The Direct Assay for Platelet-Associated IgG (PAIgG): Lack of Association Between Antibody Level and Platelet Size

By J. G. Kelton, P. B. Neame, J. Bishop, M. Ali, J. Gauldie, and J. Hirsh

Elevated levels of platelet-associated IgG (PAIgG) found by direct assay in patients with immune thrombocytopenia, may in part reflect passive absorption of IgG to megathrombocytes present in increased proportions in thrombocytopenia caused by increased platelet destruction. Using a direct assay for PAIgG based on the inhibition of lysis of IgG-coated sheep red blood cells, we correlated these levels with two platelet volume parameters, median platelet volume and percentage megathrombocytes, using a Coulter Channelizer. The mean PAIgG was 4.7 fg IgG/platelet in 30 controls, 6.6 fg IgG/platelet in 15 patients with nonimmune thrombocytopenia, and 41.6 fg IgG/platelet in 32 patients with immune thrombocytopenia. Only in the immune thrombocytopenic patients was there a correlation between platelet count and PAIgG ($r = 0.66, p < 0.001$); however, there was no correlation between the PAIgG and either of the two parameters of platelet volume. Furthermore, all 4 patients with nonimmune thrombocytopenia who had an increase percentage megathrombocytes (20%-36%) had normal levels of PAIgG. These results indicate that the elevated levels of PAIgG in patients with immune thrombocytopenia is specific for this disorder and not due to passive absorption of IgG to megathrombocytes, which are present in increased proportions in these conditions.

DIOPATHIC THROMBOCYTOPENIC PURPURA (ITP) is a disorder caused by autoimmune-mediated platelet destruction of unknown etiology. There is evidence from studies in vivo and in vitro that antibodies coat these platelets and is responsible for their rapid removal by the reticuloendothelial system. Demonstration of these antibodies by testing in vitro has been surprisingly difficult. In general, two types of assays for platelet autoantibodies have been used, those based on the disruptive effects of the patient’s serum (presumably immunoglobulins) on nonautologous platelets and those that specifically assay the immunoglobulins on the patient’s platelets.

Although more complex, the direct methods for quantitating antibodies on the platelet’s surface are more sensitive and specific than the serum assays. These direct assays quantitate the concentration of surface-bound IgG contributed to both by platelet antibodies that bind specifically to the platelet membrane and by nonspecific IgG that remains passively absorbed to the membrane after the platelets are washed in preparation for their testing in vitro. This nonspecifically absorbed IgG could potentially produce an artifact leading to falsely high levels of platelet-associated IgG (PAIgG) in patients with increased numbers of large platelets (megathrombocytes), since these megathrombocytes have a large surface area to which the IgG could be absorbed. This consideration is of practical
importance because the increased platelet production in immune thrombocytopenia results in an increased number of megathrombocytes that could contribute nonspecifically to the increased concentrations of PAIgG documented in these conditions.

Using the method of Dixon and Rosse for directly assaying PAIgG we studied patients with immune and nonimmune thrombocytopenia as well as control groups of normal individuals and hospitalized patients and correlated these levels with platelet volume and the percentage of megathrombocytes.

To enhance the reliability of the assay, we developed a method for producing a highly reproducible positive control that could be stored for weeks and assayed each time a sample was tested.

**MATERIALS AND METHODS**

**Patients**

Platelets were studied from the following groups: (1) healthy adult volunteers with normal platelet counts who were not taking any medication (normal control); (2) hospitalized patients who had no hematologic abnormality but who were taking a variety of medication; (3) patients with thrombocytopenia secondary to a variety of “nonimmune” disorders (Table I); and (4) patients with thrombocytopenia of a presumed immune origin. Two groups of patients with immune thrombocytopenia were studied, those with ITP and those with systemic lupus erythematosus (SLE). Patients were classified as having 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. Some of these patients were being treated with corticosteroids or immunosuppressive agents, and some had had a splenectomy. Patients with SLE had thrombocytopenia and both clinical and serologic evidence of SLE.

**Assay of PAIgG**

1. **Preparation of IgG-coated sheep cells.** Sheep cells were washed three times in 0.9% NaCl, and 1 ml of the packed cells was added to 0.5 ml stock IgG solution (14 mg/mL) (Sigma Chemical, St. Louis, Mo.). To this was added 0.14 ml 0.8% glutaraldehyde. After 45 mm incubation at 37°C, the cells were washed and diluted to a final concentration of 4.4 X 10^9 cells/mL in veronal-buffered saline (VBS). These cells were stable for approximately 1 wk.

