Autoantibodies Against Platelet Glycoprotein Ib in Patients With Chronic Immune Thrombocytopenic Purpura

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The present studies provide direct evidence that some patients with chronic immune thrombocytopenic purpura (ITP) have autoantibodies reactive with platelet glycoprotein Ib (GPIb). Microtiter wells coated with a monoclonal antibody that recognized GPIb were reacted with either platelet extract or a control cell extract. After washing and incubating with test plasma, well-bound IgG was quantitated using radioactive anti-IgG. When compared to plasma from normal subjects, plasma from 3 of 106 patients with chronic ITP had significantly increased quantities of IgG bound to microtiter wells reacted with platelet extracts. Negative results were obtained with the remaining 103 patients with chronic ITP and 59 patients with a variety of other platelet disorders. Plasma from two of the three positive patients precipitated a protein from 125I-surface-labeled platelet extract with a molecular weight similar to GPIb. One of the three patients with anti-GPIb antibody also had demonstrable autoantibodies to the platelet glycoprotein Ib/IIa complex.

Chronic immune thrombocytopenic purpura (ITP) is a clinical syndrome characterized by the destruction of platelets due to an antiplatelet antibody. It has been postulated for several years that this is an autoimmune disease, but until recently, there were no data to support this hypothesis. In 1981, Van Leeuwen et al noted that serum antibody or antibody eluted from the platelets of patients with chronic ITP reacted with normal target platelets but not with platelets from patients with Glanzmann’s thrombasthenia. Because thrombasthenic patients are severely deficient in platelet glycoproteins IIb and IIa (GPIIb and GPIIIa), these investigators suggested that the antiplatelet antibody in some ITP patients may be specific for GPIIb and/or GPIIIa. More recently, Woods and coworkers have demonstrated directly the presence of anti-GPIIb/GPIIIa autoantibodies in the serum of patients with chronic ITP.

In this study, we have used similar methods to evaluate ITP plasmas for the presence of autoantibodies reactive with platelet glycoprotein Ib (GPIb). This glycoprotein is an integral platelet membrane protein, consisting of two glycoprotein chains linked by an intermolecular disulfide bridge, which plays a critical role in the adhesion of platelets to vascular subendothelium, platelet agglutination by ristocetin, and the ristocetin-associated binding of factor VIII-related antigen (VIII:Ag). Glycoprotein Ib is markedly reduced in patients with the Bernard-Soulier syndrome, which is an inherited disorder of hemostasis associated with large platelets having a markedly reduced capacity to adhere to the vascular subendothelium. This glycoprotein has a small membrane-bound region and a large portion that is released from the platelet surface after cleavage by a calcium-dependent protease.

Using a monoclonal antibody specific for this glycoprotein, we have developed a specific and sensitive assay for the detection of autoantibodies against GPIb. Sera from 3 of 106 patients with chronic ITP had demonstrable autoantibodies reactive with GPIb; one of these also had GPIIb/GPIIIa autoantibodies. In two of the three patients, molecules of molecular weight similar to GPIb were precipitated using the patient’s plasma.

Materials and Methods

Plasma samples were obtained from 106 patients with chronic ITP (75 adults and 31 children), 14 patients with systemic lupus erythematosus (SLE) and thrombocytopenia, 10 patients with acute childhood ITP, 8 patients with non-Hodgkin’s lymphoma and thrombocytopenia, 9 patients with qualitative platelet defects, 3 patients with drug-related immune thrombocytopenia, 2 patients with thyroiditis and thrombocytopenia, 4 patients refractory to transfused platelets who had anti-HLA antibodies, 12 patients with thrombocytopenia due to hypoproduction, and 22 normal subjects. The diagnosis of chronic ITP was made as previously described. Briefly, all patients with ITP had thrombocytopenia, increased or normal numbers of megakaryocytes, increased levels of platelet-associated IgG, and no other demonstrable causes of destructive thrombocytopenia.
Assay for Anti-GPIb

