Western Blot Identification of Platelet Proteins That Bind Normal Serum Immunoglobulins. Characteristics of a 95-Kd Reactive Protein

By Diane M. Reid, Charles E. Jones, Jaroslav G. Vostal, and N.R. Shulman

We have found that Western blots (WBs) of whole platelets exposed to normal autologous or homologous sera commonly have bands at 90 to 95 (95) Kd, and less often at 100 to 110, 80 to 85, 60 to 75, and 50 to 60 Kd when developed with antiglobulins. The percentages of normal sera producing a 95-Kd band with anti-immunoglobulin G (IgG) -IgA, and -IgM are 85, 50, and 30, respectively. Antiglobulin reagents alone also produce background bands on WBs that we have shown correspond to levels of platelet-associated lgs (PALgs) or their derivatives. Titers of 95 Kd-reactive IgG in normal sera range from 10 to 1,280 (85% ≤ 50), and the reaction appears to be partially F(ab')₂-mediated. The 95-Kd protein is internal and differs in many respects from surface glycoproteins III, IV, V of similar apparent molecular weight. In thrombocytopenic patients there was no correlation between severity of thrombocytopenia or PALg of platelet eluates and corresponding serum titers of 95 Kd-reactive IgG. Some WB reactions previously reported as evidence of autoimmune may represent normal variations in reactions of lgs with internal platelet proteins. These reactions may be immunospecific or analogous to nonspecific, partially F(ab')₂-dependent binding of lgs by certain bacterial proteins.

R E A C T I O N S O F alloantibodies with Western blots (WBs) of platelets have been a reliable basis for diagnosing neonatal and posttransfusion disorders, identifying new platelet alloantigen systems, and defining glycoproteins (GPs) that contain specific epitopes. WB techniques also have been used to identify apparent antiplatelet autoantibodies in sera of thrombocytopenic patients with a variety of disorders, including systemic lupus erythematosus (SLE), idiopathic thrombocytopenic purpura (ITP), acquired immunodeficiency syndrome (AIDS), sepsis, posttransfusion purpura, and drug purpura. However, in the process of screening sera of similar groups of patients for antiplatelet antibodies, we found that control normal sera often produce WB reactions resembling those reported as hallmarks of autoimmune disease. In addition, antiglobulin reagents alone produce bands that have been noted previously, but are not well-characterized. We have evaluated experimental parameters of WB assays that affect the number and intensity of bands and have concluded that controls for normal sera and antiglobulins have often been inadequate. For this reason, some WB patterns previously interpreted as evidence of autoimmunity may represent quantitative variations of normal rather than qualitatively pathologic reactions. Normal autologous or homologous immunoglobulins (lgs) were found to react in 85% of instances with a 90 to 95-Kd internal platelet protein, and less commonly with proteins of 100 to 110, 80 to 85, 60 to 75, and 50 to 60 Kd. We have established the differences that distinguish the 95-Kd protein from other platelet proteins of similar molecular weight.

MATERIALS AND METHODS

Sera. Sera were obtained from normal adult National Institutes of Health (NIH) Blood Bank donors (sex distribution approximately 55% male, 45% female) and stored at −20°C. Samples were centrifuged at 12,000 x g 5 minutes before use. Baboon and rhesus monkey sera were obtained from the NIH animal center.

Reagents. Purified human IgG was obtained from Cappel, Cochraneville, PA; Sigma, St. Louis, MO; and Miles, Elkhart, IN. IgM and IgA were obtained from Cappel.

Platelet preparations. Platelets from normal and thrombocytopenic donors were isolated from EDTA-whole blood by differential centrifugation, washed once in 1% ammonium oxalate and three times in phosphate-buffered saline, pH 7.4, containing 5 mmol/L EDTA (PBS-EDTA).

Some platelet preparations were obtained by elutriation (Beckman JE-10X elutriator, Palo Alto, CA) of citrate platelet-rich plasma (PRP) obtained by apheresis.

