**Quantification of Platelet-Bound IgG by \(^{125}\text{I}-\text{Staphylococcal Protein A}\) in Immune Thrombocytopenic Purpura and Other Thrombocytopenic Disorders**

By George M. Shaw, John Axelson, J. Gertrude Maglott, and Albert F. LoBuglio

In this report we describe the use of an \(^{125}\text{I}-\text{Staphylococcal protein A (SPA)}\) assay to measure platelet-bound IgG in the evaluation of 62 thrombocytopenic patients. Platelets from 150 normal subjects were found to bind 146 ± 112 molecules of SPA per platelet (mean ± 2 SD). Nineteen of 20 patients with untreated immune thrombocytopenia had platelet IgG values above this range, with 15 of 20 having values above 1,000 molecules of SPA per platelet. Patients with immune thrombocytopenic purpura by clinical criteria, but who had failed conventional therapy (corticosteroids or splenectomy), had a wide range of platelet IgG levels: 4 of 20 had normal values, 6 of 20 had minimally elevated levels in the range seen with nonimmune thrombocytopenia, and 10 of 20 had much higher values. Fifteen patients with thrombocytopenia of apparent nonimmune origin and 7 others with chronic stable thrombocytopenia of unknown etiology were found to have platelet IgG levels within or only slightly above the normal range. Because of its simplicity, accuracy, and clinical correlation, the \(^{125}\text{I}-\text{SPA}\) assay provides an important new approach for studying platelet IgG in thrombocytopenic states. The data obtained with this technique are similar to those found in immune hemolytic anemia and suggest that the platelet-bound IgG so measured has pathophysiologic relevance in immune thrombocytopenic purpura.

**MATERIALS AND METHODS**

**Preparation of \(^{125}\text{I}-\text{Staphylococcal Protein A (SPA)}\)**

SPA was purchased from Sigma Chemicals, St. Louis, MO, and 400-μg aliquots were iodinated using 2 mCi \(^{125}\text{I}\) by the chloramine-T method\(^1\) at approximately 3-mo intervals. Protein-bound \(^{125}\text{I}\) was separated from free \(^{125}\text{I}\) by Sephadex G25 column chromatography.

**Assay Technique for Quantifying Platelet-Bound IgG**

Venous blood was collected and anticoagulated with acid citrate dextrose-A (ACD). The whole blood was centrifuged at 200 g for 20 min and the platelet-rich plasma (PRP) collected (Fig. 1). The pH was adjusted to 6.50–6.55 with ACD and then centrifuged at 1,750 g for 10 min. The platelet button was then washed twice in PBS.
containing 1 g/dl BSA and 4 mM EDTA (final pH 6.6) and resuspended in the same buffer. A quantity of 250 x 10⁶ platelets in 250 µl was then incubated with 500 ng ¹²⁵I-SPA (2 ng/µl) for 45 min at room temperature. The amount of SPA used was based on studies that demonstrated that this amount was far in excess of that needed to achieve maximal binding (saturation binding) with platelets sensitized in vitro with P1 antibody. Aliquots of 50 x 10⁶ platelets were layered on top of a 200-µl cushion of 30% Percoll in 400 µl soft conical tubes and centrifuged at 10,000 g for 4 min. Platelets and bound ¹²⁵I-SPA were pelleted, while unbound ¹²⁵I-SPA remained above the Percoll cushion. (When platelets were directly labeled with ¹⁰⁵Cr and centrifuged through various concentrations of Percoll, it was shown that a concentration of 30% Percoll did not impair significantly the sedimentation of platelets to the tube tip.) The tips of the tubes were cut off above the platelet button and the platelet-bound ¹²⁵I-SPA counted in a Packard Autogamma Scintillation spectrometer. The number of SPA molecules bound per platelet was calculated based on its specific activity, molecular weight, and Avogadro's number. The binding ratio of SPA:IgG has been reported to be 0.5–1.5.⁵,⁶ and data from our laboratory confirm this (unpublished). We would thus interpret the number of molecules of SPA bound per platelet to be equivalent to the number of IgG molecules present.

