombocytopenic purpura; SLE = systemic lupus erythematosus; CLL = chronic lymphocytic leukemia; and TTP = thrombotic thrombocytopenic purpura.

Calculation

Alkaline P-ase activity per platelet =

\[
\frac{O.C. \text{ of test enzyme activity of standard}}{O.D. \text{ Standard}} \times \frac{\text{enzyme activity of standard}}{\text{No. of platelets in } 0.1 \text{ ml of sample}}
\]

Dixon et al complement mediated red cell lysis inhibition procedure was performed as described by the authors.4

Results

Control Subjects

The platelet associated IgG was measured in 18 control subjects. With the technique of Dixon et al, the mean level (± 1 SD) was 6.3 ± 3.1 fg of platelet with range of 0 to 12. With the ELISA technique, the mean level (± 1 SD) was 0.10 ± 0.06 ng alkP-ase per platelet with range of 0 to 0.20 (figure 1). The control subjects were tested by both procedures on several occasions. The values with both techniques were always within the normal range.

Patients

Of the 33 patients with clinical diagnosis of ITP, 24 (72 percent) were positive with the ELISA technique (figure 1) while 22 (66 percent) were positive with the technique of Dixon et al. Three patients positive with the ELISA were negative with the technique of Dixon et al, while one patient negative with the ELISA was positive with the technique of Dixon et al. In 29/33 patients, there was a close agreement between the two techniques: 4/4 patients in systemic lupus erythematosus (SLE) group, 2/3 in chronic lymphocytic leukemia (CLL) group, 1/2 with septicemia and 1/3 with thrombotic thrombocytopenic purpura (TTP) gave results in abnormal range by both techniques. Correlation coefficient (r) between the two methods was 0.82 (figure 2).

Discussion

Ever since Harrington5 established the immune nature of ITP, a search continues for a practical and reliable test for demonstrating platelet antibody. The platelet agglutination test developed by Harrington4 was shown to be positive in ap-

\[\text{fg} = \text{femtogram or } 10^{-15}\]
DETECTION OF PLATELET ANTIBODIES BY ELISA

proximately 65 percent of the patients with ITP. However, some laboratories could not reproduce Harrington's results and, indeed, some workers suggested that the antiplatelet factor was thrombin. The work of Dixon et al established a new era in the quantitation of platelet antibodies. They reported 17 of 17 patients with ITP were positive for platelet associated IgG. Luiken et al, using Fab-antiFab IgG assay, reported 29 of 31 patients with ITP positive for platelet associated IgG. Hymes et al, using a solid phase radioimmunoassay for IgG employing rabbit anti-human IgG sandwiched to radioactive 125I staphylococcal protein A which binds to the Fc domain of IgG, demonstrated high levels of platelet associated IgG in 32 of 35 patients. Cines and Schreiber, using radioactive 125I Coombs test, detected 45 of 50 patients positive for platelet antibody. Many of the present day workers employ the technique of Dixon et al as a reference procedure. However, the Dixon and Rosse technique is tedious and time consuming.

A simple technique is hereby presented which correlates well with the method of Dixon et al (r = 0.82) and takes less than four hours. The percentage of positive results in patients with ITP is comparable to other techniques. Antiplatelet antibodies have been reported in thrombocytopenic patients with chronic lymphocytic leukemia, with adequate numbers of megakaryocytes in bone marrow. In our series, 2/3 CLL patients with thrombocytopenia were positive for platelet antibody. Circulating immune complexes in ITP have been reported by Lahurna and associates. It is probable that immune complexes are present on the surface of platelet in some of the patients with SLE, septicemia, and thrombotic thrombocytopenia (TTP). Antiplatelet antibody has been detected in 4/4 cases of SLE, 1/2 cases of septicemia, and 1/3 cases of TTP. However, the number of patients in our series is too small to draw a definite conclusion. The disadvantage of the ELISA technique is that the results are expressed in terms of enzyme units of alkaline phosphatase present in anti IgG preparation rather than the amount of platelet associated IgG, as in the technique of Dixon et al. The enzyme units may vary with each batch of labeled anti IgG, and a new normal range may have to be established for each batch. Moreover, the results may not be comparable from one laboratory to another. Standardization of anti-IgG-alkaline phosphatase reagent may help to solve this problem. If desired, the result of the test may be expressed semiquantitatively instead of in unfamiliar alkaline phosphatase units.

References


