Platelet-associated IgG in Immune Thrombocytopenic Purpura

By George A. Luiken, Robert McMillan, Alton L. Lightsey, Pam Gordon, Sally Zevely, Irving Schulman, T. John Gribble, and Robert L. Longmire

A method for the measurement of immunoglobulin G associated with gel-filtered platelets is described and findings in 70 control subjects and 37 patients with immune thrombocytopenic purpura (ITP) are reported. Control platelet-associated IgG (PAIgG) levels (nanograms IgG per $10^9$ platelets) averaged ($\pm$ SD) 1231 $\pm$ 424; samples studied after 24 and 48 hr remained within the control range. PAIgG values of 19 adult and 12 childhood patients with chronic ITP averaged 4711 $\pm$ 3025 and 4923 $\pm$ 3955, respectively, and differed significantly from controls ($p < 0.001$). There was an inverse correlation between PAIgG values and the chronic ITP patient’s platelet count. Six patients with childhood acute ITP had PAIgG levels ranging from 5588 to 56,250 and appeared to represent a different statistical population from those with chronic ITP. In chronic ITP patients responding to splenectomy, there was an immediate normalization of PAIgG levels; however, a certain percentage of patients studied several months after splenectomy evidenced elevated PAIgG levels in association with normal platelet counts. These data showed that the direct measurement of platelet-associated antibody is a useful technique in the diagnosis and follow-up of patients with chronic ITP. Preliminary studies in patients with acute ITP have suggested that this method may be useful in differentiating acute and chronic childhood ITP.

Patients with chronic immune thrombocytopenic purpura (ITP) have a circulating antiplatelet factor which in most cases is an immunoglobulin of IgG type. Although the presence of antiplatelet antibody in ITP has been clearly shown using a wide variety of techniques, a clinically useful quantitative blood test is not readily available. Multiple serum assays have been described, but difficulty has been experienced either with their insensitivity, complexity, or reproducibility. Since antibody should be antigen bound, the most logical approach in this disease is the measurement of platelet-associated antibody. This approach was first applied in the early 1960s by two European groups. Using a direct antiglobulin consumption test, they showed increased levels of platelet-associated globulins in 59 of the 116 patients who were tested. In 1975, Dixon and co-workers reported increased levels of platelet surface IgG as measured by a complement lysis inhibition technique; the IgG values appeared to correlate with the patient’s clinical course.

The present report describes the results obtained in our laboratory using the Fab-anti-Fab IgG assay technique to measure platelet-associated IgG (PAIgG)
in control subjects and in patients with ITP. These results suggest that this test will be useful in the diagnosis and follow-up of patients with ITP.

MATERIALS AND METHODS

Blood was obtained with appropriate informed consent from 70 hematologically normal control subjects, 10 patients with thrombocytopenia of other causes, and 37 patients with ITP (19 adults with chronic ITP, 12 children with chronic ITP, 6 children with acute ITP).

Venous blood containing at least 2 x 10^8 platelets was drawn into acid-citrate-dextrose (blood to ACD-A, 5 volumes to 1) and centrifuged for 7 min at 400 g. The volume of blood required ranged from 10 to 100 ml depending on the platelet count; in no instance did the blood removed constitute more than 3% of the calculated blood volume. The platelet-rich plasma was aspirated and centrifuged for 15 min at 1000 g. The platelet button was resuspended in 1.0 ml of 0.05 M citrate buffer (pH 6.2) and a platelet count was determined on a Coulter Counter. The platelet suspension was washed by passage through a Sepharose 2B column using a modification of the method of Tangen and co-workers.3

The agarose beads were washed three times with distilled water and once with normal saline and were degassed by exposure to a vacuum (14 cm Hg) for 30 min. A thick slurry was made in saline and poured into a 2.5 x 30 cm previously siliconized glass column (Pharmacia, containing a 40 g outlet filter). After equilibration of the column with citrate buffer, the platelet suspension was layered on the gel surface. After attachment of a buffer reservoir, the platelet suspension was washed with passage through the column at a flow rate of 20-25 ml/hr and the first 6 ml of the platelet-rich effluent were collected. A platelet count and leukocyte count were determined and exactly 5 ml were ultracentrifuged (36,000 g, 20 min). The platelet button was resuspended in 4 ml of saline and lysed by freezing and thawing.