Monoclonal Antibodies

The monoclonal antibody specific for GPIb (AP-1) has been shown to bind to immobilized GPIb and glycoliculin, to inhibit ristocetin-induced platelet aggregation, and to block >99% of the binding to platelets of radiolaabeled VIII/Ag induced by ristocetin.4,5

Radioiodinated Fab2 Fragments of Anti-human IgG

Goat anti-human IgG prepared in our laboratory was affinity purified as previously described using purified Sepharose-linked human IgG. Fab2 fragments were prepared by pepsin digestion, and the antibody was radiolabeled (25 μg protein) using the chloramine-T method.8

Cell Extracts

Platelet-rich plasma was obtained from freshly drawn citrate dextrose, NIH formula (ACD-A)–anticoagulated blood (5 vol blood to 1 vol ACD-A) by centrifugation at 400 g for 10 minutes. After aspiration, the PRP was centrifuged for 15 minutes at 1,100 g and the plasma was discarded. The platelet button was washed six times in citrate buffer (0.5% Na3Citrate, 0.1 mol/L NaCl, 0.14 mol/L dextrose). For control cell extracts, a leukemia cell line, CEM (kindly provided by Dr Dennis Carson, Scripps Clinic and Research Foundation), was grown in suspension culture, harvested, and washed four times with Tris-buffered saline (TBS—0.01 mol/L Tris, 0.15 mol/L NaCl, pH 6.5). The washed cells were solubilized by incubation in 0.5% nonidet-P 40 (NP 40; Shell Chemical Co., Elmhurst, Ill) in Tris-buffered saline, pH 7.4, for 30 minutes at 4°C at a cell concentration of either 106 or 107 cells/mL or 100 μl CEM cells/mL. Insoluble material was removed by ultracentrifugation (100,000 g for 30 minutes) and the extracts aliquoted and stored at −70°C. Prior to use, the extracts were diluted 1:10 in Tris-buffered saline containing 0.5% NP 40, 0.5 mmol/L calcium, and 0.05% Tween 20 (wash buffer).

Assay Protocol

The assay system for anti-GPIb was identical to the assay for anti-GPIIb/GPIIIa, except for the monoclonal antibody employed. Details on the development of the anti-GPIb/GPIIIa assay have been previously published.7 Each well of a flexible, flat-bottom microtiter plate (Dynatech Laboratories, Alexandria, Va) was coated with the monoclonal antibody (AP-1) by overnight incubation with 100 μL of the purified protein at a concentration of 5 μg/mL in 0.1 mol/L NaHCO3 buffer. Preliminary studies showed that this protein concentration allowed saturation of the wells. The wells were then washed three times with 0.05% Tween 20 (J. T. Baker Chemical Co., Phillipsburg, NJ) in phosphate-buffered saline (PBS; 0.01 mol/L phosphate, 0.15 mol/L NaCl) to remove unbound monoclonal protein and then incubated for 20 minutes in this buffer to block any remaining protein-binding sites on the plastic surface. The coagulant solution, 100 μL of a 1:10 dilution of the CEM control cell extract, and to the other half of the wells, a 1:10 dilution of the platelet extract. After incubation for 90 minutes at 4°C, the wells were washed three times with wash buffer and then incubated for 60 minutes with 100 μL of a 1:10 dilution of the human plasma sample to be tested. Selected plasmas were tested at higher dilutions. Triplicate aliquots of each plasma sample were tested against both hydridoma-coated wells that had been reacted with platelet extract and hybridoma-coated wells that had been reacted with control extract. After washing three times, radioiodinated affinity-purified Fab2 fragments of goat anti-human IgG (approximately 70,000 cpm/well) were added and incubated for 60 minutes at 4°C. The microtiter wells were then washed three times and the radioactivity of each well determined. assay values are expressed in terms of the percent change in radioactivity above or below the radioactivity associated with control wells using the following formula:

\[
\text{Percent change} = \frac{\text{cpm Platelet extract wells} - \text{cpm Control wells}}{\text{cpm Control wells}} \times 100
\]

Immunoprecipitation of Radiolabeled Antigen With ITP Plasma Containing Autoantibodies

Platelets were surface labeled as follows: 10 μL of lactoperoxidase (4 mg/mL) and 1.0 mCi 125I were added to a capp tube containing 106 washed normal platelets in 1.0 mL PBS. After mixing, 3 aliquots (5 μL) of 0.03% H2O2 were added at 0.2 seconds intervals. After centrifugation for 15 minutes at 1,100 g, the platelets were washed three times with 0.1% bovine serum albumin (BSA) and PBS and then suspended in 1.0 mL of 0.2% BSA-PBS.

