Comparison of Two Direct Assays for Platelet-Associated IgG (PAIgG) in Assessment of Immune and Nonimmune Thrombocytopenia

By John G. Kelton, Alan R. Giles, Peter B. Neame, Peter Powers, Nina Hageman, and Jack Hirsh

A number of methods have been developed to measure platelet-associated IgG (PAIgG). The antiglobulin consumption assay directly quantitates IgG on the platelet and is sensitive and specific. A fluorescent anti-IgG assay has recently been described and has the advantage of simplicity. We compared the results of these two PAIgG assays in immune and nonimmune thrombocytopenia and nonthrombocytopenic controls. The antiglobulin consumption assay was negative in 61 of 62 and the fluorescent negative in 54 of 62 assays in nonthrombocytopenic controls, and they were negative in 11 of 13 and 8 of 13 assays, respectively, in nonimmune thrombocytopenic patients. The antiglobulin consumption assay was positive in 54 of 58 and the fluorescent positive in 24 of 58 assays of patients with immune thrombocytopenia (ITP and SLE). The overall sensitivity and specificity of the antiglobulin consumption assay was 94% and 95% and of the fluorescent assay was 44% and 82%.

In his classic study, Harrington demonstrated that idiopathic thrombocytopenic purpura (ITP) was caused by a plasma factor, subsequently identified as an immunoglobulin, which binds to platelets and leads to their rapid clearance. Subsequent studies have demonstrated that immune mechanisms also contribute to thrombocytopenia of systemic lupus erythematosus (SLE), certain lymphoproliferative disorders, and septicemia.

Unlike hemolytic anemia, in which IgG coating of red cells can usually be detected with the Coombs test, considerable difficulty has been encountered in developing an in vitro assay for platelet antibodies. The assays initially developed were indirect and either measured the platelet release reaction or platelet damage following incubation of normal platelets with sera from patients with suspected immune thrombocytopenia. While initially promising, they subsequently were shown to lack sensitivity. More recently, specific assays, such as the antiglobulin consumption assay and the competitive binding radioimmunoassay, have been developed and applied to the diagnosis of immune thrombocytopenia. These assays directly quantitate IgG on the surface of the platelet, and their sensitivity and specificity for platelet-associated IgG (PAIgG) has been confirmed. Several recent studies have suggested that fluorescent labeling on anti-IgG may provide a rapid assay for platelet-associated IgG. In their original description of this assay, the authors reported a 54% positivity rate with ITP platelets. This assay has now been optimized by the use of paraformaldehyde fixation; however, the results using the modified assay have not yet been reported. This technique has the potential advantage of simplicity and can be established in most laboratories. We have therefore compared the antiglobulin consumption assay with the modified fluorescent anti-IgG assay in patients with immune and nonimmune thrombocytopenia, in normal volunteers, and in nonthrombocytopenic hospitalized patients.

Materials and Methods

Patient Material Studied

Blood samples for measurement of PAIgG were obtained from the following groups of patients and controls.

Patients With ITP

Patients were considered to have ITP if they had thrombocytopenia, a nonpalpable spleen, a normal or increased number of megakaryocytes in the bone marrow, and clinical features and laboratory investigations that excluded other causes of thrombocytopenia. These patients had not received platelet transfusions prior to study. Some were receiving corticosteroids at the time of testing or had undergone splenectomy.

Patients With Systemic Lupus Erythematosus (SLE)

These patients had thrombocytopenia in association with systemic lupus erythematosus (SLE) and were not receiving immune suppressive agents, except some were receiving corticosteroids. All had positive antinuclear antibodies and other clinical features compatible with SLE.

Immune Thrombocytopenia Other Than ITP or SLE

These patients had other forms of immune thrombocytopenia and included posttransfusional thrombocytopenia, acute childhood ITP, and thrombocytopenia complicating infectious mononucleosis.

Patients With Nonimmune Thrombocytopenia

These patients had thrombocytopenia associated with aplastic anemia, chemotherapy, multiple myeloma, B12 deficiency, alcoholism, malignant hypertension, and following massive transfusions.
Normal Hospitalized Patients

The hospital controls were nonthrombocytopenic patients with various illnesses.

Normal Control Population

The normal controls were a group of laboratory personnel. None were taking medication (with the exception of several females taking oral contraceptives).