2. **Standardization of anti-IgG.** Anti-human IgG was produced from sheep injected with pooled human IgG that had been purified by column chromatography with DEAE-cellulose and Sephadex G200 (Pharmacia Fine Chemicals, Dorval, Quebec). The specificity of the anti-IgG was verified by immunodiffusion and immunoelectrophoresis. The prime reactivity of the antisera was to the Fc fragment of the IgG. Dilutions of the anti-IgG was made in VBS solution; then 0.1 ml of each dilution was added to 0.1 ml of the IgG-coated sheep cells. After a 30-mm incubation at 37°C, 0.2 ml of a 1:20 dilution of guinea pig complement (GIBCO, Grand Island, N.Y.) was added to each dilution. After a further 30-mm incubation at 37°C, the reaction was terminated with the addition of 5 ml VBS. The samples were centrifuged for 10 mm, and the optical density (OD) of the supernatant was read at 412 nm.

**Table 1. Nonimmune Thrombocytopenia**

<table>
<thead>
<tr>
<th>Etiology</th>
<th>Number</th>
<th>Mean Platelet Count (per μl)</th>
<th>Mean PAIgG (fg IgG/Platelet)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aplasia</td>
<td>4</td>
<td>71.1</td>
<td>53.0</td>
</tr>
<tr>
<td>Leukemia</td>
<td>4</td>
<td>71.1</td>
<td>53.0</td>
</tr>
<tr>
<td>Myelodysplastic</td>
<td>2</td>
<td>23.5</td>
<td>23.5</td>
</tr>
<tr>
<td>Hypersplenism</td>
<td>3</td>
<td>61.6</td>
<td>61.6</td>
</tr>
<tr>
<td>Multiple myeloma</td>
<td>1</td>
<td>88.0</td>
<td>88.0</td>
</tr>
<tr>
<td>After surgery (multiple transfusions)</td>
<td>1</td>
<td>68.0</td>
<td>68.0</td>
</tr>
</tbody>
</table>

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nm on a spectrophotometer (Gilford Labs, Oberlin, Ohio). The percentage lysis of the sheep cells was calculated as

\[
\text{Percentage lysis} = \frac{\text{OD test} - \text{OD background}}{\text{OD 100\%} - \text{OD background}} \times 100.
\]

OD background was the lysis produced by the addition of complement only, and OD 100\% was calculated by substituting water for the anti-IgG and complement.

**Standard curve for IgG.** A 1:10,000 dilution of stock IgG (100 mg/ml) was prepared, and varying dilutions of this solution were incubated with the standardized anti-IgG for 30 min at 37\(^\circ\)C. Then 0.1 ml IgG-coated sheep cells was added to each mixture and incubated for 30 min at 37\(^\circ\)C; 0.2 ml complement (1:20) was then added to each and incubated for 30 min at 37\(^\circ\)C. To stop lysis 5 ml VBS solution was added. After centrifugation, the OD was read at 412 nm. Percentage inhibition was calculated as

\[
\text{Percentage inhibition} = \frac{\text{OD sample} - \text{OD background}}{\text{maximum OD with anti-IgG} - \text{OD background}}.
\]

A standard curve was plotted as ng IgG versus percentage inhibition, on semilog paper. Only the linear part of the curve was used for calculations (Fig. 1).

**Testing platelets.** Blood (20 ml) was drawn into a plastic tube containing 0.3 ml 10% EDTA solution. Platelet-rich plasma was prepared by centrifugation at 240 g for 10 min at 22\(^\circ\)C in a small RC 3 centrifuge (Dupont Industries, Newtown, Conn.). The platelet pellet was then isolated by centrifugation at 2230 g for 15 min at 22\(^\circ\)C, and the platelets were washed twice with 0.015 M EDTA and then once in saline. The platelets were counted in a Coulter counter and resuspended in saline to a final concentration of 200,000–600,000/ml. The test was then carried out in the same way as the standard curve for IgG except that the platelet dilutions were substituted for the IgG dilutions.

**Preparation of a positive control.** A pooled sample of platelets was washed twice in 0.015 M EDTA and then in saline. Equal amounts of platelet suspension (500,000/\mu l) and a 2% formalin solution were mixed and left to stand 12–24 hr at 4\(^\circ\)C. The platelets were then washed twice with saline and resuspended in saline. Then 10 ml IgG (5 mg/ml) was added to 10 ml platelet suspension (500,000/\mu l) at 37\(^\circ\)C for 1 hr. The platelets were then washed twice with 0.015 M EDTA solution, washed one more time in saline, and resuspended in saline and kept at 4\(^\circ\)C.

