Autoantibodies Against the Platelet Glycoprotein IIb/IIIa Complex in Patients With Chronic ITP

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Chronic idiopathic thrombocytopenic purpura (ITP) is a syndrome caused by a circulating antibody reactive with the platelet membrane; the antigenic specificity of these antibodies is unknown. Recently, van Leeuwen et al. reported that serum antibody (14 of 42) or platelet eluates (35 of 42) from patients with ITP would bind to normal platelets but not to the platelets of patients with Glanzmann's thrombasthenia. Since thrombathenic patients have absent or markedly reduced amounts of platelet glycoproteins IIb (GPIIb) and IIIa (GPIIIa), these authors suggested that some ITP antiplatelet antibodies might be directed at platelet GPIIb and/or GPIIIa. However, thrombathenic platelets were recently noted to have additional glycoprotein deficiencies and alterations. Moreover, Kunicki et al. found that ITP antiplatelet antibodies are equally cytotoxic toward the platelets of both normal and thrombathenic patients. It is therefore not clear whether these ITP antibodies reacted preferentially with GPIIb/GPIIIa or with other platelet membrane proteins.

Platelet GPIIb and GPIIIa are major constituents of the platelet plasma membrane and are critically involved in the formation of the cell surface fibrinogen receptor on activated platelets. When these glycoproteins are extracted from the platelet membrane with nonionic detergents, they exist in solution as a calcium-dependent complex. We have produced monoclonal antibodies specific for the platelet GPIIb/IIIa complex and have used these antibodies to develop a sensitive and specific assay for human autoantibodies to GPIIb and GPIIIa. We have found that some ITP patients have circulating antibodies to these glycoproteins and that some of these ITP plasma immunoprecipitate molecules similar in molecular weight to GPIIb and GPIIIa.

MATERIALS AND METHODS

Patient Population

Plasma samples were obtained from 56 patients with chronic ITP (39 adults, 17 children), 16 normal subjects, and 34 patients with other diseases, including 2 with posttransfusion purpura (anti-PI antibodies), 1 with recurrent Hodgkin’s disease and an ITP-like syndrome, 3 refractory to platelet transfusions (anti-HLA antibodies), 2 with postviral immune thrombocytopenia (monkeypox virus), 7 with systemic lupus erythematosus (SLE) and thrombocytopenia; 5 with non-Hodgkin’s lymphoma (4 of these with immune thrombocytopenia), 2 with thrombocytopenia associated with immune complex nephritis, 1 with autoimmune hemolytic anemia (IgG type), 3 with cirrhosis and hypersplenism; 2 with drug-induced thrombocytopenia (quinidine and propranolol); 2 with Crohn’s disease and thrombocytopenia, 2 with platelet aggregation disorders; 2 with acute childhood ITP, and 1 each with Wiscott-Alldrich syndrome, thyroiditis and thrombocytopenia, immune thrombocytopenia after bone marrow transplantation, pancytopenia of unknown cause, and the mother of a patient with neonatal purpura.

All patients with chronic ITP satisfied the usual clinical criteria: thrombocytopenia with normal or increased numbers of megakaryocytes, increased levels of platelet-associated IgG, and absence of other etiologies, such as drugs, SLE, or lymphoproliferative disorders.

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 Assay for Plasma Antibody to Platelet GPIIb/GPIIa

Monoclonal Antibodies Specific for GPIIb/GPIIa

The production and characterization of the specificity of these murine monoclonals are described in detail elsewhere and are summarized below.