Bernard-Soulier platelets were obtained with the help of Dr Margaret Johnson, Christiana Hospital, Newark, DE. Erythrocytes were separated from freshly drawn Bernard-Soulier blood by gravity sedimentation, and white blood cells (WBCs) were removed by repeated centrifugation at 150 x g. Subsequent processing of platelets was identical to that described above. WBC contamination per 10,000 platelets was consistently less than 10 in normal donor preparations, less than 100 in Bernard-Soulier preparations, and less than 4 in elutriated platelet preparations.

Platelets for electrophoresis were solubilized in an equal volume of treatment buffer containing 0.125 mol/L Tris-Cl pH 6.8, 4% sodium dodecyl sulfate (SDS), 20% glycerol, and 0.1% bromphenol blue, and used immediately or stored at −70°C. When protein reduction was desired, samples were additionally treated with dithiothreitol (Boehringer-Mannheim, Indianapolis, IN) and iodoacetamide (Sigma). Otherwise, platelets were treated with sodium azide to a final concentration of 0.02% and stored at 4°C.

Platelet membrane preparation. Membranes were prepared by glycerol-loading, hypotonic lysis, and sedimentation through a sucrose density step gradient. Membranes were collected at the interface between the 0.25 mol/L and 27% sucrose solutions, washed twice in PBS, and stored at −70°C.

Mechanical lysis of platelets. A saline suspension of washed platelets (3 x 10⁶/μL), containing 125 μg/mL leupeptin, 2 mmol/L phenylmethylsulfonyl fluoride (PMSF), and 10 mmol/L EDTA, was sonicated on ice, freeze-thawed three times, and centrifuged at 61,500 x g for 30 minutes. The supernatant was filtered through a 0.2 μm filter and analyzed for protein. The membrane pellet was washed once with water, solubilized in 2% Triton X-100 (Pierce, Rockford, IL), stirred for 1 hour at room temperature, centrifuged at

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100,000 x g for 30 minutes to remove the Triton-insoluble fraction, concentrated, and subjected to protein determination.

**WBC preparation.** Hetastarch, 6% in 147 mmol/L NaCl containing 0.03% sodium citrate (American McGaw, Irvine, CA) was added to citrated whole blood in a ratio of 1 part per 8 parts whole blood. Red blood cells (RBCs) were sedimented by gravity and WBCs in the supernatant plasma were pelleted by centrifugation at 170 x g for 10 minutes and washed three times with normal saline (NS). Differential counts indicated 50% to 60% polys, 40% to 50% lymphs, and 1% platelets. Protein was measured before WBCs were solubilized in electrophoresis treatment buffer (see platelet preparations) and stored at -70°C.

**GP IIb/IIIa preparations.** Solubilized platelet membranes were applied to a Concanavalin A-Sepharose 4B column (Pharmacia, Piscataway, NJ). Triton X-100 was removed by washing the column with 0.1% CHAPS (Pierce), a detergent that does not absorb at 280 nm, in PBS. The column was eluted with 10% a-methyl-D-mannopyranoside (Sigma) and the eluate was dialyzed versus PBS-EDTA buffer with 0.1% SDS, applied to a Sephacryl S-300 column (Pharmacia), and fractions containing a single band at 85 to 90 Kd (GP IIb/IIIa) by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (7.5% T) were stored at -70°C.

**F(ab')2 preparations.** IgG from serum of a normal donor containing 95 Kd-reactive IgG that titered 1,280 (dilution (-1) was prepared by chromatography using CM-Affigel Blue (BioRad, Richmond, CA) and Protein A-tris-acyl (Pierce) matrix, following manufacturer's instructions. The purified IgG was digested with Immobilized Pepsin (Pierce) at pH 4 for 4 hours at 37°C with constant agitation. F(ab')2 fragments were separated from residual IgG by sequential passage over Sephacryl S-300, Protein A-tris-acyl, and anti-human Fc affinity columns, the latter prepared by coupling anti-Fc (Caltag, South San Francisco, CA) to a Reacti-Gel 6-X activated resin (Pierce) per manufacturer's instructions. SDS-electrophoresis of the final product showed a single band at 120 Kd by Coomassie staining, and an immunoblot assay in which 30 μg of F(ab')2 were loaded per lane was free of IgG. The lowest quantity of IgG detectable by this immunoblot assay was approximately 10 ng.