Reproducibility of the SPA assay was assessed by comparing triplicate determinations of bound ¹²⁵I-SPA in each of 25 consecutive patients having platelet-bound IgG values ranging from 182 to 5,505. The mean coefficient of variation (standard deviation x 100/mean) in this group was 5.3%. The day-to-day variability of the assay could not be so readily addressed, since, in patients with immune thrombocytopenia, platelet lifespan is often very short (hours) and treatment may result in marked changes in platelet-associated IgG (Table 1).²,³,¹² However, in normal subjects, the SPA values were quite reproducible, even over a 2-yr period: T.M., 123 ± 51, n = 14; G.C., 124 ± 37, n = 9; L.V., 263 ± 103, n = 5; M.S., 148 ± 45, n = 4 (mean ± SD, n = number of determinations).

General Characteristics of the Patients Studied

Sixty-two adult thrombocytopenic patients with at least three platelet counts less than 100,000/cu mm and 150 normal subjects comprised the study group. Thirty-three of the patients carried the diagnosis of chronic idiopathic (immune) thrombocytopenic purpura (ITP) based on conventional clinical criteria (adult onset thrombocytopenia of less than 50,000/cu mm, normal or increased megakaryocytes on bone marrow examination, lack of evidence for a secondary cause of thrombocytopenia, such as SLE, infectious mononucleosis, lymphoproliferative disease, hypersplenism, or drug ingestion). Seven patients have thrombocytopenia of apparent secondary immune etiology, including drug-induced (2), SLE (2), rheumatoid arthritis (1), infectious mononucleosis (1), and lymphoma (1). Fifteen patients had thrombocytopenia not considered immune in origin, including thymic thrombotic thrombocytopenic purpura (TTP) (3), hypersplenism (3), bone marrow failure (8), and pulmonary hypertension with microangiopathic hemolytic anemia (1). Seven patients with chronic stable thrombocytopenia had a benign clinical course never requiring therapy, and these patients were considered separately.

The sixty-two thrombocytopenic patients were categorized based on clinical criteria and then evaluated in regard to platelet-bound IgG. Group I included newly diagnosed and untreated patients with thrombocytopenia believed by clinical criteria to be immune in etiology. This included idiopathic and secondary causes. The ¹²⁵I-SPA studies on these patients were performed before they received any form of therapy or at least not more than 24 hr of corticosteroids.

Table 1. Effect of Splenectomy on Platelet Count and Platelet-Bound IgG in Idiopathic Thrombocytopenic Purpura

<table>
<thead>
<tr>
<th></th>
<th>Presplenectomy</th>
<th>Postsplenectomy</th>
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<tr>
<td>SPA Assay</td>
<td>Platelet Count</td>
<td>SPA Assay</td>
</tr>
<tr>
<td>H.H.</td>
<td>4,159*</td>
<td>10,000†</td>
</tr>
<tr>
<td>R.L.</td>
<td>3,219</td>
<td>2,000</td>
</tr>
<tr>
<td>P.N.</td>
<td>2,508</td>
<td>23,000</td>
</tr>
<tr>
<td>J.S.</td>
<td>2,505</td>
<td>2,000</td>
</tr>
<tr>
<td>F.D.</td>
<td>2,230</td>
<td>1,000</td>
</tr>
<tr>
<td>W.S.</td>
<td>1,598</td>
<td>11,000</td>
</tr>
<tr>
<td>A.S.</td>
<td>1,354</td>
<td>4,000</td>
</tr>
<tr>
<td>D.S.</td>
<td>1,235</td>
<td>11,000</td>
</tr>
<tr>
<td>A.B.</td>
<td>986</td>
<td>22,000</td>
</tr>
<tr>
<td>J.R.</td>
<td>233</td>
<td>50,000</td>
</tr>
</tbody>
</table>

*Number of molecules SPA bound per platelet.
†Platelet count per cu mm whole blood.
Group 2 was comprised of patients with presumably immune thrombocytopenic purpura resistant to conventional therapy, namely, corticosteroids or splenectomy or both. In contrast to group 1, these patients were under treatment when studied. Group 3 consisted of patients who were referred to us for evaluation of moderate thrombocytopenia of many years' duration, never requiring therapy. In contrast to idiopathic thrombocytopenic purpura, which often presents with purpura, bleeding, and extremely low platelet counts, these patients were discovered incidentally, were never symptomatic, were never treated, and generally had platelet counts above 50,000/cu mm. Group 4 included patients with presumed nonimmune thrombocytopenia. Disease categories within this group are detailed above. None of these patients were receiving corticosteroids when studied. Group 5 contained 150 normal adults who served as controls.

