Identification of Platelet Proteins That Bind Alloantibodies and Autoantibodies

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We have used the techniques of radioimmunoprecipitation (RIP) and Western blot to identify the membrane proteins that bind certain alloantibodies. Anti-Pl\textsuperscript{A1} sera precipitated two bands, corresponding to platelet glycoproteins Iib and III, whether or not calcium was present during the procedure. By Western blot, this antibody bound only glycoprotein III. Anti-Pl\textsuperscript{A1} serum does not precipitate proteins from the platelets of a patient with Glanzmann's thrombasthenia. Two monoclonal antibodies reacting with lymphocyte HLA antigens, as well as sera from highly allosensitized patients, precipitated bands of 38,500 and 13,500 daltons. These bands correspond to the molecular weights of the two subunits of the HLA antigen, as it has been described for other cell types. The patients' sera also precipitated a protein of 72,000 daltons from some platelets. The sera of two patients with quinidine-induced thrombocytopenia precipitated a 138,000-dalton band (glycoprotein Iib-alpha) in the presence of quinidine. The purified IgG antibody from one patient did not require other plasma factors to bind to platelets in the presence of quinidine, while purified antibody from a second patient required plasma factors other than, or in addition to von Willebrand factor. Although several sera from patients with idiopathic thrombocytopenic purpura (ITP) were tested, only one precipitated membrane proteins by the RIP method; this serum identified binding proteins corresponding to glycoproteins Iib and III.

In recent years, techniques have been developed to detect the binding of IgG to the platelet surface. In the case of alloantibodies, the specificity of the binding antigens have, in many cases, been defined serologically. More recently, direct evidence has been reported that demonstrates the binding of the most common platelet-specific alloantibody, anti-Pl\textsuperscript{A1} (Zw), to glycoprotein III of the platelet surface.\textsuperscript{1-3} Furthermore, indirect evidence has suggested that glycoprotein Ib is important in the binding of quinidine-induced thrombocytopenia.\textsuperscript{4,5} The HLA antibodies appear to bind to proteins similar to HLA-bearing proteins on other cells.\textsuperscript{6}

In this work, we have analyzed immunochemically some of the membrane proteins to which alloantibodies bind. We have relied primarily upon immunoprecipitation and the Western blot technique to demonstrate these proteins.

**MATERIALS AND METHODS**

**Platelet Preparation**

Platelets were isolated from blood that was anticoagulated in EDTA (0.2 mL 10% EDTA/10 mL whole blood). Platelet-rich plasma was prepared by centrifugation for 15 minutes at 110 g. The isolated platelets were washed three times in phosphate-buffered saline, pH 7.4, containing 0.015 mol/L EDTA (PBS-EDTA). Platelets were prepared as described above.

Blood from a patient with type I Glanzmann's thrombosthenia was kindly provided by Dr Gilbert C. White II, University of North Carolina, Chapel Hill. The characteristics of the membrane surface proteins of this patient's (J.W.) platelets have been reported.\textsuperscript{7} Platelets were prepared as described above.

**Immunoglobulin Sources**

Anti-Pl\textsuperscript{A1} antibody was obtained from two patients with posttransfusion purpura who had been plasmapheresed. Apheresis units were recalcified, incubated at 56°C for 30 minutes to inactive complement, aliquoted, and the serum was stored frozen at −90°C. Serum from patients with idiopathic thrombocytopenia purpura (ITP) was screened for detectable circulating antiplatelet antibodies by the radiolabeled monoclonal antibody technique described below. Positive samples were aliquoted and stored frozen at −90°C. Blood from patients with drug-induced thrombocytopenia was collected for serum. This serum was screened for reactivity of antibody with platelets in the presence and absence of the appropriate drug(s). Sera from allosensitized patients with high titer anti-HLA antibodies, either single antigen specificity or broad spectrum, were kindly provided by Dr Emily Reisner, HLA Typing Laboratory, Duke University Medical Center. Two monoclonal antibodies recognizing lymphocyte HLA antigens were provided by Dr Barton Haynes, Duke University Medical Center. Antibody 3F10, a murine monoclonal antibody of the IgG\textsubscript{1} subclass, recognizes the nonpolymorphic backbone of HLA, and antibody 4D12, a murine monoclonal antibody of the IgG\textsubscript{1} subclass, binds to lymphocytes containing antigens of the B5 cross-reactive group.\textsuperscript{8}