Adult female BALB/c mice were immunized intraperitoneally with 104 freshly prepared Ficoll-Hypaque-purified human peripheral blood mononuclear cells, also containing platelets (PBM-platelets) at monthly intervals. One month after the fourth immunization, the mice were injected intravenously with the same preparation, and 4 days later, splenocytes were fused with the myeloma cell line SP 2/0-Ag14, hybrids were selectively grown in HAT media, and immunoglobulin (lg) producing hybrids were identified. Culture supernatants from these hybrids were screened for their ability to bind to PBM-platelets, and their cellular specificity was determined by indirect immunofluorescence against PBM-platelets. Four hybrids were found that produced Ig that bound exclusively to platelets. Three of these hybrids, 2G12, 3F5, and 4F10, were cloned by limiting dilution and were used to generate ascites in pristane-primed BALB/c mice. Monoclonal antibodies were purified from ascites by adsorption to protein-A-Sepharose (Pharmacia Fine Chemicals, Upsala, Sweden), followed by sequential pH elution. Mouse Ig subclass was determined by double radial immunodiffusion of 20-fold concentrated cloned hybridoma culture supernatants against subclass-specific antisera. Monoclonal 3F5 is an IgG1 lg, 4F10 is an IgG2b Ig and 2G12 is an IgG2a lg. IgG-containing fractions were pooled, dialyzed against phosphate-buffered saline (PBS), and stored in aliquots at -20°C. IgG concentrations were determined spectrophotometrically, assuming an estimated E of 14.3, and molarities were calculated using a molecular weight for IgG of 150,000 kilodaltons.

Triton X-100 extracts of lactoperoxidase-catalyzed cell surface radiiodinated platelets were immunoprecipitated with each of these purified monoclonals, and the precipitates were examined on SDS-PAGE with autoradiography. Only molecules of molecular weight (mol wt) 100 and 120 kilodaltons (kd) were visualized in reduced samples (Fig. 1), and molecules of mol wt 90 and 130 kD were seen with unreduced samples (data not shown). In sequential immunoprecipitation experiments, labeled platelet extracts were first exhaustively precipitated with a goat antiserum specific for platelet GPIIb/GPIIa (gift from Dr. Mark Ginsberg), then precipitated with each of the monoclonals, and precipitates examined as above. After precipitation with goat anti-GPIIb/GPIIa, no radiolabeled platelet molecules were seen in monoclonal precipitates, indicating that the monoclonals were reactive with platelet GPIIb/GPIIa. The results of one representative study using 2G12, the antibody used in our assay, are shown in Fig. 1. Similar results were noted using 3F5 or 4F10. This was confirmed in platelet binding studies, in which the monoclonals were found to be unreactive with platelets from two patients with Glanzmann's thrombasthenia, though quite reactive with normal platelets. These monoclonal antibodies do not react with purified fibrinogen or the neuronal cell line SK-N-SH; the latter is used in this assay as a platelet antigen-negative control (see below).

Cross-binding experiments were performed to determine the relative epitopic specificity of the monoclonals. Flexible microtiter plate wells were coated with each of the purified monoclonal antibodies, washed, and then reacted with a Triton X-100 extract of unlabeled platelets. After further washing, radiolabeled monoclonal antibodies were incubated with each well, and well-bound radioactivity was determined. It was found that monoclonal antibodies 2G12 and 4F10 are directed at sterically related determinants on the GPIIb/GPIIa complex, whereas hybridoma 3F5 is directed at a sterically unrelated antigenic determinant.

Other Antibodies and Immunoglobulins

The control hybridoma-produced monoclonal IgG 10.4.22 (mouse anti-murine lgD antibody, unreactive with platelets) was prepared with cells obtained from the Salk Institute Cell Distribution Center, La Jolla, CA and was purified on protein-A-Sepharose (see above). We obtained purified mouse IgG2a immunoglobulin UPC-10 from Litton Bionetics, Kensington, MD; Fab, fragments of goat anti-human IgG (gamma-chain-specific) from Cappell Laboratories, Cochranville, PA; human IgG and mouse IgG by DEAE chromatography of serum; and goat anti-mouse IgG serum from Cappell Laboratories. This latter protein was affinity purified by incubation with mouse IgG (10.4.22) linked to Sepharose 4B (see below); specific antibodies were eluted with 3.5 M MgCl2, dialyzed against PBS, and stored at -20°C.