RESULTS

The SPA values in 62 thrombocytopenic patients and 150 normal controls are depicted in Fig. 2. Normal subjects had 146 ± 112 molecules of SPA bound per platelet (mean ± 2 SD). Within group 1, 19 of 20 patients with presumed immune thrombocytopenia, including 12 of 13 with “idiopathic” thrombocytopenia, had SPA values above the normal range. Only 1 of 20 patients had SPA levels within the range observed in patients with presumed nonimmune thrombocytopenia (group 4). This one patient, with an assay result within the normal range, was a 30-yr-old woman with idiopathic thrombocytopenic purpura whose platelet count increased from 59,000/cu mm to 200,000/cu mm while receiving prednisone, 60 mg/day, but who relapsed when corticosteroids were tapered and then failed to maintain a remission following splenectomy. Group 2 patients, all with presumably immune thrombocytopenic purpura resistant to steroids or splenectomy, had a wide range of SPA values. Four patients had normal levels, another 6 had levels above normal but within range seen in nonimmune thrombocytopenia (group 4), and another 10 had higher values. None of the patients with chronic stable thrombocytopenia (group 3) had SPA levels significantly above the range found in nonimmune thrombocytopenia (group 4). Finally, 15 patients had clinical diagnoses implicating thrombocytopenia of nonimmune origin (group 4). Four of these patients had SPA values within the normal range, and all 15 had values less than 600 molecules/platelet—only slightly higher than the normal controls.

The relationship between platelet count and number of SPA molecules bound per platelet was examined (Figs. 3–5). Previously untreated patients with the clinical diagnosis of immune thrombocytopenia demonstrated no correlation between degree of thrombocytopenia and number of molecules of SPA bound per
Fig. 3. Platelet counts and SPA values in patients with newly diagnosed and untreated immune thrombocytopenic purpura (group 1). (○) Idiopathic; (●) secondary.

Fig. 4. Platelet counts and SPA values in patients with therapy-resistant idiopathic thrombocytopenic purpura. (○) Corticosteroid-resistant; (●) splenectomy-resistant.
platelet (Fig. 3). This was true for patients with both idiopathic and secondary etiologies (Fig. 3). Patients with therapy-resistant idiopathic thrombocytopenic purpura (Fig. 4) also had no correlation between degree of thrombocytopenia and number of molecules of SPA bound per platelet. As can be seen, 5 of 11 patients with severe thrombocytopenia (<15,000 platelets/cu mm) had SPA values in or near the normal range. Conversely, 2 of 5 patients with much higher platelet counts (>50,000 platelets/cu mm) had SPA values that were much higher in comparison.

Figure 5 illustrates the SPA assay data and platelet counts in the patients with nonimmune thrombocytopenia of varying etiology. The values for SPA bound per platelet were low for all presumed mechanisms of thrombocytopenia, including impaired platelet production (bone marrow failure), enlarged splenic pool (hypersplenism), and peripheral platelet destruction (thrombotic thrombocytopenic purpura and microangiopathy). There was no correlation between platelet count and SPA value.