Platelet Preparation

Whole blood was collected into 10% EDTA (20 ml: 0.3 ml, v/v). The platelet-rich plasma was obtained by centrifugation (240 g for 10 min, 22°C) and a platelet pellet was obtained (2230 g for 10 min, 22°C) and washed with Veronal-buffered saline (VBS). The platelet suspension was divided into two parts for assay by each method. The sample tested by the fluorescent method was fixed with 2 ml 2% paraformaldehyde in phosphate-buffered saline. In some cases, the platelets tested with the antiglobulin consumption assay were tested before and after fixation with paraformaldehyde.

Measurement of Platelet Associated IgG

Antiglobulin Consumption Test

Platelet-associated IgG was quantitated using a modification of the method of Dixon and Rosse that has been previously described in detail. Sheep red cells are coated with human IgG, and after washing, are incubated with known concentrations of anti-human IgG (specific against all subtypes of IgG). Guinea pig complement (GIBCO, Grand Island, N.Y.) is added and the amount of lysis quantitated by measuring the free hemoglobin in the supernatant spectrophotometrically (412 nm). A standard curve is generated by adding varying dilutions of an IgG standard to the anti-IgG, followed by the addition of the IgG-coated sheep cells and complement. The binding of the anti-IgG by the IgG produces inhibition of lysis of the sheep cells and forms the basis of this assay. Varying dilutions of washed platelets are then incubated with anti-IgG, and following the addition of the sheep red cells and complement, the degree of inhibition of lysis for each platelet dilution is calculated. This is compared to the standard inhibition curve and the amount of IgG per platelet calculated.

Since the previous report, we have modified this assay, as follows. (1) Platelets are now washed five times instead of three. The two extra washings lower the level of PAIgG of normal platelets, but additional washings do not lead to a further decrease. (2) Each step has been optimized to increase the amount of lysis for each dilution of anti-IgG and to allow the use of lower concentrations of anti-IgG. This was achieved by obtaining an optimal complement dilution (1/10 final) and improving the method for coating sheep red cells with IgG (40 mg IgG/ml sheep cells).

Positive and negative controls were prepared as described and tested for PAIgG each time an assay was performed. The level of PAIgG in the positive controls is proportional to the concentration of IgG used in their preparations.

The Fluorescent Assay for PAIgG

This assay was performed as described by van dem Borne et al. The platelets to be tested were washed, and the pellet was fixed with the addition of 2% paraformaldehyde in phosphate-buffered saline (w/v) for 5 min at 22°C. Following two washes in phosphate-buffered EDTA, 100 μl platelet suspension, 50,000–150,000/μl, was incubated for 30 min at 37°C with 100 μl of a 1/20 dilution of fluorescein-labeled anti-IgG. The anti-IgG used in the antiglobulin consumption assay was labeled in the standard fashion. The platelets were washed twice in phosphate-buffered EDTA and resuspended in 80% glycerol phosphate-buffered saline. One drop of the platelet suspension was examined for fluorescence using a standard fluorescent microscope (Dialex; Wild Leitz Canada Ltd., Toronto, Ontario).

The same observer performed all fluorescent studies, grading the results as positive or negative. The decision of a positive or negative result was subjectively made after scanning a number of fields that contained from 50 to 200 platelets. To assist the observer, a laboratory positive control, produced to have a moderate level of PAIgG (20–25 fg IgG/platelet) was examined prior to each test sample. The clinical diagnosis was unknown to the technologists carrying out either assay.

RESULTS

Antiglobulin Consumption Assay

With the modification described, the sensitivity of the assay was increased so that 50% inhibition occurred with 40–50 ng IgG, whereas previously, 200 ng IgG standard was required. The normal range, which was 0–15, is now 0–5 fg IgG/platelet (mean ± 3 SD, n = 30). No assay in 50 tests of normal individuals had a PAIgG level greater than 5 fg IgG/platelet. Preliminary studies demonstrated that fixation of platelets with 2% paraformaldehyde did not change the level of PAIgG. Platelets to be tested (control or patient) are now washed 5 times instead of 3.

The additional washings of the platelets resulted in a plateau in the level of PAIgG; however, it did not change an elevated result into a normal result. The washings resulted in a linear loss in platelet recovery that could range from a low of 14.2% to a high of 37.5% (n = 5).

Fluorescent Assay

Preliminary studies demonstrated that the optimal dilution of fluorescein-labeled anti-IgG (assessed using known positive and negative controls) was 1/20 (other dilutions tested were 1/10, 1/50).

