Platelet Surface-Bound IgG in Patients With Immune and Nonimmune Thrombocytopenia

By Wayne S. Court, Joyce M. Bozeman, Seng-jaw Soong, Mansoor N. Saleh, Denise R. Shaw, and Albert F. LoBuglio

We quantitated the amount of platelet surface-bound IgG using an 125I monochlonal anti-IgG assay in 149 patients with thrombocytopenia and 260 normal donors. The normal subjects had 122 ± 5 molecules of IgG/platelet (mean ± SE). Fifty-five patients with nonimmune thrombocytopenia had 338 ± 37 molecules of IgG/platelet, whereas 67 patients with immune thrombocytopenia studied at the time of their initial evaluation had 4,120 ± 494 molecules of IgG/platelet. An analysis of the distribution of values in these two groups indicated that 90% of the patients with immune thrombocytopenia had >800 molecules of IgG/platelet, whereas only 7% of patients with nonimmune thrombocytopenia exceeded this amount. The immune thrombocytopenic patients included 39 idiopathic, 14 secondary, and 14 drug-induced disorders, and they did not significantly differ in their distribution of values for platelet IgG. The nonimmune thrombocytopenic patients included 12 cases with a platelet destructive mechanism; their platelet-bound IgG was similar to that of the other nonimmune patients. Twenty-seven patients with treatment-resistant immune thrombocytopenia were also studied; they had 2,100 ± 670 molecules of IgG/platelet. Their values were significantly greater than those of the nonimmune thrombocytopenic patients and not significantly different from those of immune thrombocytopenic group. Their distribution of values was much broader, however, with 33% of patients having <800 molecules of IgG/platelet, suggesting possible alternate mechanisms in their thrombocytopenia. Thus, patients with immune thrombocytopenia have a high frequency of elevated IgG on the platelet surface which reflects the pathophysiology of this disorder. Quantitation of platelet-bound IgG provides a useful laboratory tool in the differential between immune and nonimmune thrombocytopenia.

IMMUNE thrombocytopenic purpura (ITP) in adults is a syndrome of shortened platelet survival with reticuloendothelial sequestration. This syndrome of accelerated platelet destruction appears to be mediated primarily by IgG antibody directed toward ill-defined platelet surface antigens.1,2 In the decade since Dixon and co-workers3 first documented elevated levels of platelet-bound IgG (PBlgG) in patients with ITP, a variety of assay techniques for measuring platelet IgG has been reported.4-13 These different assays use various methodologies and report elevated values for IgG associated with platelets in ITP. Elevated values have also been noted in thrombocytopenia presumed to be due to nonimmune mechanisms, however, resulting in the interpretation that such values are a nonspecific finding unrelated to the pathophysiology of immune platelet destruction.14,20 Recent studies have clarified the limitations imposed by conventional competitive binding assays, using calibration by soluble IgG standards21,22 and the potential value of a direct binding assay using stoichiometric relationships. We have previously characterized and described such an assay using monoclonal 125I anti-human IgG to quantify platelet surface-bound IgG.23,24 By means of this quantitative technique the amount of surface IgG displayed on the platelet surface in a large group of thrombocytopenic patients was analyzed.