We had an opportunity to study 10 patients with idiopathic thrombocytopenic purpura both before and after splenectomy (Table 1). The 6 patients who had an excellent clinical response (postsplenectomy counts >200,000/cu mm) all had a dramatic fall in SPA assay values, with 4 in the normal range and 2 that were only minimally elevated (294 and 356 molecules of SPA bound/platelet). The excellent responders had presplenectomy values that varied from very high (H.H. and R.L.) to only moderately elevated (A.B.). Similarly, the four patients who had less than a complete response to splenectomy had pretreatment SPA values ranging from 2,230 to 233 molecules/platelet. Two of the patients (F.D. and W.S.) had a partial response to splenectomy, with postsplenectomy SPA values distinctly elevated although lower than presplenectomy values. One patient (D.S.) had a partial response to splenectomy, and postsplenectomy, the SPA value had fallen to the normal range. Patient J.R. was interesting in that she was the only patient in our study with idiopathic thrombocytopenic purpura on clinical grounds who had a normal pretreatment SPA value. She had an initial complete response to prednisone therapy (platelet counts >200,000/cu mm), but relapsed when the dose was decreased below 20 mg/day. She then underwent splenectomy, with subsequent platelet counts reaching 700,000/cu mm, only to have these fall to 38,000/cu mm 7 mo after the operation and remain low thereafter. The postsplenectomy values listed in Table 1 for J.R. were obtained 18 mo after splenectomy.
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DISCUSSION

Staphylococcal protein A has been used to detect surface immunoglobulin on a variety of cell types, including human erythrocytes, tumor cells, and fibroblasts. This report is the first to describe the use of $^{125}$I-SPA to measure membrane-bound IgG on whole platelets. SPA is a 42,000 mol wt protein with 4 potential receptor sites for the Fc fragment of IgG. It binds all human IgG subclasses, with the possible exception of IgG3, although even this IgG subclass may be bound in some instances. An important characteristic of SPA is its molecular homogeneity and high binding affinity for the Fc portion of IgG, which allows the reagent to achieve saturation binding kinetics. The binding stoichiometry of SPA and membrane-bound IgG has been reported to be approximately 1 (0.5-1.5), although this may be affected by characteristics of the cell membrane, the arrangement of surface IgG, and content of IgG3 subclass. Results from our laboratory (unpublished) have produced binding ratio data between 0.5 and 1.0 (SPA/IgG). Furthermore, we have recently developed an entirely independent method for measuring cell-bound IgG using $^{125}$I-mouse monoclonal anti-human IgG. Like SPA, this reagent is monoclonal, specific for IgG, does not cross-react with platelet antigens, and shows saturation binding kinetics. Determination of the number of molecules of monoclonal anti-IgG bound/platelet in these same groups of patients gave results similar to those reported here for molecules of SPA bound/platelet. Thus, the congruent data with two independent and highly specific immune probes suggest that they are a reasonable estimate of platelet surface-bound IgG.

Measurement of IgG bound to the platelet surface membrane has, in the past, been difficult. This may be a reflection of the inherent "stickiness" of the platelet, the tortuous nature of its membrane, the nonspecific adsorption of various plasma proteins to the platelet surface, the intracellular content of plasma proteins in platelets or resealed platelet fragments, or the polyclonal nature of many anti-IgG reagents. Notwithstanding, the different assay methods employed previously have been valuable in defining the immune etiology of idiopathic thrombocytopenic purpura, as well as certain secondary thrombocytopenic conditions. The data reported here generally corroborate these earlier studies, but differ in several respects, most notably in the absolute amount of IgG detected on platelets in both normal and pathologic states. Most earlier studies indicated that normal platelets had large amounts of surface IgG (5,000–40,000 molecules/platelet) and that platelets from ITP patients had even greater amounts. Our findings demonstrate that normal platelets have relatively little surface IgG, approximately 150 molecules/platelet. It is of interest that Leporrier et al. found similarly low values for platelet-associated IgG in 10 normal subjects using a polyclonal enzyme-linked anti-IgG assay. It has been suggested that platelet fragmentation and nonspecific membrane binding of plasma proteins may have contributed to high platelet IgG values reported in certain cases of presumably "nonimmune" thrombocytopenia. While the Percoll gradient used in this study could have eliminated these fragments from the platelet button in certain patients, such as those with TTP, this presumably does not account for the 10–20-fold lower values for platelet IgG found in all groups, particularly normal controls. For example, when platelets were directly labeled with $^{51}$Cr, greater than 95% of the radioactivity ($^{51}$Cr-labeled platelet protein) was found in the tube tip following our standard Percoll gradient centrifugation. A more tenable hypothesis is that certain assays may be purposely or inadvertently causing platelet lysis, resulting in measurement of both surface and intracellular IgG. Our finding of low amounts of IgG on the normal platelet surface is further supported by our studies with monoclonal anti-IgG.