**Determination of Platelet Size**

Blood samples were collected in vacutainer tubes (Becton-Dickinson, Rutherford, N.J.) containing EDTA. The platelets were counted and sized within 2 hr after collection. After sedimentation at 1 g for

![Fig. 1. Typical standard curve determined for each group of test samples.](image-url)
30 min, 3.3 µl PRP was diluted in 10 ml Isoton (Coulter Electronics, Hialeah, Fla.). Platelet volume distributions were determined on a Coulter Channelizer model 1000: The machine was standardized with 2.02- and 3.4 µm latex particles (Coulter). The window width was set at 100, allowing particle sizing from 1.4 to 28 fl. Each window was made to equal 0.27 fl. Using a base channel threshold of 4, electrical noise was minimized. One hundred channels size platelets according to volume. After one thousand platelets are counted within one channel (the mode), a histogram is produced. From this the median platelet volume is determined, and the percentage of megathrombocytes was calculated. A megathrombocyte is defined as a platelet greater than 13 fl in volume,4,15 and this corresponded to those platelets counted from windows 45–100.

Statistical Analysis

Statistical analysis was carried out by least-squares linear regression and product moment correlation.

RESULTS

The results of a typical IgG standardization curve is shown in Fig. 1. Only the linear part of the curve is used for calculations. To standardize the reading of results, the platelet counts of each platelet dilution were plotted against its inhibition on semilog paper and the percentage inhibition read at a platelet count of 100,000/µl. The percentage inhibition was then used to calculate the concentration of IgG per platelet from the standard curve.

The mean (± 1 SD) PAIgG for 30 healthy controls was 4.7 ± 3.1 fg IgG/platelet. Accordingly, the normal range of PAIgG was defined as 0–14 fg IgG/platelet (mean ± 3 SD). The mean PAIgG (± 1 SD) of 10 hospitalized nonthrombocytopenic controls was 5.35 ± 2.8 fg/platelet. The mean PAIgG (± 1 SD) of 30 consecutive positive controls was 32.5 ± 10.7 fg IgG per platelet. This concentration of PAIgG in the positive controls remained constant for up to 30 days of storage. The level of PAIgG in 15 patients with nonimmune thrombocytopenia was 6.6 fg IgG/platelet, and the mean platelet count was 62,133/µl (Table 1, Fig. 2). The mean (± 1 SD) PAIgG in 32 patients with immune thrombocytopenia (24 with ITP, 8 with SLE) was 41.6 ± 32 fg/platelet and the mean platelet count was 49,726/µl; all but 3 had elevated levels of PAIgG. There was an inverse
correlation between the antibody level and platelet count ($r = 0.66, p < 0.001$; \(y = -4.3x + 60.8\); Fig. 3).

The percentage megathrombocytes in 108 healthy individuals was $13\% \pm 6.8\%$ (mean $\pm 1$ SD). Platelet-associated IgG and the percentage of megathrombocytes were determined on aliquots of the same blood sample in 55 healthy or hospitalized controls, 11 patients with nonimmune thrombocytopenia, and 13 patients with immune thrombocytopenia (Table 2). There was no correlation between the percentage megathrombocytes or median platelet volume and the PAIgG in any of these groups (Table 2). The patients with immune thrombocytopenia had a higher mean percentage of megathrombocytes (31%) and a higher mean PAIgG (32 fg IgG/platelet) than either of the other two groups. These patients showed a significant inverse relationship between platelet count and percentage megathrombocytes.

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**Table 2. Relationship Between Platelet Size and Platelet-Associated IgG**

<table>
<thead>
<tr>
<th>No.</th>
<th>Platelet Count (Mean ± 1 SD) (per μl)</th>
<th>Platelet Volume (Mean ± 1 SD) (per fl)</th>
<th>Percentage Megathrombocytes (Mean ± 1 SD)</th>
<th>PAIgG (Mean ± 1 SD) (fg IgG/Platelet)</th>
<th>Correlation Between Percentage Megathrombocytes and PAIgG*</th>
<th>Correlation Between Median Platelet Volume and PAIgG*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>55</td>
<td>287.158 ± 69.400</td>
<td>7.35 ± 1.1</td>
<td>15.73 ± 4.71</td>
<td>8.6 ± 3.9</td>
<td>0.06</td>
</tr>
<tr>
<td>Immune thrombocytopenia</td>
<td>17</td>
<td>59.530 ± 38.590</td>
<td>9.05 ± 3.5</td>
<td>31.3 ± 18.8</td>
<td>32.3 ± 17.2</td>
<td>0.22</td>
</tr>
<tr>
<td>Nonimmune</td>
<td>13</td>
<td>72.857 ± 41.057</td>
<td>6.79 ± 1.71</td>
<td>20.2 ± 5.9</td>
<td>7.5 ± 2.3</td>
<td>0.07</td>
</tr>
</tbody>
</table>