PAIgG was determined using the Fab-anti-Fab assay system. The principles, specificity, and details of this system and its application to the assay of platelet IgG have been previously reported in detail.2,8,14,15 Briefly, the assay depends on the fact that the antigen, human Fab fragments, is soluble in 50% saturated ammonium sulfate (SAS), while antigen complexed to rabbit anti-human Fab precipitates. This difference allows the separation of free from bound antigen. The anti-Fab is standardized by dilution in 10% normal rabbit serum, so that 0.5 ml of antibody solution precipitates 50% of the radioactivity in 0.5 ml of 125I-Fab containing 20.0 ng nitrogen. The ability of the platelet or leukocyte samples to inhibit this reaction is compared to that of known amounts of human IgG.

All procedures were performed at 5°C. On the first day, 0.5 ml of the standardized anti-Fab was incubated for 24 hr with 0.5 ml of the material to be assayed. Triplicate samples of serial dilutions of the platelet or leukocyte samples were assayed, as well as serial dilutions of purified human IgG (0.8-50.0 ng nitrogen). If the study material has antigenic determinants recognized by the antibody, fewer antibody molecules remain available and proportionally less 125I-Fab is precipitated. On day 2, 0.5 ml of 125I-Fab, containing 20.0 ng nitrogen, is added and the mixture is incubated an additional 24 hr. On the third day, 1.5 ml of SAS are added to each tube and incubated for 30 min. After centrifugation for 30 min at 850 g, the supernatant fluid is discarded and the precipitate is washed once with 3 ml of 50% SAS and recentrifuged. The radioactivity of the precipitate is determined and the percentage of 125I-Fab precipitated is calculated as previously described.14 An IgG inhibition curve is constructed by plotting the percentage 125I-Fab precipitated against the amount of IgG added. The platelet concentration and the IgG dilution that cause blocking at the 25% level (the center of the linear portion of the inhibition curve) are determined and the PAIgG is calculated and expressed as nanograms IgG per 10^9 platelets.

In selected instances (10 control subjects and thrombocytopenic patients with platelet counts less than 20,000/cu mm), leukocyte-associated IgG was determined. A leukocyte-rich fraction was prepared by dextran sedimentation and washed four times with citrate buffer. After lysis, the leukocyte-associated IgG was determined in an identical manner as that described above for the platelets.

RESULTS

Preliminary studies using labeled serum proteins suggested that washing by gel filtration was both faster and more complete than mechanical washing.
Platelet suspensions from five normal subjects were washed in parallel by either gel filtration or by five separate washes with citrate buffer followed by centrifugation. In each instance PAIgG values were lower in the gel-filtered platelets, indicating a more adequate wash.

To evaluate the feasibility of applying this assay to transported specimens, the stability of PAIgG levels was evaluated. Platelet-rich plasma from normal subjects was divided into three aliquots, which were processed either immediately or after incubation at 5°C for 24 or 48 hr. The results of eight studies using ACD-A are shown in Table 1. The mean differences between the immediate and the 24- and 48-hr samples were +4.2% (range +3.7% to −28.1%) and −12.6% (range +38.3% to −51.8%), respectively. In every instance the PAIgG levels remained within the normal range. Results obtained using EDTA-anticoagulated blood were similar if processed immediately, but they varied considerably after incubation for more than a few hours.

Control Subjects

PAIgG was measured in 70 normal control subjects. The mean level (± SD) was 1231 ± 424 ng IgG/10⁹ platelets with a range of 356–1956. The percentage variation of replicate platelet samples averaged 13.0% ± 9.5% (25 observations); the results were not affected by the number of platelets assayed. The effect of leukocyte contamination was evaluated in 10 control subjects. The mean percentage of the total assay IgG due to the contaminating leukocytes was 0.8% ± 1.2%. Similar values were noted in thrombocytopenic subjects. In no case did the contaminating leukocytes contribute more than 4% of the total IgG in the test suspensions.