MATERIALS AND METHODS

Assay for platelet surface-bound IgG. The preparation of monoclonal 125I anti-IgG reagent as well as the standard assay conditions are reported in detail elsewhere.24 This antibody was originally generated by J. Donald Capra and was purchased from Bethesda Research Laboratory (MD), but is no longer available from this source. More recently, the hybridoma producing this antibody is available through the American Type Culture Bank (ATCC-HB-43), and purified antibody is commercially available. (Southern Biotechnology Associates, Birmingham, AL, catalogue no. 9040). In brief, venous blood was collected in plastic syringes and anticoagulated with acid citrate dextrose A. This method allows shipment of whole blood on wet ice with minimal effect on platelet levels.25 The whole blood was centrifuged at 100 for 30 minutes, and the platelet-rich plasma was collected. The pH was adjusted to 6.5 with acid citrate dextrose A and then centrifuged at 1,750 g for 20 minutes. The platelet button was washed thrice in platelet-suspension buffer (Dulbecco’s phosphate-buffered isotonic saline containing 1 g/dL of bovine serum albumin (BSA) and 4 mmol/L of EDTA. pH 6.7). Ten million washed platelets were incubated with 300 ng of 125I-labeled mouse monoclonal anti-IgG in a total volume of 300 µL for 20 minutes at room temperature. Triplicate aliquots of 75 µL (2.5 x 10⁵ platelets) were transferred to Percoll gradient tubes containing 250 µL of isotonic 30% Percoll in saline, centrifuged at 7,500 g for 5 minutes, and the tube tip containing the platelet button was transected with a hemostat and razor blade into γ counting tubes. The number of 125I monoclonal antibody molecules bound per platelet was calculated on the basis of Avogadro’s number, the antibody’s specific activity, and mol wt. We previously showed that with these assay conditions the stoichiometry of the binding reaction approaches unity and that the “nonspecific” binding of the monoclonal anti-IgG is <5%.26 Thus, the number of molecules of platelet-bound 125I monoclonal antibody is equal to the number of molecules of platelet surface-bound IgG.24

Patient characteristics. One hundred forty-nine thrombocytopenic patients with at least three platelet counts <100,000/µL and 260 normal subjects comprised the study group. Fifty-seven of the patients were referred for evaluation at the time of initial diagnosis of immune thrombocytopenic purpura based on conventional clinical
criteria (adult onset thrombocytopenia with normal to increased megakaryocytes in the bone marrow and no other explanation for thrombocytopenia found). Of this group, 39 were classified as idiopathic, 14 were secondary (7 systemic lupus erythematosus, 3 rheumatoid arthritis, and single cases of lymphoma, infectious mononucleosis, Evans syndrome, and AIDS), and 14 were drug-induced (11 quinidine and single cases with reserpine, mezlocillin, and chlorpropamide). These patients were studied prior to therapy or after no more than 48 hours of initiation of corticosteroids.

Fifty-five patients had thrombocytopenia that was considered non-immune in origin, including nonimmune destructive thrombocytopenia in 12 patients (Table 1), aplastic anemia in 13, acute leukemia in 11, hypersplenism in 6, multiple myeloma in 3, metastatic cancer in 3, hereditary thrombocytopenia in 2, sepsis in 2, renal disease in 2, and paroxysmal nocturnal hemoglobinuria in 1 patient.

A third group of thrombocytopenic patients was classified as treatment-resistant ITP. These 27 patients had the criteria for idiopathic thrombocytopenic purpura, were treated extensively elsewhere, and were referred for evaluation because of persistent thrombocytopenia. All these patients had received weeks to months of corticosteroids, many had been splenectomized, and some had received immunosuppressive therapy.

Table 1. Platelet Counts and Surface-Bound IgG in 12 Patients With Nonimmune Destructive Thrombocytopenia

<table>
<thead>
<tr>
<th>Patient</th>
<th>Diagnosis*</th>
<th>Platelet Count (μL)</th>
<th>Molecules IgG/Platelet</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. J.H.</td>
<td>TTP</td>
<td>5,000</td>
<td>723</td>
</tr>
<tr>
<td>2. R.M.</td>
<td>TTP</td>
<td>5,000</td>
<td>158</td>
</tr>
<tr>
<td>3. T.H.</td>
<td>TTP</td>
<td>8,000</td>
<td>1,612</td>
</tr>
<tr>
<td>4. D.E.</td>
<td>TTP</td>
<td>9,000</td>
<td>136</td>
</tr>
<tr>
<td>5. M.G.</td>
<td>TTP</td>
<td>16,000</td>
<td>489</td>
</tr>
<tr>
<td>6. D.S.</td>
<td>TTP</td>
<td>18,000</td>
<td>149</td>
</tr>
<tr>
<td>7. I.G.</td>
<td>TTP</td>
<td>57,000</td>
<td>423</td>
</tr>
<tr>
<td>8. D.D.</td>
<td>TTP</td>
<td>63,000</td>
<td>211</td>
</tr>
<tr>
<td>9. R.M.</td>
<td>MHA</td>
<td>12,000</td>
<td>274</td>
</tr>
<tr>
<td>10. J.O.</td>
<td>DIC</td>
<td>29,000</td>
<td>341</td>
</tr>
<tr>
<td>11. J.K.</td>
<td>DIC</td>
<td>33,000</td>
<td>228</td>
</tr>
<tr>
<td>12. C.C.</td>
<td>DIC</td>
<td>73,000</td>
<td>239</td>
</tr>
</tbody>
</table>

* TTP, thrombotic thrombocytopenic purpura; MHA, microangiopathic hemolytic anemia; DIC, disseminated intravascular coagulation.