**Protein determinations.** Biochimiconic acid (BCA) reagent was used according to the manufacturer's directions (Pierce). Standards consisted of solutions of bovine serum albumin (BSA) (Fraction V, Sigma) in PBS (with or without detergent) having concentrations ranging from 0.03 to 2.0 mg/mL. IgG and F(ab')2 proteins were also quantitated specifically by microtiter sandwich enzyme immunoasays (EIAs).

**ELISAs.** Microtiter plates (Immulon I, Dynatech, Chantilly, VA) were coated with goat anti-human IgG, γ chain-specific (Kirkegaard & Perry Laboratories [KP], Gaithersburg, MD), 750 ng/well in 100 μL of coating buffer. The coating, washing, and blocking buffers were prepared from stock reagents contained in an ELISA Mate™ kit (KP). Plates were blocked with buffer containing 1.0% BSA. Samples, diluted in blocking buffer, were incubated for 30 minutes at room temperature; plates were washed three times; and alkaline phosphatase-conjugated goat anti-human IgG, γ specific (KP), was applied. After 60 minutes, plates were washed three times. p-nitrophenyl phosphate substrate in diethanolamine buffer (KP) was added, and absorbance measured with a Dynatech MR 650 plate reader. Sensitivity was linear in the 2 to 65 ng range. All samples and controls were done in triplicate.

**Thrombin treatment of platelets.** Fresh, washed platelets (1 x 10^9/mL) in PBS-EDTA were incubated with or without 5 U/mL human thrombin (Sigma), final concentration, at 37°C for 60 minutes. Without centrifugation, samples were immediately solubilized, electrophoresed, transferred to nitrocellulose, and developed after exposure to normal serum.

In a separate experiment, 5 x 10^9 fresh washed platelets in PBS-EDTA were exposed to thrombin (5 U/mL) or saline for 30 minutes at 37°C, pelleted at 1,700 x g for 10 minutes, and the supernatants were passed through a 0.2-μm filter. The quantity of 95-Kd protein present in filtrates was determined by WB titration and compared with that in a 0.2-μm filtrate of an equal number of the same platelets lysed by sonication and freeze-thawing (see mechanical lysis of platelets).

**Electrophoresis.** Discontinuous SDS-PAGE was performed according to the method of Laemmli on a BioRad Mini Protein II electrophoresis cell. Unless otherwise stated, resolving gels contained 7% T acrylamide and had dimensions of 8 x 5.2 cm x 1.5 mm. Gel reagents were prepared as outlined in the Hoefer electrophoresis guide (Hoefer Scientific Instruments, San Francisco, CA). BioRad high molecular weight (mol wt) standards (3 μL per lane) were used. Unless otherwise stated, fresh platelets were loaded on the basis of count (4 x 10^7 per lane) and gels were electrophoresed under nonreducing conditions at a constant voltage of 150 V for 60 to 75 minutes.

Protein blotting was performed according to the guidelines of Burnette using 0.45-μm pore nitrocellulose paper (Schleicher & Schuell, Keene, NH) on a BioRad Mini-Transblot cell with a modified buffer containing 10% methanol, at constant current of 150 mA for 2 hours or 90 mA overnight. Dried blots were stored at 4°C in zip-lock bags up to 1 month before development.