Several of the control subjects were studied on multiple occasions, and although considerable day-to-day variation occurred, the values were always within the control ranges. Of the 10 thrombocytopenic control subjects, 8 had PAIgG values within the normal range (4 patients with solid tumors on chemotherapy, 2 patients with chronic lymphocytic leukemia who had marrow infiltration, 1 patient with drug-induced marrow aplasia, and 1 patient with myeloproliferative syndrome). Two patients with acute granulocytic leukemia who had recently received extensive platelet transfusions showed moderately elevated levels.
Patients With Immune Thrombocytopenia

Of the 31 patients with chronic ITP who were studied, 25 were on high-dose prednisone therapy at the time of study. PAIgG values of 19 adults and 12 children with chronic ITP averaged 4711 ± 3025 and 4923 ± 3955 ng IgG/10⁹ platelets respectively. The difference between the mean PAIgG levels of either adult or childhood chronic ITP patients and control subjects was highly significant. Of the 31 with chronic ITP, values for 29 were greater than 2 SD above control values. Patients with platelet counts of less than 50,000/μl in every case had levels greater than 3 SD above mean control values.

The relationship between PAIgG values and the patient’s platelet count is shown in Fig. 1. Adult and childhood ITP values were evaluated together since their mean and standard deviations were almost identical. A significant correlation was noted (r = 0.506; t = 3.66; p < 0.001). In two subjects, PAIgG values were much greater than those of the others (15,043 and 16,800 ng IgG/10⁹ platelets). If these two patients are excluded from the analysis, the regression line appears more representative (Fig. 1) and the correlation is improved (r = 0.612).

Six patients with acute childhood ITP were studied during the course of their thrombocytopenia; all showed elevated PAIgG levels, ranging from 5588 to 56,250 (Table 2). If these values are plotted in the identical manner shown in

<table>
<thead>
<tr>
<th>Patient</th>
<th>Platelet Count (per μl)</th>
<th>PAIgG (ng IgG/10⁹ platelets)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2,200</td>
<td>10,312</td>
</tr>
<tr>
<td>2</td>
<td>3,000</td>
<td>30,556</td>
</tr>
<tr>
<td>3</td>
<td>12,000</td>
<td>56,250</td>
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<td>4</td>
<td>25,000</td>
<td>5,588</td>
</tr>
<tr>
<td>5</td>
<td>30,000</td>
<td>6,531</td>
</tr>
<tr>
<td>6</td>
<td>78,000</td>
<td>5,868</td>
</tr>
</tbody>
</table>
AII patients were at least 6 mo postsplenectomy.

Fig. 1. only one of the six falls within the standard error of the estimate (SY.X = 1235), suggesting that acute ITP patients represent a different statistical population, possibly reflecting a different mechanism of immune destruction. Each of the acute childhood ITP patients went into spontaneous remission. Four were restudied after remission, and normal PAIgG values were seen.

Effect of Therapy

The immediate effect of splenectomy on PAIgG levels has been studied in six patients with chronic ITP (Fig. 2). In five, the platelet count normalized within 1 wk of surgery; this was associated with a return of PAIgG levels into the normal range. One patient developed subsequent thrombocytopenia and his PAIgG level again became abnormal. The sixth patient, who was severely thrombocytopenic before surgery, responded only partially to splenectomy.