RESULTS

Our observations of platelet-bound IgG in various patients and normal groups is summarized in Table 2. In a group of 260 normal volunteers studied for a 2-year period, the mean amount of platelet-bound IgG was 122 ± 5 (mean ± SE) with a median of 92 molecules of IgG per platelet. The highest value recorded was 400 molecules of IgG per platelet. The 55 patients with nonimmune thrombocytopenia had higher mean (338 ± 37) and median (260 molecules per platelet) amounts of platelet-bound IgG that were significantly (P < .001) above that seen in normal controls by the Wilcoxon two-sample test (Table 3). There was a modest negative correlation of platelet count and platelet-bound IgG with a Spearman coefficient of correlation of −0.285 (P = .037).

As shown in Table 2, the patient groups of immune thrombocytopenia at the time of initial evaluation as well as the group of treatment-resistant immune thrombocytopenia had dramatically greater amounts of platelet-bound IgG than did either the normal donors or nonimmune thrombocytopenic patients. The patients with idiopathic, secondary, and drug-induced immune thrombocytopenia did not differ significantly from each other in regard to platelet-bound IgG values (Table 3). As a group or individually, however, they had significantly greater amounts of platelet-bound IgG than did patients with nonimmune thrombocytopenia or normal donors (Table 3).

Figure 1 examines the proportion of patients having various amounts of platelet-bound IgG. To differentiate between patients with immune thrombocytopenia v nonimmune, a cutoff value of 800 molecules of IgG per platelet provided the best sensitivity and specificity values. Sixty of 67 patients with immune thrombocytopenia at the time of initial evaluation had >800 molecules of IgG per platelet (sensitivity 90%), whereas 51 of 55 patients with nonimmune thrombocytopenia had <800 molecules of IgG per platelet (specificity 93%). Values of <400 molecules of IgG per platelet adequately describe the population of normal donors.

Table 2 provides a breakdown of all patient groups according to these cutoff values. Thus, 90% of patients with immune thrombocytopenia had platelet-bound IgG >800 molecules of IgG per platelet whereas 7% of nonimmune thrombocytopenic patients had values above this cutoff.

The group of patients with nonimmune thrombocytopenia included 12 patients with thrombocytopenia due to premature destruction of platelets (nonimmune) and presumably increased platelet production similar to the platelet kinetics...
that exist in immune thrombocytopenia. As listed in Table 1, eight of these patients had thrombotic thrombocytopenic purpura. Their distribution of platelet-bound IgG was similar to that of the other nonimmune thrombocytopenic patients, with 67% in the normal range (<400), 25% between 400 to 800, and 8% (one patient) >800 molecules of IgG per platelet. This patient had received large volumes of plasma prior to referral to our institution, which may have influenced the result at the time of our study.

The group of patients with treatment-resistant immune thrombocytopenia had all been extensively treated prior to referral to our institution. This is obviously a highly selected subgroup of immune thrombocytopenia. As a group, their platelet-bound IgG was significantly greater than that of nonimmune thrombocytopenic patients or normal donors (Table 3). Their amount of platelet surface-bound IgG was almost significantly lower than that of the group of patients with immune thrombocytopenia, however (P = .09). In addition, the values for platelet-bound IgG were more widely distributed than those of the patients studied at the time of initial diagnosis (Table 2 and Fig 2). A third of these patients had <800 molecules of IgG per platelet, whereas some of the patients had large amounts of IgG on their platelet surfaces (Fig 2).

Table 4 lists the characteristics of the patients whose platelet-bound IgG did not correlate with the clinical diagnosis of immune v nonimmune etiology. We were unable to discern any clinical parameters that characterized the patients with immune thrombocytopenia who had low platelet antibody values as compared with their counterparts with elevated values, including appropriate response to therapy. Three of the patients with nonimmune thrombocytopenia had values minimally >800 molecules of IgG per platelet cutoff, and the fourth patient had TTP with substantial plasma infusions prior to study.