Control Subjects

Normal Controls

Thirty-nine normal controls (laboratory personnel) were assayed by both methods. Six had both assays performed twice for a total of 45 assays. The fluorescent assay was positive in four of these individuals, and one of these was positive on two separate determinations. The antiglobulin consumption assay was elevated in one person on one occasion (7 fg IgG/platelet). The result was normal when repeated. The mean PAIgG for this group was (2.2 ± 0.1 fg IgG/platelet; mean ± SD).

Nonthrombocytopenic Hospital Controls

Seventeen nonthrombocytopenic hospitalized controls were tested by both assays. One patient was...
tested on two separate occasions. The fluorescent assay was positive in four of these patients. Using the antiglobulin consumption assay, the mean PAIgG for this group was $3.1 \pm 1.3$ fg IgG/platelet (mean ± SD) with no PAIgG determination outside the normal range.

**Nonimmune Thrombocytopenia**

Thirteen patients had thrombocytopenia defined as nonimmune (Table 1). The platelet count ranged from 10,000/μl to 130,000/μl, with a mean of 55,000/μl. The PAIgG level was elevated in two of these patients (levels of 7 and 8 fg IgG/platelet); both of these patients had multiple myeloma. The mean PAIgG for this group was $3.8 \pm 2.0$ fg IgG/platelet (mean ± SD). The fluorescent assay was positive in five patients.

**Immune Thrombocytopenia**

**ITP**

Forty-six studies were performed on 37 adult patients with ITP (Fig. 1). Using the antiglobulin consumption assay, the PAIgG level was elevated in 42 of 46 studies to a mean of $20.0 \pm 25$ fg IgG/platelet (mean ± SD). One of the patients with normal PAIgG levels was unresponsive to steroids and splenectomy and had an illness atypical for ITP. The fluorescent assay was positive in 21 of these studies. In one patient with ITP, the fluorescent assay was positive and the antiglobulin consumption assay was negative. The relationship between platelet count and PAIgG level and positive or negative fluorescence is shown in Fig. 1. There was no relationship between the level of PAIgG quantitated by the antiglobulin consumption assay and the results of the fluorescent assay.

**Thrombocytopenia Complicating SLE**

Twelve studies were carried out on 10 patients who had thrombocytopenia and SLE. None were receiving cytotoxic agents at the time of testing. Using the

<table>
<thead>
<tr>
<th>Table 1. Nonimmune Thrombocytopenia</th>
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<tr>
<td><strong>Diagnosis</strong></td>
</tr>
<tr>
<td>Normal</td>
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<tr>
<td>Aplastic anemia</td>
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<tr>
<td>L.Z.</td>
</tr>
<tr>
<td>L.S.</td>
</tr>
<tr>
<td>J.H.</td>
</tr>
<tr>
<td>Multiple myeloma</td>
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<tr>
<td>H.B.</td>
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<tr>
<td>U.A.</td>
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<tr>
<td>Chemotherapy for leukemia</td>
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<tr>
<td>M.C.</td>
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<td>A.F.</td>
</tr>
<tr>
<td>A.B.</td>
</tr>
<tr>
<td>C.L.</td>
</tr>
<tr>
<td>B12 Deficiency</td>
</tr>
<tr>
<td>A.R.</td>
</tr>
<tr>
<td>Alcohol thrombocytopenia</td>
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<tr>
<td>B.S.</td>
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<tr>
<td>Malignant hypertension</td>
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<tr>
<td>A.G.</td>
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<td>Multiple transfusions</td>
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<tr>
<td>R.F.</td>
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</table>
antiglobulin consumption assay, the PAIgG level was elevated in all assays to a mean of 29 ± 25 fg IgG/platelet (mean ± SD). The fluorescent assay was positive in 3 of the 12 assays. The relationship between platelet count, PAIgG level, and the result of the fluorescent assay for this group of patients is illustrated in Fig. 2. Once again, there was no relationship between the level of PAIgG as assayed by the antiglobulin consumption assay and the results of the fluorescent assay. The relationship between the results of both assays for all ITP and SLE patients and the platelet count is illustrated by Fig. 3.

Other Immune Thrombocytopenia

Five patients had other causes of immune thrombocytopenia, and the PAIgG level was elevated in all. The fluorescent assay was positive in three of the five patients (Table 2).