All sera were screened using a radiolabeled monoclonal anti-IgG binding assay.\textsuperscript{9} Normal platelets were prepared as described above and resuspended in PBS-EDTA to a concentration of 500 × 10\textsuperscript{6} /mL. Two hundred microliters of platelets were then incubated for 30 minutes at 37°C with equal volumes of patient plasma or normal plasma (with or without drug, depending on the suspected etiology of the thrombocytopenia). After three washes in PBS-EDTA, 100 μL of platelets and 100 μL \textsuperscript{125}I-labeled monoclonal anti-IgG (Bethesda Research Laboratories, Bethesda, Md), which had been purified from ascitic fluid, were incubated for 30 minutes at 37°C. Radiolabeled anti-IgG was used at a concentration of 10 μg/mL. Following incubation, three 50-μL aliquots of the reaction mixture were then spun through phthalate oils (1.5 parts n-butyl phthalate and 1 part bis[2-ethylhexyl] phthalate) to separate bound radiolabel from unbound. The pelleted cells were then counted in a
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scintillation type gamma counter, and the molecules of anti-IgG bound were calculated from the specific activity of the radiolabel. Controls were normal platelets incubated with autologous plasma, with and without the drug, as well as normal platelets incubated with buffer only. A similar assay has been described by LoBuglio and co-workers. The reactivity of the murine monoclonal antibodies was assayed by the technique described above, but the radiolabeled anti-IgG used was a 125I-labeled F(ab')2 fragment of a sheep antimouse IgG (Amersham, Arlington Heights, Ill).

All sera were tested for the presence of immune complexes using a modification of the Clq-binding method of Glikmann and Svehag. 125I-labeled staphylococcal protein A was used to detect Clq-bound aggregated IgG. 121-I-labeled aggregates of IgG prepared using the method of Segal and Hurwitz, as modified by Kurlander and Barker, were used for calibration.

Radioimmunoprecipitation (RIP) Procedure

Washed platelets prepared as described above were labeled with 125I-sodium iodide (Amersham). Platelets (10⁷) were suspended in PBS-EDTA and labeled with 1 mCi of 125I, using the IodoGen method, as modified from Fraker and Speck. Briefly, a 1.5-mL polystyrene microfuge tube was filled with 1 mL of dichloromethane containing 300 µg IodoGen (Pierce Chemicals, Rockford, Ill). This solution was then evaporated under a stream of dry nitrogen. After incubating the platelets and 125I in this tube for 15 minutes at room temperature with mixing, the platelets were placed in a clean tube and washed three times with phosphate-buffered iodide (0.02 mol/L PO₄, 0.003 mol/L KCl, 0.14 mol/L NaI), containing 0.015 mol/L EDTA, followed by three washes in PBS-EDTA. Labeled platelets were spun down and resuspended in 1 mL 10 mmol/L Tris-HCl, pH 7.2, containing 0.15 mol/L NaCl, 0.02% NaN₃, and 1% Nonidet P-40 (Sigma, St Louis, Mo). After incubating for 30 minutes at 4 °C with mixing, the platelet lysate was centrifuged for 30 minutes at 50,000 g at 4 °C. The amount of trichloroacetic acid (TCA)-precipitable protein in the platelet lysate was generally greater than 80%.

IgG sorb (The Enzyme Center, Boston) (formalin-fixed protein A-containing Staphylococcus aureus in 10% suspension) was washed three times in PBS containing 0.5% Nonidet P-40 (SAC buffer) and resuspended to starting volume. The platelet lysate was precleared of nonspecifically adsorbing platelet membrane components by incubating 600 µL of bacterial suspension, which had been centrifuged and resuspended in 1 mL of platelet lysate. After a 30-minute incubation at 4 °C, the bacteria were centrifuged out of the lysate.