Finally, as illustrated in Fig 3, we found no correlation between platelet count and platelet-bound IgG values in the 67 patients with immune thrombocytopenia (r = −.063, P = .612).

DISCUSSION

The controversy regarding the amount and pathophysiological role of immunoglobulin associated with platelets from normal and immune thrombocytopenic patients is gradually being clarified. Using a variety of assay methods, most earlier studies suggested that normal platelets had large amounts of platelet-associated IgG (5,000 to 80,000 molecules per platelet) and even higher levels in immune thrombocytopenia. These very large and seemingly nonphysiological amounts of platelet-associated IgG have led some investigators to question the role of the IgG so measured in the pathophysiology of immune destruction. Some stud-
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Fig 2. Platelet surface-bound IgG in 94 patients with immune thrombocytopenia. Patients evaluated at the time of initial diagnosis, upper bar; patients referred because of treatment-resistant immune thrombocytopenia, lower clear bar at each level of platelet-bound IgG.

Fig 3. Correlation of platelet count and amount of platelet surface-bound IgG in 67 patients with immune thrombocytopenia at the time of initial diagnosis.

ies have explored the result of differences in assay and calculation technique on estimates of platelet IgG. Rosse and colleagues21 and Blumberg and colleagues22 recently suggested that anti-IgG has 40 to 80 times the affinity for surface-displayed IgG as compared with soluble IgG, thus accounting for the very high values of platelet IgG when competitive binding assays calibrated with soluble IgG standards are used. Another tenable hypothesis is that certain assays purposely or inadvertently6,12,26 cause platelet lysis or release of canalicular entrapped plasma, resulting in measurement of both surface and "intracellular" IgG. In this regard, we used the monoclonal anti-IgG assay to demonstrate that the normal platelet surface displays only a few hundred IgG molecules but that platelet lysates have large amounts (20,000 to 80,000 molecules of IgG per platelet), comparable to results of prior reports.24 To provide truer estimates of platelet-bound IgG, certain assay techniques directly measure anti-IgG binding and use stoichiometric calculations to quantify platelet surface-bound IgG. These reports include the monoclonal anti-IgG assay,21,22,24,27 staphylococcal protein A assay,24 and an assay using a polyclonal anti-IgG.21 All these studies demonstrate that normal platelets have relatively little surface IgG, and amounts ranging from 500 to 13,000 molecules of IgG per platelet are present in immune thrombocytopenia. These amounts of platelet surface-bound IgG are similar to the values seen in the well-studied pathophysiology of warm immune hemolytic anemia.29

Of these techniques, we believe that the monoclonal anti-IgG assay has several advantages. First, the monoclonal anti-IgG reacts equally with all four subclasses of IgG24, whereas staphylococcal protein A reacts poorly or not at all with IgG, and the polyclonal reagent has not been characterized and may vary with each antiserum produced. Second, the monoclonal anti-IgG has a very high binding affinity,24,27

<table>
<thead>
<tr>
<th>Patient</th>
<th>Diagnosis</th>
<th>IgG/Platelet</th>
<th>Platelet Count</th>
</tr>
</thead>
<tbody>
<tr>
<td>R.S.</td>
<td>Idiopathic</td>
<td>251</td>
<td>50,000</td>
</tr>
<tr>
<td>E.E.</td>
<td>Idiopathic</td>
<td>350</td>
<td>12,000</td>
</tr>
<tr>
<td>M.H.</td>
<td>Idiopathic</td>
<td>380</td>
<td>38,000</td>
</tr>
<tr>
<td>R.M.</td>
<td>Idiopathic</td>
<td>671</td>
<td>16,000</td>
</tr>
<tr>
<td>J.W.</td>
<td>Idiopathic</td>
<td>702</td>
<td>89,000</td>
</tr>
<tr>
<td>E.H.</td>
<td>Drug, chlorpropamide</td>
<td>766</td>
<td>22,000</td>
</tr>
<tr>
<td>D.H.</td>
<td>Infectious mononucleosis</td>
<td>790</td>
<td>10,000</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>J.C.</td>
<td>Aplastic anemia</td>
<td>852</td>
<td>20,000</td>
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<tr>
<td>J.H.</td>
<td>Non-Hodgkin's lymphoma</td>
<td>861</td>
<td>26,000</td>
</tr>
<tr>
<td>R.W.</td>
<td>Breast cancer, marrow metastasis</td>
<td>893</td>
<td>22,000</td>
</tr>
<tr>
<td>T.H.</td>
<td>TTP</td>
<td>1812</td>
<td>8,000</td>
</tr>
</tbody>
</table>