**WB analysis.** Nitrocellulose strips were subjected to the following sequential manipulations on a rocker platform using 5 mL of reagent per strip: (1) Nonspecific protein blocking for 60 minutes; solutions in PBS tested included 1% gelatin, 1% normal goat serum, 0.5% BSA Penetx Fraction V (Miles), and Blotto, a 5% nonfat dry milk solution, containing 0.01% antifoam A emulsion (Sigma). Diffuse background color was least with Blotto, but otherwise the results were the same. (2) Thirty-minute incubation with test serum diluted in Blotto; (3) two five-minute Blotto washes; (4) sixty-minute incubation with biotin-labeled affinity-purified goat anti-human IgG, -IgA, or -IgM, heavy chain specific (KP) diluted 1/500 in Blotto. This dilution was chosen after comparing dilutions from 1/100 to 1/500 for line clarity and interfering background color. (5) Two Blotto washes; (6) sixty-minute incubation with KP peroxidase-labeled streptavidin (SP) diluted 1/330 in Blotto; (7) two biotinylated goat anti-human IgG, F(ab')2 fragment, fragment, (Caltag). Reagents used for the assay to determine purity of the human F(ab')2 preparation were sheep anti-human IgG, F(ab')2 specific antibody (ICN, Lisle, IL), each diluted 1/250.

Other reagents that were tested and found to give nearly indentical results to the KP biotinylated antiglobulin reagent at comparable dilutions were biotinylated goat anti-human IgG, F(ab')2, fragment, γ specific (Tago, Burlingame, CA) and horseradish peroxidase (HRPO)-labeled goat anti-human IgG, Fc specific, (Caltag). Reagents used for the assay to determine purity of the human F(ab')2 preparation were sheep anti-human IgG, F(ab')2 specific antibody (Cappel) and HRPO-labeled donkey anti-sheep IgG, H + L specific (ICN, Lisle, IL), each diluted 1/250.

**Immunofluorescence studies.** A total of 1.5 x 10^7 fresh saline-washed platelets were incubated with test sera diluted 1/10, 1/100, 1/1000, and 1/10,000 in a total volume of 0.5 mL of PBS-5 mmol/L EDTA-0.5% BSA for 60 minutes, washed once, incubated 60 minutes with fluorescent-labeled goat γ-specific anti-human IgG (FITC-anti-IgG, KP) diluted 1/500 in the same buffer, washed once, and then resuspended in 0.5 mL buffer. Cell fluorescence was measured in a Becton Dickinson Fluorescence Activated Cell Sorter (FACS; Mountain View, CA).

**Adsortion studies.** Aliquots, 2.0 mL, of one normal serum containing IgG reactive in a titer of 320 with the 95-Kd protein were
exposed for 1 hour at 37°C to 8 x 10⁸ platelets that had been freshly washed and pelleted. In addition, 2 mL of the same normal serum was mixed with an equal volume of the initial supernatant of 4 x 10⁶ lysed platelets after it had been filtered through a 0.2-µm filter. Adsorbed aliquots were titered by serial dilution against WBs of whole platelets obtained from the donor of the serum.

Platelet-associated IgG (PAIgG) measurement. PAIgG determinations were performed according to the technique of Hotchkiss et al.¹ using microtiter EIA rather than RIA to quantitate IgG. Serum samples obtained concurrently were titrated for overdeveloped (lane 2), also at 75 and faintly at 44 Kd. Anti-IgA (lane 3), even with overdevelopment, produced only a faint band at 200 to 210 Kd, and no bands were produced with anti-IgM (lane 4). The minimum number of platelets per lane needed to produce the 200 to 210-Kd background band was 0.6 to 0.8 x 10⁶, whereas other background bands required three- to fourfold more platelets per lane. Platelet blots exposed only to SP and 4CN-H₂O₂ did not produce bands. WBs of commercially purified IgG (Fig 1B, lane 5), IgA (lane 6), and IgM (lane 7) exposed to the respective biotinylated antiglobulin reagents, SP, and 4CN-H₂O₂ yielded bands indistinguishable from those background bands produced on blots of whole platelets. Quantities of purified Ig yielding patterns equivalent to background reactions of 2.4 x 10⁹ platelets corresponded approximately to the expected amount of total platelet-associated IgG, IgA, or IgM from the same number of platelets,³⁶ i.e. 0.06 to 0.12 µg IgG, 0.01 to 0.02 µg IgA, and <0.001 µg IgM.