Before use, plasma samples were ultracentrifuged for 60 minutes at 100,000 g at 5°C. For each test, 100 μL of test plasma was incubated with 100 μL of the 125I-labeled platelet suspension for 60 minutes at room temperature. The mixture was then centrifuged for 5 minutes at 3,000 g, and the platelets and plasma were washed five times in 0.2% BSA-PBS. After the third wash, the tubes were changed. The antibody-sensitized platelets were solubilized in TBS, containing 1% Triton X-100, 10 mmol/L EDTA, and 5 mmol/L KI, by incubating for 60 minutes at 4°C, and the insoluble material was removed by ultracentrifugation for 20 minutes at 100,000 g (4°C).

One-milliliter aliquots of a 10% suspension of formalin-fixed Staphylococcus aureus bacteria (Staph A, Calbiochem-Behring, La Jolla, Calif) were centrifuged at 1,100 g for 10 minutes and the pellets washed three times with 1.0 mL of TBS containing 5 mmol/L KI. Next, the pellet was resuspended in 200 μL of TBS containing 5 mmol/L KI, 1.0 mmol/L EDTA, and 0.3% Triton X-100 and incubated for 10 minutes on ice with 200 μL of a Triton X-100 extract of unlabeled platelets (109/mL) from the same platelet donor. Then, 50 μL of antibody-sensitized 125I-labeled platelet extract was added, and the mixture was incubated for an additional 15 minutes. The Staph A bacteria were then washed five times with 1.0 mL of TBS containing 10 mmol/L EDTA, 2.5 mmol/L KI, and 0.3% Triton X-100; the tubes were changed after the third wash. To each pellet was added 50 μL of 2% sodium dodecylsulfate (SDS) and 5% mercaptoethanol (ME) and, after boiling for 2 minutes, the solutions were electrophoresed in 5% polyacrylamide gels using the method of Laemmli. Parallel wells containing either molecular weight standards or solubilized surface-labeled platelet membranes were included to allow localization of the major labeled platelet glycoproteins. The gels were fixed overnight, dried, and then applied to x-ray film.

RESULTS

Assay of Plasma for Anti-GPIb Autoantibodies

Normal Subjects

Plasma from 22 normal subjects was studied. Ten of these normal subjects were tested on multiple occasions (8–15 times each). Mean (±SD) percent change values from each person's platelets ranged from −5.4% ± 9.4% to 1.4% ± 6.4%; the difference among these subjects was not significant (P = .295). The

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mean (±SD) of all normal data (136 studies on 22 subjects) was −1.94% ± 6.4% (see Table 1).

*Chronic ITP Patients*

Of 106 patients tested, 103 were within 2 SD of the mean of normal subjects. Mean (±SD) percent change values of these negative patients were: −1.95% ± 6.2% (73 adults) and −3.7% ± 8.2% (31 children). Three patients had significantly abnormal percent change values (>3 SD above the normal mean); patient 1, 121.0% ± 54.8% (8 studies); patient 2, 64.5% ± 30.4% (7 studies); and patient 3, 18.9% ± 5.8% (6 studies). Positive results were obtained in patient 1 at a plasma dilution of 1:160, in patient 2 at a plasma dilution of 1:40, and in patient 3 at a plasma dilution of 1:10. One of these (patient 2) has been previously shown to have anti-GPIIb/IIIa autoantibodies (titer 1:80), whereas the other two did not.1

Inhibition experiments in this assay system, using purified GPIb as an inhibitor, would have been desirable controls. Unfortunately, only source of GPIb available to us, in sufficient quantity, is obtained by elution from insolubilized AP-1 monoclonal antibody. We did not feel that GPIb obtained by this method would be appropriate for inhibition studies. Alternatively, platelet extracts from Bernard-Soulier patients, which have markedly reduced GPIb concentrations, could have been used as a control, but unfortunately, we have no access to such patients.