Table 3. Long-Term Effect of Splenectomy on PAIgG Values in Patients with Chronic ITP*

<table>
<thead>
<tr>
<th>Condition</th>
<th>Platelet Count (per μl)</th>
<th>PAIgG (ng IgG/10^9 platelets)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical remission</td>
<td>250,000</td>
<td>938</td>
</tr>
<tr>
<td></td>
<td>350,000</td>
<td>856</td>
</tr>
<tr>
<td></td>
<td>430,000</td>
<td>1,822</td>
</tr>
<tr>
<td></td>
<td>300,000</td>
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</tr>
<tr>
<td></td>
<td>250,000</td>
<td>1,369</td>
</tr>
<tr>
<td></td>
<td>320,000</td>
<td>1,742</td>
</tr>
<tr>
<td>Thrombocytopenic</td>
<td>10,000</td>
<td>14,206</td>
</tr>
<tr>
<td></td>
<td>20,000</td>
<td>3,688</td>
</tr>
<tr>
<td></td>
<td>35,000</td>
<td>3,381</td>
</tr>
<tr>
<td></td>
<td>6,000</td>
<td>1,582</td>
</tr>
<tr>
<td></td>
<td>45,000</td>
<td>1,543</td>
</tr>
</tbody>
</table>

*All patients were at least 6 mo postsplenectomy.
†Normal values: 1231 ± 424 ng IgG/10^9 platelets.
Her platelet count increased from 1000 to 85,000/μl with a concurrent reduction in her PAIgG levels from 16,800 to 3,300 ng IgG/10⁹ platelets.

The long-term effect of splenectomy has been evaluated in 13 patients—8 who achieved a complete clinical remission and 5 who remained thrombocytopenic (Table 3). All had been splenectomized at least 6 mo prior to study (6 mo to 6 yr). In patients who had attained a complete remission, 6 had normal and 2 had abnormal PAIgG values; 1 of the latter relapsed clinically 6 mo later. Of the thrombocytopenic patients, 3 had elevated levels as expected but, surprisingly, 2 patients had repeatedly confirmed normal values.

Two ITP patients were studied during vincristine therapy (Fig. 3). Blood was obtained prior to treatment and at weekly intervals for 3 wk. One patient whose PAIgG levels remained elevated during therapy showed no response to vincristine. The second patient's PAIgG levels began to decrease after the second vincristine injection and were within the normal range by the third week. This change was associated with an increase in his platelet count from 25,000 to 185,000/cu mm.

**DISCUSSION**

These studies suggest that direct quantitation of PAIgG provides a useful clinical tool for the diagnosis and follow-up of ITP patients. The technique is quantitative, reproducible, and applicable to the study of transported specimens. As previously reported, platelets from normal subjects contain a large quantity of IgG when compared to red cells.⁸⁻¹⁴ This fact may explain the difficulty experienced by earlier investigators in the use of nonquantitative immunologic tests in the evaluation of antiplatelet antibodies. The nature of this IgG on normal platelets is unknown but it resists multiple washes and is sensitive to trypsin. Approximately one-half is expressed on the surface of intact platelets, with the remainder becoming manifest after platelet lysis.⁵

The PAIgG levels of chronic ITP platelets differ significantly from control values obtained from either normal subjects or from patients with thrombocytopenia of other causes. There is an inverse correlation between the PAIgG levels of chronic ITP patients and their platelet counts. In every case, platelet counts of less than 50,000/cu mm are associated with significantly increased PAIgG values (>3 SD). It is possible that the PAIgG levels of many of these patients may have been higher if specimens had been studied prior to cortico-
steroid therapy. Dixon and co-workers have noted a decrease in platelet surface IgG in their patients in conjunction with steroid therapy.\textsuperscript{11,12} Unfortunately, most of our patients had been on corticosteroids at the time of the study.

It is assumed that the difference between the PAIgG levels of chronic ITP patients and control subjects reflects the antiplatelet antibody bound to the circulating platelets. The similarities between the magnitude of these differences and antigen-saturating quantities of platelet-binding IgG, as determined from ITP spleen cultures,\textsuperscript{2} support this thesis. Additional evidence is provided by the inverse relationship between the patient's platelet count and the PAIgG levels. If the increase in PAIgG seen in ITP patients reflects platelet-bound antibody, these results provide evidence that the quantity of antibody bound to the platelet is directly related to the magnitude of platelet destruction and the severity of the patient's disease.