Sepharose-4B Conjugation of Proteins

Monoclonal antibody 10.4.22, 4F10, DEAE-purified human IgG, and mouse IgG were conjugated to CNBr-activated Sepharose (Pharmacia Fine Chemicals), according to the manufacturers' instructions, at 2 mg protein/ml of Sepharose.
Radioiodination of Antibodies

The GP1Ib/GPIIa-specific monoclonal antibody 3F5 (150 µg) was radioiodinated by the chloramine-T procedure to a specific activity of 2,100 cpm/ng. The Fab, fragments of goat anti-human IgG (γ-chain-specific) were incubated with 20 µl of human IgG-sepharose for 30 min at room temperature. After 3 washes with PBS, the Sepharose–goat antibody conjugate was labeled by a modified chloramine-T method. Goat anti-mouse IgG was similarly radiolauled using mouse IgG-Sepharose. Estimated specific activity for the goat anti-human IgG antibody was 1.6 x 10^6 cpm/µg, and, for the goat anti-mouse IgG antibody, was 3 x 10^6 cpm/µg.

Preparation of Cell Extracts

Normal platelets were obtained from outdated platelet packs by differential centrifugation, and the platelets were washed 3 times with Tris-buffered saline (TBS-0.01 M Tris, 0.15 M NaCl, pH 6.5). Control cell extracts were prepared from a human neuroblastoma line (SK-N-SH) grown in monolayer culture, harvested, and washed as previously described. The washed cells were solubilized by incubation in 0.5% nonidet P40 (NP40; Shell Chemical Co., Elmhurst, IL) in TBS, pH 7.4, for 30 min at 4°C at a final concentration of 10^6 platelets/ml or 10^5 SK-N-SH cells/ml. Insoluble material was removed by centrifugation at 100,000 g for 30 min, and the extracts stored in aliquots at -70°C.

Assay Protocol

Each well of a flexible, flat-bottomed microtiter plate (Dynatech Laboratories, Alexandria, VA) was coated with GP1Ib/GPIIa-specific monoclonal antibody 2G12 by overnight incubation with 100 µl of the purified hybridoma protein at 5 µg/ml in 0.1M NaHCO3 buffer. The microtiter wells were washed 3 times with 0.05% Tween 20 (JT Baker Chemical Co., Phillipsburg, NJ) in PBS to remove unbound monoclonal antibody and incubated with this buffer for 20 min to block remaining protein-binding sites. To one-half of the monoclonal antibody-coated microtiter wells were added 100 µl of a 1:10 dilution of platelet extract in TBS, pH 7.4, containing 0.5% NP40, 0.5 mM calcium, and 0.05% Tween 20 (wash buffer). The other half of the antibody-coated microtiter wells was incubated with a 1:10 dilution of the SK-N-SH neuronal cell extract in wash buffer. After 90-min incubation at 4°C, the microtiter wells were washed 3 times with wash buffer and then incubated for 1 hr with 100 µl of human plasma samples diluted 1:10 in wash buffer. Plasma from either EDTA- or ACD-A-anticoagulated blood gives identical results. Additional dilutions were studied in selected experiments. Each plasma sample was incubated with two hybridoma antibody-coated wells previously reacted with platelet extract and two wells previously reacted with control extract. After washing, radioiodinated, affinity-purified Fab, fragments of goat anti-human IgG gamma-chain antibody were incubated with each microtiter well for 1 hr at 4°C (approximately 70,000 cpm/well in wash buffer). The microtiter wells were then washed 3 times with wash buffer, individual wells were cut out, and well-bound radioactivity was determined. Assay values are expressed as a ratio of the mean counts per minute (cpm) of radioiodinated Fab, goat anti-human IgG bound to the GP1Ib/GPIIa-coated hybridoma monolayers, divided by the mean cpm of radioiodinated goat antibody bound to the control cell extract-coated monolayers. As a further control, plasma samples found to be positive in the assay (greater than 3 SD above control ratios) were incubated with microtiter wells coated with control murine myeloma UPC-10, followed by either platelet or neuronal cell extract. The assay was then carried out as described above.

Establishment of Assay Conditions

Preliminary experiments with radioiodinated goat anti-mouse IgG showed that overnight incubation of the microtiter wells with a 5 µg/ml solution of monoclonal antibody 2G12 resulted in saturation binding of this antibody to the microtiter well.