The clinical characteristics of the three patients with anti-GPIb antibodies are summarized in Table 2. Patient 1 developed mild disease (platelet count ~60,000/μL) at age 80. Three years later, his symptoms increased markedly, and he was hospitalized for severe mucosal bleeding (epistaxis, hematuria, GI bleeding). His platelet count ranged from 1,000 to 3,000/μL despite high-dose corticosteroids, multiple transfusions (48 U of platelets; 14 U of RBC), emergency splenectomy, vincristine, and cyclophosphamide. He died of uncontrolled bleeding 3 weeks after admission. Blood was obtained for study 13 days after his first transfusion.

Patient 2 developed the acute onset of thrombocytopenia at age 19, which required emergency splenectomy. After a partial response (platelet count 80,000–135,000/μL) for several years, he developed severe thrombocytopenia and GI bleeding in 1958. Since that time, he has remained severely thrombocytopenic despite continuous corticosteroid therapy. His platelet count has been less than 10,000/μL for the past 5 years. He received RBC transfusions in 1945, 1958, and 1977 for GI bleeding. Blood for study was obtained in August 1982.

Patient 3 is an 81-year-old woman with a long history of bullous pemphigus, requiring prednisone therapy (5–20 mg/d) since 1981. In January 1983, she was hospitalized for severe epistaxis and oral bleeding; her platelet count was 7,000/μL. She had never been pregnant and received no transfusions prior to study. She has responded partially to high-dose corticosteroids and cyclophosphamide (platelet count ~70,000/μL).

*Miscellaneous Patient Groups*

Of 62 patients in other disease categories, none had values outside the normal range. Mean (±SD) percent change values for the major groups were: SLE, 1.94% ± 10.8%; acute ITP, −8.0% ± 5.4%; lymphoma, −3.7% ± 6.0%; platelet qualitative defects, −1.7% ± 7.4%; and production defects, −2.0% ± 6.3% (Table 1).

**Precipitation of Platelet Antigens With ITP Plasma**

Plasma from the ITP patients, giving positive tests using the microtiter well assay (patients 1, 2, and 3; Table 1), was evaluated for its ability to precipitate platelet antigens. The plasma was incubated with surface-labeled platelets and, after washing and solubilization, the IgG and any bound radiolabeled antigens were precipitated with Staph A. The Staph A-bound proteins were electrophoresed on 5% SDS-PAGE and the antigens localized by autoradiography. Both patients 1 and 2 showed a distinct band with a
molecular weight of 145,000, which correlated in position with platelet GPIb (Fig 1). The intensity of this band was greater using plasma from patient 1, who had a more positive result (titer of 1:160) using the microtiter well assay. Patient 2 had two additional radiolabeled bands that have previously been shown to be due to autoantibodies against GPIIb/GPIIIa.3 Faint bands in the location of GPIIIa are seen using plasma from patient 1 and with control plasma. This probably represents mild nonspecific precipitation of the more heavily labeled GPIIb/GPIIIa. When the intensity of the radiolabeled bands was evaluated, using laser densitometry scanning, the visual impressions were confirmed (data not shown). Similar studies were performed on 7.5% gels, and no significant bands of lower molecular weight were noted on autoradiographs (data not shown).

Plasma from 5 additional normal subjects and 3 patients with chronic ITP, who gave negative results in the microtiter well assay, was also studied and gave similar immunoprecipitation results to those of the control plasma shown in Fig 1 (data not shown). The plasma from patient 3, who gave only moderately positive results with the microtiter well assay, did not give clear-cut positive results when immunoprecipitation was performed, probably because of the low titer (1:10).