One hundred microliters of precleared lysate was incubated with 10 µL of serum (approximately the optimum amount for this amount of platelet lysate) or buffer for one hour at 4 °C. Two hundred microliters of washed bacterial suspension was added after platelet lysis. Immunoprecipitations were performed (1) in the presence of 15 mmol/L EDTA in platelet washing buffer, (2) with 15 mmol/L EDTA in all buffers, and (3) with 5 mmol/L Ca²⁺ added after platelet lysis. Lanes A show bands precipitated by anti-PiA⁺ serum, and lanes B show autologous serum controls. Lane C is platelet lysate not reacted with serum or precipitated. As this lane is somewhat underloaded, refer to Fig 4, lane I, for a more representative example of the labeling pattern of surface proteins.

Western Blot Procedure

The procedure followed was modified from Towbin et al. Washed platelets (10⁷) were resuspended in 200 µL nonreducing SDS-PAGE sample buffer and boiled for two minutes. Twenty-five microliters per lane was loaded on a 5% to 15% gradient gel. After electrophoresis was complete, the proteins were blotted onto nitrocellulose paper using a TransBlot protein blotting system (BioRad Laboratories). The transfer buffer was 20% methanol, 20 mmol/L Tris base, 150 mmol/L glycine, and the transfer was made at 150 mA constant current for 15 hours. Following an overnight incubation in Tris-saline containing 3% bovin serum albumin (BSA), the nitrocellulose paper was incubated with a 1:10 dilution of serum for 30 minutes at room temperature with mixing. After six to eight washes with Tris-saline, the paper was then incubated for one hour with 125I-labeled affinity-purified goat anti-human IgG (Tago, Inc, Burlingame, Calif), diluted with Tris-saline containing 3% BSA to a concentration of 10⁶ cpm/mL. After extensive washing with Tris-saline, the paper was dried and autoradiographed.

RESULTS

Anti-PiA⁺ Antibodies

When the sera or the IgG fraction of sera from patients with known anti-PiA⁺ that had caused post-transfusion purpura was used in the RIP assay, proteins that had the characteristics of glycoprotein (gp) IIb and gpIII were precipitated. These proteins were found to be 132,000 daltons and 89,000 daltons,
proteins were immunoprecipitated (Fig 3). The radioactivity precipitated from these platelets using antisera positive on normal platelets was the same as autologous serum. Using the radiolabeled monoclonal anti-IgG assay, Glanzmann's thrombasthenia platelets also failed to bind anti-P(I)A antibody and four of the seven ITP antisera that were positive on normal platelets.

HLA Antibodies

Three types of antibody reacting with HLA antigens were used:

1. Monoclonal antibody 3F10, which was previously shown to be directed against either the nonpolymorphic backbone common to all HLA antigens, or monoclonal antibody 4D12, which detects antigens of the B5 cross-reactive group.

2. Alloimmune sera from patients who had received multiple transfusions of platelets.

3. Alloimmune sera from patients specifically immunized against single HLA antigens.

With the monoclonal antibody 3F10, two proteins were precipitated of 38,500 and 13,500 daltons (Fig 4). The monoclonal antibody directed against the B5 antigen family immunoprecipitated primarily the low molecular weight component from platelets of a person with lymphocytes known to bear the B5 antigen.