It was also shown that incubation of the 2G12-coated microtiter wells with 100 µl of a 1:10 dilution of platelet extract resulted in binding of 3F5 to the 2G12 monolayer. Microtiter wells coated with 2G12 were incubated with various dilutions of platelet extract and washed. The ability of radioiodinated anti-GP1Ib/GPIIa monoclonal antibody 3F5 to bind to the GP1Ib/GPIIa adherent to the 2G12-coated microtiter well was determined by incubation for 1 hr with 100 µl of a 0.05 µg/ml solution of radioiodinated 3F5 in 1% bovine serum albumin (BSA)-PBS followed by washing and quantitation of well-bound radioactivity. As 3F5 is directed at an antigenic determinant distinct from the determinant reactive with 2G12, the amount of 3F5 bound to the well reflects the amount of GP1Ib/GPIIa complex bound to the well. An average of 18 ng of 3F5 (3.6 x 10^6 cpm) was bound to each microtiter well. Assuming that 1 mole of 3F5 antibody reacts with 1 mole of GP1Ib/GPIIa complex, approximately 7 x 10^6 molecules of GP1Ib/GPIIa complex are bound to each microtiter well. When control microtiter wells coated with the murine IgG2a protein UPC-10 were reacted with platelet extract, only 200 cpm of radioiodinated 3F5 was bound to the microtiter wells.

Immunoprecipitation of Radiolabeled Antigen With ITP Plasma Containing Anti-GP1Ib/GPIIa Antibody

Platelets were surface labeled by combining 10^4 washed normal platelets in 1.0 ml of PBS, 10 µl lactoperoxidase (4 mg/ml), and 1.0 mCi I^125. After mixing, 3 µl aliquots of freshly diluted 0.03% H2O2 were added at 30-sec intervals. After centrifugation for 15 min at 1,100 g, the platelets were washed 3 times with 0.1% BSA-PBS and resuspended in 1.0 ml of 0.2% BSA-PBS.

To the pellets were added 50 µl 2% sodium citrate, incubated for 1 hr at 4°C in TBS, pH 7.4, containing 1 Triton X-100 and 5 mM KI. These samples were then ultracentrifuged for 20 min at 100,000 g (4°C). For each sample tested, 100 µl of plasma was incubated with 100 µl of the I^125-labeled platelet suspension for 1 hr at room temperature. After centrifugation for 5 min at 3,000 g, the platelets were washed 3 times with 0.1% BSA-PBS and, after changing tubes, washed an additional 2 times. These antibodiesensitive platelets were solubilized by incubation for 1 hr at 4°C in TBS, pH 7.4, containing 1 Triton X-100 and 5 mM KI. These samples were then ultracentrifuged for 20 min at 100,000 g (4°C). These samples will be referred to as "I^125-sensitized platelet extracts."
(40 experiments). The gels were then fixed overnight in 25% isopropyl alcohol-10% acetic acid, dried, and applied to x-ray film and exposed at ~70°C for 3–5 days.

Absorption of ITP Plasma With Insolubilized GPIIb/GPIIa Complex

Purified GPIIb/GPIIa-specific monoclonal 3F5 was coupled with CNBr-activated Sepharose-4B following the manufacturer’s instruction (5 mg/ml). Aliquots of this conjugate were then reacted with a 1.0% Triton X-100 extract of either platelets (10^9 platelets/ml, 1.0 cc) or the neuroblastoma cell line, SK-N-SH (10^5 cells/ml, 1.0 cc). The Sepharose-monoclonal antibody conjugate specifically absorbs GPIIb/GPIIa molecules from the platelet extract. The Sepharose conjugates were extensively washed and then each incubated overnight at 5°C with 0.5 ml of ITP plasma. The absorbed plasmas were then studied in the immunoprecipitation system.

RESULTS

Assay of Human Plasmas for Anti-GPIIb/GPIIa Antibodies

Normal Subjects

Plasma from 16 normal subjects was tested. Mean (±SD) ratios were 0.93 ± 0.13 (Fig. 2). Variation between replicate samples was <10%.