TTP, thrombotic thrombocytopenic purpura.
allowing saturation kinetics to be reached at low protein concentrations (1 ng/μL) under our assay conditions, resulting in little or no nonspecific anti-IgG association with the platelet surface.24 Finally, the use of a single Percoll gradient centrifugation to separate platelet-bound from free anti-IgG precludes loss of platelets and disassociation of anti-IgG from IgG during repeated washing steps. The major drawback with this technique has been the relative unavailability of the monoclonal anti-IgG reagent. This is no longer a problem because the hybridoma cell line is available from the American Type Culture Bank (ATCC-HB-43) and from a commercial source of the purified antibody (Southern Biotechnology Associates).

In this study, we examined the amount of platelet surface-bound IgG in patients with thrombocytopenia due to immune and nonimmune etiologies. We showed that 90% of patients with immune thrombocytopenic purpura have increased amounts of surface-bound IgG (>800 molecules of IgG per platelet) at the time of initial diagnosis with a range of 87% to 93% for the various subgroups (Table 2). In contrast, patients with nonimmune thrombocytopenia infrequently had elevations of this degree (7%) indicating that quantitation of platelet surface-bound IgG can be a valuable adjunct in the evaluation of thrombocytopenic patients. Prior reports have indicated that patients with TTP have elevated platelet-associated IgG.14,15 Our experience in eight patients failed to confirm this observation: only one patient had an elevated value, which was suspect because of prior exposure of the patient to large amounts of pooled plasma before being referred for study. Indeed, the patients with nonimmune thrombocytopenia associated with enhanced platelet destruction (and presumably enhanced platelet production) had low amounts of platelet-bound IgG, similar to those of other nonimmune thrombocytopenic disorders.

The group of patients with treatment-resistant immune thrombocytopenia differed from their counterparts at initial diagnosis in that a larger proportion of them (33%) had values of platelet surface IgG comparable to those of normal donors or nonimmune thrombocytopenia patients. This may reflect the role of other pathophysiologic processes, including IgM, complement, or nonimmune mechanisms.

Two caveats to interpretation of platelet-bound IgG values in thrombocytopenic patients should be noted. Patients with immune thrombocytopenia who have been on therapy for >48 hours, usually with rising platelet counts, may have normal platelet-bound IgG values. Conversely, patients with acute leukemia who are severely ill and are receiving multiple antibiotics and platelet transfusions often have elevated values, the mechanisms of which are unknown but may be multifactorial. We have excluded both of these groups from our analysis and do not recommend interpretation of platelet-bound IgG values in these circumstances.

Finally, studies carried out in our laboratory25 using normal platelets sensitized in vitro with an IgG anti-P1A antibody indicated that human monocytes have a threshold of ~1,100 molecules of IgG per platelet for recognition and binding of IgG-coated platelets through their Fc receptors. This is certainly compatible with the degree of sensitization observed in this study of platelets sensitized in vivo (ITP) resulting in presumed macrophage binding in splenic sinusoids.

Thus, this study indicates that patients with immune thrombocytopenia have a high frequency of elevated amounts of platelet surface-bound IgG as compared with patients with nonimmune thrombocytopenia. Quantitation of surface-bound IgG represents a clinically useful tool for delineating an immune etiology related to IgG on the platelet surface. The finding of elevated platelet-bound IgG by this monoclonal anti-IgG technique certainly appears to be of pathophysiologic relevance.

REFERENCES

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is a non-specific consequence of platelet destruction. Blood 60:191a, 1982 (abstr)
Platelet surface-bound IgG in patients with immune and nonimmune thrombocytopenia

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