When platelets or purified Iggs were electrophoresed under reducing conditions before transfer to nitrocellulose, bands only at 50 to 55 Kd were seen with anti-IgG and anti-IgA; no bands were visible with anti-IgM. Moreover, all background bands produced on WBs of platelets or of Iggs with anti-IgG or anti-IgA could be blocked by preincubating the antiglobulin for 60 minutes with an equimolar amount of purified human IgG or IgA, respectively. The platelet WB background reactions, all of which appeared to be attributable to platelet-associated Iggs or their derivatives, were duplicated with two different biotinylated goat anti-human IgG reagents, one HRPO-labeled goat anti-human IgG reagent (KP. Tago, Caltag, respectively, as listed in Materials and Methods), and one rabbit anti-human IgG (Cappel) used in combination with biotinylated goat anti-rabbit IgG (KP).

Patterns on WBs of platelets exposed to normal sera or purified human Iggs before development with antibiogen reagents. When 100 different normal sera at 1/10 dilution were each incubated with a WB of 10 different platelets before development with antihuman reagents, 85% produced bands in addition to the background bands seen with antihuman reagents alone. Examples of normal serum reactions are shown in Fig 2 (lane 1, no bands other than background; lane 2, 95-Kd band; lane 3, 95- and 55-Kd bands; lane 4, 95- and 100-Kd bands; lane 5, 95- and 70 to 75-Kd bands; lane 6, 95-Kd band). Serum titers, based on the reciprocal of the highest dilution producing a discernable band, varied from less than 10 to 1,280 with anti-IgF, from less than 10 to 640 with anti-IgA, and less than 10 to 20 with anti-IgM. Serum dilutions less than 1/10 were not routinely checked because they tended to produce background color that obscured identification of bands. The varying frequencies with which normal sera produced different bands are shown in Table 1. A 90 to 95-Kd band, which we will refer to as the 95-Kd band, was observed most frequently, irrespective of the class specificity of antiglobulin used. Serum titers of 95 Kd-reactive IgG were the same with WBs of autologous and homologous platelets based on testing eight different normal sera, each with autologous and four different homologous platelets.

Two chromatographically pure IgG preparations, substituted for normal serum in blot development, produced a 95-Kd band at a minimum concentration of 0.125 mg IgG/mL. Based on normal serum IgG concentrations, this is equivalent to a serum dilution of 1/50 to 1/130. Purified IgA at a concentration of 0.10 mg/mL, corresponding to a serum dilution of 1/6.5 to 1/42, did not produce a 95-Kd band.

Both rhesus and baboon sera produced 95-Kd bands, each with titers of 100, when anti-human IgG was used as the second antibody.
Because the reaction of IgG in normal serum with the 95-Kd platelet protein was the most common, it was studied in detail.

**Attributes of IgG reactions with the 95-Kd platelet protein using normal sera, purified IgG, and F(ab')₂.** The minimum platelet number per lane that produced a detectable 95-Kd band when exposed to serum containing 95 Kd-reactive IgG (titer 10) was 6 × 10⁶ (15 µg platelet protein), and the number that produced bands of maximum strength was approximately 2.4 × 10⁷ (60 µg protein). Approximately 85% of random normal donor sera produced a 95-Kd band at titers ≥10 with 2.4 × 10⁷ platelets per lane. Titers of 10 to 20 were found in 35%, 30 to 50 in 30%, 100 to 300 in 15%, and 640 to 1,280 in 5%. Individuals maintained the same titer from year to year over 3 to 5 years based on multiple sampling of high- and low-titered donors. Serum could be stored for more than 2 years without change in titer. Reactions of sera with platelets were not influenced by ABO type of the donor from which serum or platelets were obtained.