**Table 2. Immune Thrombocytopenia (Other Than SLE, ITP)**

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Platelet Count (per μl)</th>
<th>Antiglobulin Consumption (fg IgG/platelet)</th>
<th>Fluorescent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>150,000–450,000</td>
<td>0–5</td>
<td>–</td>
</tr>
<tr>
<td>Childhood ITP</td>
<td>15,000</td>
<td>11</td>
<td>+</td>
</tr>
<tr>
<td>A.C.</td>
<td>12,000</td>
<td>18</td>
<td>+</td>
</tr>
<tr>
<td>L.W.</td>
<td>10,000</td>
<td>21</td>
<td>–</td>
</tr>
<tr>
<td>M.S.</td>
<td>16,000</td>
<td>30</td>
<td>+</td>
</tr>
<tr>
<td>Posttransfusion purpura</td>
<td>70,000</td>
<td>11</td>
<td>–</td>
</tr>
<tr>
<td>Infectious mononucleosis</td>
<td></td>
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<td>J.R.</td>
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</table>

**DISCUSSION**

The development of sensitive assays that quantitate PAIgG have demonstrated that increased levels of platelet-associated IgG (PAIgG) occur in thrombocytopenia complicating SLE, lymphoproliferative disorders, septicemia, as well as in ITP.4–8

However, the optimal assay for measuring PAIgG is as yet unknown. The recently described fluorescent anti-IgG assay offers the potential advantage of simplicity.15,16 We therefore compared the results obtained using the antiglobulin consumption assay and the fluorescent assay on platelets obtained from patients with immune and nonimmune thrombocytopenia. To allow as direct a comparison as possible between these two techniques, the same antisera was used, and all assays using each technique were performed by a single technologist. The results indicate that the antiglobulin consumption assay is more sensitive and specific than the fluorescent assay for the diagnosis of immune thrombocytopenia. The antiglobulin consumption assay was positive in 92% of assays for ITP, and the fluorescent assay was positive in 46% (Fig. 1). In two of the four patients in whom the antiglobulin consumption assay was negative, the...
thrombocytopenia was mild (platelet counts of 100,000 and 150,000/ml, respectively). The distribution of positive and negative results of the fluorescent assay for PAIgG was not related to the level of PAIgG. The antiglobulin consumption assay was positive in 100% of thrombocytopenic patients with SLE, while the fluorescent assay was positive in 25% (Fig. 2). Again, there was no relationship between the results of the fluorescent assay and the level of PAIgG. Combining the results of all assays for immune thrombocytopenia, the antiglobulin consumption assay was positive in 59 of 63 (94%) and the fluorescent assay positive in 27 of 63 (43%).

The fluorescent assay was positive in 14% of the normal and hospitalized controls, and the antiglobulin consumption assay was positive in 2%. In the patients with nonimmune thrombocytopenia, the fluorescent assay was positive in five, and the antiglobulin consumption assay was positive in two patients. Both of these latter patients had multiple myeloma, and it is conceivable that the elevation in PAIgG level was of pathogenic importance, or alternatively, reflected the elevated level of gamma globulin in these patients. However, if these are considered to be false positive results, the overall frequency for the falsely positive results for the antiglobulin consumption assay was 6%. In summary, the sensitivity and specificity of the antiglobulin consumption assay was 94% and 95%, respectively, and for the fluorescent assay was 44% and 82%, respectively.

The sensitivity we observed using the modified fluorescent assay is similar to that reported by van Boxtel et al., using the original method. Our results suggest that despite the modification of this method by using paraformaldehyde fixation, it is less sensitive and specific than the antiglobulin consumption assay. We postulate that the better results obtained with the antiglobulin consumption assay reflect the basic differences between these two assays; the antiglobulin consumption assay is an immunologic assay measuring IgG on all surfaces of the platelet, while the fluorescent assay is a visual assay and the observer can examine only two dimensions of the platelet. This, theoretically, can be overcome with the use of microfluorometry.

Of interest were the results of a patient with severe thrombocytopenia who was not responsive to corticosteroids or splenectomy. This patient had a normal level of PAIgG by either assay, and clinically, had ITP with markedly reduced recovery of homologous platelet transfusions. He is similar to several other patients we have studied who also had severe ITP but normal levels of PAIgG. These patients may represent a subset of ITP in whom the platelet destruction is mediated through other mechanisms than IgG coating of the platelets.

REFERENCES

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