**DISCUSSION**

Plasma from 3 (all adults) of 106 patients with chronic ITP had demonstrable autoantibody reactive with GPIb. The presence of this autoantibody was demonstrated by showing binding to antigen immobilized on microtiter wells, and in two of the three patients, by immunoprecipitation of the antigen from extracts of surface-labeled platelets. One of the three chronic ITP patients having anti-GPIb autoantibodies also had anti-GPIIb/GPIIIa autoantibodies.3 In previously reported studies, we noted anti-GPIIb/IIIa autoantibodies in 5 of 56 patients with chronic ITP;3 since that time, we have completed studies on the same 106 patients with chronic ITP presented in this study and have noted 9 patients with anti-GPIIb/IIIa antibodies.

It seems likely to us that the thrombocytopenia in these patients was due to the presence of the demonstrable autoantibodies (anti-GPIb in patients 1–3 and also anti-GPIIb/IIIa in patient 2). Patient 3 had no exposure to blood products and had not been pregnant. Although patients 1 and 2 had received transfusions prior to study, these were required for treatment of their thrombocytopenia. Therefore, if transfusion-induced alloantibodies were responsible for the anti-GPIb antibodies, another explanation would be required to explain the cause of their low platelet count. In addition, essentially all well documented transfusion-induced antibodies are against the HLA locus, and immunoprecipitation studies showed no evidence of a molecule compatible in size with the HLA protein. Further evidence against anti-HLA antibodies is provided by the negative results in the microtiter well assay, which were seen using plasma from patients with known high-titer anti-HLA antibodies. One could also postulate an antibody response to a common platelet antigen by patients lacking this

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**Table 2. Clinical Characteristics of Patients With Anti-GPIb Autoantibodies**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Onset</th>
<th>Study</th>
<th>Sex</th>
<th>Transfusions</th>
<th>Pregnancies</th>
<th>Platelet Count* (mm$^3$)</th>
<th>Autoantibody Titer</th>
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<td>83</td>
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<td>No</td>
<td>7,000</td>
<td>1:10</td>
<td>Steroids, cyclophosphamide</td>
</tr>
</tbody>
</table>

*Platelet count at the time of study.
antigen, such as that seen in posttransfusion purpura; but thrombocytopenia due to this cause would be unlikely to persist for several months (patient 2), and both patients 1 and 2 were thrombocytopenic prior to transfusion. For these reasons, it seems likely that the antibodies were causative and were not the result of previous transfusions.

The percentage of chronic ITP patients with demonstrable circulating antibodies to these platelet glycoprotein antigens (Ib and IIb/IIIa) is small, suggesting three possible explanations:

1. The detection of autoantibodies to these glycoproteins is limited by either the antibody titer or by the assay sensitivity, so that we are recognizing only patients with severe disease; alternatively, the absence of plasma antibody may reflect the removal of such autoantibodies by adherence to circulating platelets.

2. Most patients with chronic ITP have autoantibodies against other platelet autoantigens.

3. The disease termed chronic ITP is a syndrome with multiple causes, one of which is the production of autoantibodies reactive with platelet antigens. It seems likely that more than one of these possibilities may be correct. The observation of Van Leeuwen et al\(^2\) that a greater percentage of eluates from the platelets of patients with chronic ITP react with normal platelets when compared to the percentage of reactive plasmas suggests that the evaluation of plasma may not truly reflect the incidence of autoantibody production in this disorder. On the other hand, previous studies from our laboratory, showing that IgG produced in culture by ITP splenic cells reacted with multiple platelet proteins, some of similar size to that of the contractile proteins,\(^3\) suggest that autoantibodies reactive with antigens other than GPIb or GPIIb/GPIIIa may be demonstrable when techniques become available for their measurement.

The present study also demonstrates that patients with chronic ITP may have more than one autoantibody to platelet glycoproteins. Patient 2, who had been previously shown to have anti-GPIIb/GPIIIa autoantibodies,\(^3\) was also noted to have autoantibodies reactive with GPIb. This is consistent with our earlier studies showing binding of radiolabeled splenic IgG from chronic ITP patients to multiple platelet protein bands.\(^13\)

REFERENCES

Autoantibodies against platelet glycoprotein Ib in patients with chronic immune thrombocytopenic purpura

VL Jr Woods, Y Kurata, RR Montgomery, P Tani, D Mason, EH Oh and R McMillan