* All correlations not significant.
bocytes \( (r = 0.50, p < 0.01; y = -x + 85.8) \) and between platelet count and PAIgG \( (r = 0.53, p < 0.01; y = -0.6x + 76.7) \). The 13 patients with nonimmune thrombocytopenia also had a significant inverse relationship between platelet count and percentage megathrombocytes \( (r = 0.47, p < 0.05; y = -2.6x + 126.3) \); however, unlike the patients with immune thrombocytopenia, they showed no relationship between platelet count and PAIgG \( (r = 0.19) \). This group of patients included 4 patients with an elevated percentage megathrombocyte count (20%–36%); 2 of these patients had myelodysplastic disorders, 1 had thrombocytopenia complicating malignant hypertension and 1 had hypersplenism. The inverse relationship between platelet count and percentage megathrombocytes is shown in
Fig. 4 for both thrombocytopenic groups. The lack of relationship between PAIgG and percentage megathrombocytes for either group is illustrated in Fig. 5. A similar lack of relationship between PAIgG and median platelet volume was also noted.

DISCUSSION

The results of this study confirmed the report by Dixon and Ross that the direct assay of platelet-bound IgG is both sensitive and specific for immune thrombocytopenia. With this method, patients with immune thrombocytopenia are clearly separated from normal controls, hospital controls, and patients with nonimmune thrombocytopenic disorders. In five patients who were followed serially during treatment, the antibody level fell at the same time or before a rise in platelet count was obtained in response to therapy, indicating that the IgG bound to the platelet surface was causally related to the thrombocytopenic state. Three patients with clinical features consistent with ITP had normal levels of antibody despite severe thrombocytopenia. It is of interest that two of these patients did not respond to treatment with steroids and splenectomy, and it is possible the immune thrombocytopenia in these patients was mediated through IgM or IgA autoantibodies.

A potential major criticism of all of the direct assays for platelet antibodies is that the immunoglobulins are determined per unit number of platelets and therefore could show falsely high levels of IgG due to passive adherence of IgG to megathrombocytes, which are known to be present in increased numbers in immune thrombocytopenia.

A significant inverse correlation was found between platelet count and percentage megathrombocytes in both immune and nonimmune thrombocytopenia; however, the PAIgG was correlated with the platelet count only in those patients who had immune thrombocytopenia. In no group was there any correlation between PAIgG and either parameter of platelet size (percentage megathrombocytes, median platelet volume). Since numerous factors can influence platelet size and volume, it is important to consider how these might affect our results.

The blood for platelet studies was taken into EDTA (final concentration 1.5 mg/ml). At this concentration EDTA produces some degree of platelet swelling, which, however, would be expected to affect equally platelets from patients with immune and nonimmune thrombocytopenia. The platelet volume distribution measurements were performed on platelet-rich plasma prepared from sedimented blood, while the platelet-bound IgG levels were measured on washed platelets prepared from platelet-rich plasma obtained by centrifugation of blood for 10 mm at 22°C. It is likely that a small number of large dense and small dense platelets would have been lost during the initial centrifugation procedure. However, this is unlikely to invalidate the interpretation of our results, since a clear difference was found in the levels of PAIgG between the nonimmune thrombocytopenic and the immune thrombocytopenic patients. Furthermore, this difference was just as marked when the immune group were compared with those nonimmune thrombocytopenic patients who showed an increase in the percentage megathrombocytes or the median platelet volume (Fig. 5).

These findings plus the lack of correlation between megathrombocytes and PAIgG in patients with immune thrombocytopenia indicate that the elevated levels...
of platelet-bound antibody in ITP are not caused by an increase in the percentage of megathrombocytes and are likely to be specifically related to the immune thrombocytopenia.

REFERENCES

The direct assay for platelet-associated IgG (PAIgG): lack of association between antibody level and platelet size

JG Kelton, PB Neame, J Bishop, M Ali, J Gauldie and J Hirsh