F(ab')₂, prepared from IgG of a normal reactive serum (titer 640) elicited the 95-Kd band in WB analysis (see Fig 3). A minimum concentration of 31 µg F(ab')₂, mL was required to produce a band compared with 8.5 µg/mL of the parent IgG. The amount of F(ab')₂ used contained less than 0.0093 µg of IgG. Purified commercial Fₐ at a concentration of 1.0 mg/mL did not produce a 95-Kd band.

**Localization of 95-Kd platelet protein.** WBs of RBC membranes (40 µg protein/lane) and of whole WBCs (35 µg protein/lane), from two different donors whose platelets reacted with four different normal sera at 95 Kd, did not produce a band at this level when exposed to the same sera. Serum titers of 95 Kd-reactive IgG were the same with elutriated as with routinely prepared platelets.

The cytosolic fraction of mechanically lysed platelets, filtered through a 0.2-µm filter after centrifugation at 61,500 x g to remove sedimentable components, contained 80% to 90% of the 95-Kd protein (see Fig 4). Minimum material required to produce the 95-Kd band with either mechanically lysed or glycerol-loaded and hypotonically lysed platelets was the filtered supernatant from 7.5 to 10 × 10⁶ platelets, or the sedimented membranes from 4 to 7.5 × 10⁶ platelets.

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**Table 1. Ig Reactions With WBs of Platelet Proteins**

<table>
<thead>
<tr>
<th>Specificity of Anti-Globulin</th>
<th>Normal Sera Tested, No.</th>
<th>Mol Wt (Kd) of Reactive Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100-110 No. (%)</td>
<td>90-95 No. (%)</td>
</tr>
<tr>
<td>Anti-IgG</td>
<td>40</td>
<td>4(10)</td>
</tr>
<tr>
<td>Anti-IgM</td>
<td>30</td>
<td>—</td>
</tr>
<tr>
<td>Anti-IgA</td>
<td>30</td>
<td>2(7)</td>
</tr>
</tbody>
</table>

*WBs of 2.4 × 10⁷ platelets per lane from a single O⁺ donor were exposed to normal sera at 1/10 dilution and developed with specific biotinylated antiglobulin reagents, SP, and 4CN-H₂O₂.*
troughbasthemic platelets contain at least 50% as much 95-Kd protein as normals. Moreover, no reaction was observed when WBs of purified GPIIIa (20 μg per lane) were exposed to normal sera that produced high titer 95-KD lines with WBs of whole platelets (data not shown).

Blots of Bernard-Soulier (BS) platelets exposed to a reactive normal serum had the 95-Kd band. The minimum quantity necessary for this reaction was approximately 1.2 x 10^6 platelets/lane, which was similar to that of normal platelets although the BS platelets lack GPV.10,21 The supernatant of platelets treated with more than enough thrombin to completely hydrolyze GPV12 and the supernatant of intact platelets incubated in saline both contained only about 1% of the 95-Kd protein in the supernatant of mechanically lysed platelets. Lysates of thrombin-treated platelets contained normal amounts of 95-Kd protein.

Patterns on WBs of platelets exposed to sera of various patients before development with anti-IgG. Sera from 10 patients with chronic stable ITP (platelet counts ranging from 3,000 to 60,000/μL), 5 patients with acute quinidine- or quinine-purpura (counts <10,000/μL), 3 patients with human immunodeficiency virus associated thrombocytopenia (HIV-TP; counts 5,000 to 71,000/μL), 1 patient with thrombocytopenia due to myelodysplasia (20,000/μL), 5 patients with posttransfusion purpura (PTP; counts <10,000/μL), and 4 patients with systemic lupus erythematosus (counts of <10,000, 20,000, 80,000, and 275,000/μL) were incubated with WBs of normal platelets before development

In adsorption tests, no change in the initial titer of 320 occurred when 8 x 10^6 fresh platelets were preincubated with 2 mL serum, whereas the soluble supernatant fraction of only 4 x 10^5 lysed platelets reduced the 95-Kd-reactive IgG titer of 2 mL of serum to 80. IgG reactions with the 95-Kd protein could not be detected on the platelet surface, when 10 normal sera with reactive titers up to 1,280 were used in FACS studies (see Fig 5).