Figure 2 summarizes the results of the SPA assay in 62 thrombocytopenic patients and 150 normal control subjects. An important question addressed in this figure is what proportion of patients who have a newly diagnosed and untreated apparent "immune" thrombocytopenia (group 1) actually have elevated platelet IgG levels. Nineteen of 20 patients, including 12 of 13 with "idiopathic" thrombocytopenic purpura, had results above the normal range. Moreover, 19 of 20 patients had SPA values above the range seen in patients with nonimmune thrombocytopenia. In this regard, it is notable that one patient in group 1 had a normal SPA level. Although this patient may have had increased levels of IgG3 not detectable with SPA, this may also reflect the role of other immune components, such as IgM or complement, or possibly a nonimmune etiology. This observation is more apparent in group 2 patients, all of whom had presumably immune (idiopathic) thrombocytopenic purpura resistant to conventional therapy. SPA values ranged from a high of 9,645 molecules/platelet to a low of 140 molecules/platelet. Interestingly, 2 of the 4 patients with the lowest SPA values (140 and 215 molecules/platelet) also had platelet counts of less than 10,000/cu mm. Ten of 20 patients in this group had SPA values within the range of nonimmune thrombocytopenia (group 4),...
again implicating factors other than IgG number in the etiology of the thrombocytopenia in these treatment-resistant patients. The 7 patients in group 3 were categorized together because all presented with asymptomatic mild thrombocytopenia (mean platelet count of 68,000/cu mm), never requiring therapy. All of these patients had SPA levels similar to those in normals or patients with nonimmune thrombocytopenia. Their asymptomatic presentation and benign course is different from the typical patient with ITP, and the pathophysiologic processes leading to thrombocytopenia in these patients remain to be determined.

The values for platelet-bound IgG in patients with ITP are said to mirror the clinical picture. For example, inverse correlations have been noted between "platelet-associated" IgG level and platelet count. Furthermore, some investigators have suggested that platelet IgG levels may be predictive of response to corticosteroids or splenectomy. We did not find such correlations (Figs. 3 and 4 and Table 1) using the SPA assay for surface-bound IgG. We did, however, observe, as have others, that platelet IgG levels generally fell as platelet counts rose in our 10 patients who were evaluated both before and after splenectomy. More patients will need to be studied before the prognostic implications of pre- and posttherapy platelet IgG values can be adequately evaluated.

We believe that the 125I-SPA assay technique reported here, along with the 125I-monoclonal mouse anti-human IgG method alluded to above, represent important new tools for studying immune mechanisms in thrombocytopenic states. The recent report by Kelton et al. underscores the limitations of conventional methodologies and thus the potential value of more sensitive and specific assays. They concluded from their studies that "the presence of increased PAIgG [platelet-associated IgG] provides little information in the diagnosis of ITP," and the PAIgG is high in a wide variety of clinical disorders not conventionally considered to be associated with "immune" thrombocytopenia. Our findings, on the other hand, would suggest that the 125I-SPA assay is both sensitive and specific, discriminating in more than 90% of cases between patients with newly diagnosed and untreated immune thrombocytopenic purpura (group 1) and those with nonimmune thrombocytopenia (group 4).

Finally, the data reported here for platelet-bound IgG in immune thrombocytopenia is in keeping with the well studied pathophysiology in warm immune hemolytic anemia. In the latter disorder, fewer than 500 molecules of IgG bound per red cell results in hemolysis, with presumed splenic macrophage binding (so-called Coomb's-negative immune hemolytic anemia). The more typical case of warm immune hemolytic anemia has several thousand IgG molecules per red cell (2+ to 4+ Coomb's test), and there is no correlation between the strength of the direct Coomb's test and the severity of the hemolytic anemia. The results reported in this article are consistent with these observations. It seems unlikely that the reticuloendothelial system would deal with IgG-coated platelets in a fashion dramatically different from IgG-coated red cells.

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