Anti-Pl''1 Antibodies

As anti-Pl''1 antibodies bind to an antigenic site on platelet glycoprotein IIa, plasma from two patients with posttransfusion purpura was used as a positive control and to evaluate the sensitivity of the binding assay. Both plasmas, when tested in a 1/10 dilution against Pl''1-positive platelet extracts, showed markedly elevated ratios (ratios >30); neither plasma reacted when Pl''1-negative platelet extract was used. Additional dilutions of one of these anti-Pl''1 plasmas were evaluated. A dilution of 1/300 resulted in saturation of all GPIIa-binding sites, and positive results were detected at dilutions up to 1/10,000. This antiserum contains 25 μg/ml of specific antibody. As 100 μl of a 1/10,000 dilution contains only 0.25 ng of specific antibody, this assay is sensitive at a level of 0.1–1.0 ng of specific antibody.

Chronic ITP

Plasma from 56 patients with chronic ITP was studied. Five patients (4 adults, 1 child) had assay values (ratios 1.36–3.14) greater than 3 SD above the mean of the 16 normal plasmas (Fig. 2); the remaining 51 patients had normal values. One other positive value (ratio 1.53) was noted in a patient with recurrent Hodgkin’s disease, who presented with an ITP-like syndrome manifested by isolated thrombocytopenia (platelet count 10,000/cu mm), increased numbers of marrow megakaryocytes, and a markedly elevated platelet-associated IgG value of 21,452 ng IgG/10^6 platelets (control values <3,500). As our anti-Pl''1 antiserum, at a dilution of 1/2,700 (about 1 ng of specific antibody), had an assay ratio of 2.97, we estimate that our most positive chronic ITP patient had a plasma antibody concentration in this general range.

To confirm that the binding of autoantibodies to the platelet extract-treated microtiter wells was dependent on the presence of GPIIb/GPIIa, we removed GPIIb/GPIIa from platelet extracts and determined if 2G12-coated microtiter wells, reacted with such a depleted extract, would bind ITP autoantibodies. Platelet extract (3.0 ml) was incubated overnight at 4°C with either 50 μl of the GPIIb/GPIIa-specific monoclonal 4F10 bound to Sepharose or with the control protein 10–4.22 bound to Sepharose. The degree of GPIIb/GPIIa depletion of these treated extracts was measured by incubating the absorbed extracts with 2G12-coated microtiter wells, washing, and determining the ability of radiiodinated monoclonal anti-GPIIb/GPIIa 3F5 to bind to the microtiter wells. Using a 1/10 dilution of platelet extract, greater than 95% GPIIb/GPIIa was removed by this procedure. When
Precipitation of Radiolabeled Platelet Antigens With ITP Plasma

Plasma from the 5 chronic ITP patients, showing anti-GPIIb/GPIIIa antibodies, was incubated with \(^{125}\)I-labeled platelets and, after washing and solubilization with Triton X-100, the IgG and any bound radiolabeled antigens were precipitated with Staph-A bacteria. The Staph-A-associated proteins were then electrophoresed on SDS-PAGE, and the antigens were determined from autoradiographs of the gels.

Using plasma from patients 1 and 2, who had the most positive binding ratios, 2 distinct bands were noted with molecular weights of 100 and 120 kDa (reduced gels); under nonreduced conditions, 2 bands with molecular weights of 90 and 135 kDa were seen (Figs. 3 and 4). This behavior under reduced and nonreduced conditions is consistent with that of GPIIb and GPIIIa. In patients 3–5, whose ratios were less positive, autoradiographs were not definitely positive, although they were suggestive. Similar studies on 5 control subjects and 5 patients with chronic ITP who had negative assay values showed no evidence of GPIIb/GPIIIa precipitation.

An additional prominent radiolabeled band with a molecular weight of 150,000 kDa was also noted when plasma from patient 2 was studied; a similar band of lesser intensity was also seen in some of the control subjects, as were some faint bands of lower molecular weight. Immunoprecipitation of these low intensity bands probably represents a nonspecific background seen in our system.