However, another possible explanation of these findings must be considered—platelet size. If platelets released during periods of increased marrow production are larger in volume and surface area, as suggested by the studies of most investigators,\textsuperscript{17,18} they may contain proportionally more IgG. Evidence against platelet size as a significant factor in these studies is the observation that PAIgG levels in ITP patients may normalize within 24-48 hr after splenectomy—at a time when large numbers of large platelets should be circulating.

The immediate effect of splenectomy on patients responding to this form of therapy was normalization of PAIgG values, probably due to the abrupt removal of one large antiplatelet antibody production site.\textsuperscript{2} Since a certain percentage of ITP patients in long-term clinical remission have evidence of continued antiplatelet antibody production and platelet destruction,\textsuperscript{1,4,19} it was anticipated that elevated PAIgG levels would also be seen in some of these patients. This observation was noted in two of eight such patients; both had normal platelet counts in association with distinctly abnormal PAIgG values.

Of the five ITP patients who were thrombocytopenic when studied several months after surgery, two had normal PAIgG results. Both patients were elderly men (75 and 85 yr old) with extremely low platelet counts prior to surgery. No improvement after surgery was noted in one and only a transient increase in the platelet count was seen in the other. Several explanations could account for these observations: the thrombocytopenia was not due to an antiplatelet antibody; the platelet depression resulted from adsorbed antigen or antigen-antibody complexes which eluted during the wash procedure; or the antibody was IgM or IgA, which were not assayed by our method. Further studies are required on such patients to determine the pathogenesis.

The results noted in two patients on vincristine therapy suggest that, in producing a response, this drug in some way affects the quantity of platelet-bound antibody. Whether this is due to depression of antibody synthesis, interference with antibody binding to the platelet, or stimulation of thrombopoiesis resulting in less available antibody per platelet cannot be determined by these studies.

The observations on childhood ITP are of interest. The range of PAIgG values in children with chronic ITP is similar to that in adults. On the other hand, children with acute ITP appear to have PAIgG levels which are much higher for a given platelet count. These data not only provide evidence for an immune etiology in acute childhood ITP, but also suggest that this method may provide
a means of distinguishing children with self-limited acute ITP from those who will manifest the chronic form. If further studies prove this to be the case, this determination would provide a powerful tool in the evaluation of childhood ITP. The differences in magnitude between acute and chronic ITP values also suggest a difference in the mechanism involved in platelet destruction. Since many of these children develop thrombocytopenia several days following a viral illness, it is tempting to postulate that circulating complexes develop involving viral antigens, which then are adsorbed onto the platelet surface with subsequent removal of both the complexes and the platelets by the reticuloendothelial system. The spontaneous recovery seen in these patients and the normalization of PAIgG values probably reflect the eradication of the infection by the child’s immune system with removal of the antigen.

The results in patients with chronic ITP confirm and extend those reported by Dixon and co-workers. The ability of the two laboratories using different assays to obtain a positive result in essentially all untreated patients with ITP is important and suggests that the direct measurement of platelet-bound antibody is preferable to the evaluation of ITP sera. The advantages are (1) a high percentage of positive results, (2) quantitative data, and (3) correlation of the data with the patient’s clinical picture. Further studies are required to evaluate the potential of this assay in predicting the response of chronic ITP patients to therapy and in differentiating between acute and chronic childhood ITP.

It is anticipated that this type of assay will be of considerable importance in the further study of the pathogenesis of the immune thrombocytopenias, particularly if performed in concert with the study of platelet kinetics, antibody synthesis, and phagocytic mechanisms.

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REFERENCES

7. Aster RH, Enright SE: A platelet and granulocyte membrane defect in paroxysmal
PLATELET-ASSOCIATED IgG IN ITP


Platelet-associated IgG in immune thrombocytopenic purpura

GA Luiken, R McMillan, AL Lightsey, P Gordon, S Zevely, I Schulman, TJ Gribble and RL Longmire