Serum from a patient highly alloimmunized precipitated the same two proteins (Fig 4); in addition, a third protein of approximately 72,000 daltons was also precipitated. When antisera of a single HLA specificity were used, no proteins were specifically precipitated.
Table 1. Inhibition of Binding of Monoclonal Antibody 4D12 by Human Serum Containing Polyspecific Anti-HLA Antibodies

<table>
<thead>
<tr>
<th>Dilution of 4D12 Ascitic Fluid</th>
<th>Net Molecules of 125I-Anti-Mouse Ig Bound per Platelet</th>
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<tbody>
<tr>
<td>1:1,000</td>
<td>789</td>
</tr>
<tr>
<td>1:2,000</td>
<td>885</td>
</tr>
<tr>
<td>1:4,000</td>
<td>589</td>
</tr>
<tr>
<td>1:8,000</td>
<td>643</td>
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To demonstrate that the alloantibodies and monoclonal antibodies were reacting with the same molecule, platelets were reacted with either autologous serum or polyspecific alloimmunized serum, were washed, and were then reacted with monoclonal antibody 4D12 in various dilutions. Alloimmune serum inhibited the binding of the monoclonal antibody, whereas autologous serum did not (Table 1).

Drug-Dependent Antibodies

Antibodies directed against drugs were detected in the serum of patients with the clinical syndrome of drug-induced immune thrombocytopenia using the radiolabeled monoclonal antibody test. Those antisera demonstrating binding in the presence of the drug but not in its absence were selected for study (Table 2); radioimmunoprecipitations were performed in the presence and in the absence of the drug. In two patients, a glycoprotein of 138,000 daltons, corresponding to gpIb-alpha was immunoprecipitated when quinidine and antibody-containing serum were present (Fig 5). Because these immunoprecipitations were performed without preclearing the platelet lysate, they show the presence of glycoproteins IIb and III as well as some fainter contaminating bands. One example of serum from a patient with quinidine-induced purpura and all other examples of sera from patients with drug-induced thrombocytopenia did not specifically precipitate any proteins.

To demonstrate the role of von Willebrand factor (vWF) or other proteins in the binding of quinidine and antiquinidine antibodies, platelets were extensively washed until free of vWF, as demonstrated by lack of aggregation with ristocetin, and were incubated with buffer, autologous serum, patient serum, or the IgG fraction of patient serum (shown to be lacking in vWF by platelet aggregation studies) with and without the addition of 40 μg of purified vWF (kindly donated by Dr Patrick McKee, Duke University Medical Center) and with and without 2 mmol/L quinidine. The results are shown in Table 3. The activity of the vWF was verified by assessing its ability to aggregate vWF-free platelets in the presence of ristocetin. Antibody in the IgG fraction of serum from patient Gl. binds to the platelet in the presence of the drug without the media-
tion of any other protein; this binding is not increased by added vWF. Antibody in the IgG fraction of serum of patient Tr. does not bind in the presence of the drug, even when purified vWF is added. However, in the presence of autologous plasma, the IgG fraction, and drug, IgG is bound to platelets from normal donors. This indicates that a protein other than vWF present in normal plasma is necessary for the binding of the quinidine-antibody complex.

**ITP Antibodies**

Sera were collected from patients with ITP. Seven sera showing elevated antiplatelet antibody levels of greater than 6,000 molecules per platelet by the radio-labeled antiglobulin binding assay described above were examined. Only one of these seven sera precipitated labeled membrane proteins (Fig 3, lane E). Although this autoantibody precipitated the same bands as anti-PI\(^{A1}\) antiserum, it reacted with PI\(^{A1}\)-negative platelets in the radiolabeled anti-IgG binding assay. Glycoproteins IIb and III were precipitated by this antibody. No other serum tested specifically precipitated any labeled membrane proteins (data not shown).

**DISCUSSION**

With the development of the methodology for the assessment of IgG on the platelet surface, the detection of the binding of alloimmune and autoimmune antibodies to the platelet has become commonplace. However, little is known about the structures to which these antibodies bind and the necessary conditions for their binding. In this article, we have defined immunochromatically the surface antigens to which IgG, in various forms of alloimmune thrombocytopenia, binds.