Control Patients

Thirty-four patients with a variety of diseases of immune and nonimmune etiologies were also studied (Fig. 2); all had values within the normal range.
linked to Sepharose 4B and reacted with either GPIIb/ GPIIIa-containing platelet extracts or with control cell extracts. These Sepharose conjugates were then washed and reacted with aliquots of plasma from ITP patient no. 1. The resulting absorbed plasma was then used in the immunoprecipitation protocol. As shown in Fig. 5, the two prominent bands seen in the standard immunoprecipitation study are markedly diminished if the plasma is first reacted with platelet GPIIb/GPIIIa bound to monoclonal antibody-Sepharose. Densitometry of the autoradiographs showed an 89% reduction in the intensity of these two bands after absorption of plasma with insolubilized GPIIb/GPIIIa. Exposure of the plasma to control cell extract-treated monoclonal antibody-Sepharose did not decrease the intensity of the two bands.

**DISCUSSION**

We have determined that the plasma from some patients with ITP has autoantibodies reactive with antigenic determinants present on the platelet GPIIb/GPIIIa complex. None of the 16 normal controls and only one of 27 patients with immune-mediated thrombocytopenia due to other causes was positive for such antibodies. This patient had recurrent Hodgkin’s disease, which presented as an ITP-like syndrome. Seven patients with thrombocytopenia of nonimmune etiology also showed negative results.

It is unlikely that these GPIIb/GPIIIa-reactive antibodies were due to alloimmunization, as 4 of the 5 positive patients had no history of either transfusion or pregnancy. Additionally, several of the thrombocytopenic control patients with negative values had a history of blood transfusions, and three of these had high titers of anti-HLA antibodies.

The specific antigenic determinants on the GPIIb/ GPIIIa complex that react with these autoantibodies is as yet unclear. It is known that when GPIIb and GPIIIa are extracted from the platelet membrane with nonionic detergents, they exist as a calcium-dependent complex, which is dissociable by the action of EDTA. Some of the autoantibodies may react with antigenic determinants present on either GPIIb or GPIIIa alone, whereas others may be specific for the GPIIb/GPIIIa complex. It is also known that the GPIIb/GPIIIa complex can associate with additional glycoproteins, such as fibrinogen, or other platelet cell-surface molecules. It is therefore possible that some of the GPIIb/GPIIIa-reactive autoantibodies detected in our assay are binding to antigenic determinants on other proteins, which are in turn associated with the GPIIb/GPIIIa complex.

The low percentage of anti-GPIIb/GPIIIa antibodies demonstrated directly by us differs from the higher incidence of positive indirect findings reported by van Leeuwen et al., who noted serum antibody from 14 (33%) and platelet eluates from 35 (83%) of 42 patients with chronic ITP would bind to normal, but not to thrombasthenic, platelets. The plasma results noted in these two studies are difficult to compare for a variety of reasons: (1) the patient populations may differ in their relative distribution of children and adults, disease severity, and sampling times relative to therapy; (2) the assays vary in sensitivity, objectivity (the suspension immunofluorescent test does not result in a numerical endpoint), and specificity (their results include patients with IgM and IgA antibodies, which we did not evaluate); (3) the present studies directly measure anti-GPIIb/GPIIIa antibodies, whereas the results of van Leeuwen et al. assume that the absence of binding of thrombasthenic platelets is due to the lack of GPIIb/GPIIIa in the target platelets. Since other protein abnormalities have been noted in these patients and the absence of important membrane proteins may affect antigenic structures normally present
on the platelet surface, their results may be positive for a variety of reasons. The higher incidence of positive results, noted by these authors when platelet eluates from ITP patients were studied, is consistent with the observation by several groups that platelet-bound antibody is demonstrable in essentially all chronic ITP patients, whereas a lesser percentage (30%–60%) has detectable serum antibody. Presumably, if we had eluates available from our patients, the percentage of positive results would be greater.

It also seems likely that autoantibodies toward other platelet membrane proteins will be demonstrable in the future. Patient 2 was noted to have a prominent band of 150,000 daltons on immunoprecipitation studies. Additional studies, which will be reported in the future, show that this is due to anti-GPIIb autoantibody.

In summary, we have shown that some ITP patients have autoantibodies in their plasma that are reactive with antigenic determinants associated with platelet GPIIb/IIIa. The exact frequency and antigenic specificity of these autoantibodies is as yet unknown.

REFERENCES

Autoantibodies against the platelet glycoprotein IIb/IIIa complex in patients with chronic ITP

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