The antigen reacting with the anti-PI\(^{A1}\) antibody has been shown by other techniques to be present on gpIII.\(^{2,3}\) In the platelet membrane, gpIIb and gpIII exist in a calcium-dependent complex that is dissociated in the presence of EDTA.\(^{15,19}\) In the present study, we have confirmed by Western blotting that the PI\(^{A1}\) antigen is on gpIII because only this protein reacts with anti-PI\(^{A1}\) after separation by electrophoresis, confirming the studies of McMillan et al.\(^{1}\) However, if antibody is reacted with the protein after the physical disruption of the membrane, in the absence or in the presence of calcium, both gpIIb and gpIII are precipitated. This suggests that either the conditions were not sufficient for dissociation of the glycoprotein IIb/III complex or binding of antibody to antigen in some way stabilizes the glycoprotein IIb/III complex, even following the removal of calcium. These data do not support the reports of Kunicki and co-workers,\(^{19}\) who have demonstrated calcium-dependent association of gpIIb and gpIII using Triton X-100 extracts of platelets in a crossed-immunoelectrophoresis system. In addition to the difference in assay systems and detergents, Kunicki and co-workers were able to show chelation of the required Ca\(^{2+}\) with 1 mmol/L EDTA, whereas the RIP experiments reported here show that both gpIIb and gpIII precipitated in the presence of 15 mmol/L EDTA.

One autoimmune antibody immunoprecipitated glycoproteins IIb/III; however, this antibody does react with PI\(^{A1}\)-negative platelets, indicating that it reacts with an epitope different from the one reacting with anti-PI\(^{A1}\). All other antibodies from patients with ITP failed to immunoprecipitate labeled surface glycoproteins. This may reflect the insensitivity of the RIP procedure or the fact that the antigens lie on molecules that do not label by this procedure or contain antigens that are destroyed when the membrane is solubilized.

HLA antigens, which are widely distributed on most cells of the body, usually consist of two proteins, a heavy chain and a light chain (beta-2-microglobulin). The calculated molecular weight determined in these studies is approximately 38,500 and 13,500, respectively. Thus, although they may be slightly smaller than similar molecules in other cells, the organization of the proteins responsible for these antigens on platelets appears to be entirely similar. Of these two molecules, beta-2-microglobulin labels more readily and is therefore more easily detected. These same molecules are detected by monoclonal antibodies against epitopes on the nonpolymorphic backbone or by antigen-specific antibodies. In addition, the sera from two patients who were highly sensitized by platelet transfusion may precipitate a protein of approximately 72,000 daltons; this probably represents a non-HLA antigen, the identity of which is not known.

Drug-induced immune thrombocytopenia was first described with quinine,\(^{20}\) and quinine and quinidine remain the two most common causes of this syndrome. Controversy has existed as to the mode of attachment of the drug and antibody to the platelet. Some have maintained that the attachment was by immune complex, thus implicating the Fc receptor of the platelet in the attachment of both drug and antibody.\(^{21}\) Others have held that the quinidine was attached to a protein in a haptenic relationship, which then bound the antibody. Recent evidence has suggested that the binding protein is gpIIb and that quinidine is bound to von Willebrand factor, which in turn binds to this glycoprotein.\(^{22}\) In the present experiments, we have demonstrated that in two out of three cases of quinidine-induced thrombocytopenia, gpIIb is immunoprecipitated by antiquinidine antibody in the presence of EDTA.
of the drug but not in its absence. Careful studies, however, have shown that in neither case was von Willebrand factor necessary. In one instance, no other protein was needed, as thoroughly washed platelets free of von Willebrand factor appeared to bind the antibody to gplb in the presence of quinidine. In the other instance, a protein from plasma was required, but purified von Willebrand factor alone was not effective in providing the binding to gplb. In no other instance of drug-induced immune thrombocytopenia were we able to identify a binding protein.

These studies have demonstrated the usefulness of RIP to isolate the proteins that bind to alloimmune antibodies. Unfortunately, the technique does not appear to be as sensitive as the radiolabeled monoclonal antibody assay for the detection of antibody binding to platelet components, as with many sera, antibody binding can be demonstrated by the monoclonal antibody assay but not by RIP or Western blot techniques. Alternatively, these antibodies may bind to antigens that do not survive the procedures of isolation of membrane components. Nevertheless, in some cases, it is able to define biochemically autoantibody and alloantibody binding proteins.

REFERENCES

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