Differences between known glycoproteins and the 95-Kd protein. On WBs of normal (or thrombathenic) platelets electrophoresed under reduced conditions, the 95-Kd protein did not change in apparent molecular weight (see Fig 6, right panel). Its mol wt did not differ on gels of varying acrylamide percentages as often occurs with GPs of high carbohydrate content.19 Thrombathenic platelets that contained no GPIIIa by either silver stain analysis of reduced and nonreduced SDS-polyacrylamide gels or by anti-P1^A1 and anti-P1^A2 reactions on nonreduced WBs, produced a 95-Kd band of similar intensity to normal platelets when exposed to serum containing both anti-P1^A1 and 95 Kd-reactive IgGs (compare lanes 1 and 2 in Fig 6). The mol wts of GPIIIa, and the 95-Kd protein are so close that a strong band indicating reaction with one may obscure a weaker reaction with the other (see the 90 + 95-Kd doublet in Fig 6, lane 3). A titration of normal versus thrombathenic platelets exposed to one concentration of normal serum (Fig 7) further indicates that
with anti-IgG. None of the patients had been treated with intravenous IgG.

Eight of 10 ITP sera produced a 95-Kd band with the following titers: 10(2), 20(1), 50(2), 100(2), and 2,000(1); two of these sera produced an additional band at 100 to 110 Kd. Four of five drug purpura sera produced a 95-Kd band with titers of 20(1), 50(1), 100(2); binding was not drug-dependent and, with the exception of background, no other bands were produced. The three HIV-TP sera produced 95-Kd bands with titers of 320, 2,000, and 1,000, respectively. The myelodysplastic serum titered 640. Four of five PTP sera produced 95-Kd bands with titers of 10(1), 20(2), and 40(1). None had a 120-Kd band stronger than the 120 to 125-Kd background band commonly seen. Of four SLE patient sera, two had titers of 200, and two had titers of 50. The same 95-Kd reactivities of all these sera were demonstrated using thrombasthenic and reduced platelet blots.

No changes in serum titers of 95 Kd-reactive IgG were detected in samples of three patients tested before and after splenectomy. Of the three, two had ITP with platelet counts of <5,000/μL before splenectomy, rising to stable counts of 35,000/μL and 400,000/μL, respectively. The third patient with HIV-TP rose from <5,000/μL before to 250,000/μL after splenectomy.

PAIgG. PAIgGs of six normals ranged from 0.42 to 0.79 fg/platelet, and respective 95 Kd-reactive titers ranged from 0 to 640. PAIgGs of thrombocytopenic patients (4 with ITP, 2 with HIV-TP, and 1 with myelodysplasia, having platelet counts of 8,000 to 100,000), ranged from 1.6 to 13.7 fg/platelet and 95 Kd-reactive titers, from 0 to 1,000. There was no correlation between PAIgG values and 95 Kd-reactive IgG titers.

DISCUSSION

Table 2 summarizes reports in which WB analyses have been used exclusively to identify apparent platelet-specific autoantibodies that were considered responsible for thrombocytopenic disorders. Our studies suggest that some platelet WB reactions reported as evidence of autoimmunity may not differ qualitatively from certain antiglobulin background reactions or from reactions of normal serum Igs with intracellular platelet proteins.

Reactions of antiglobulin reagents alone with platelet WBs. Antiglobulin background bands are due to epitopes on PAIg molecules or their derivatives because: (1) WBs of purified Igs loaded in quantities equivalent to total PAIgs have patterns identical to background patterns of platelet WBs (see Fig 1); (2) preincubation of an antiglobulin reagent with the respective purified Ig blocks development of all background bands; (3) F(ab')2 preparations of antiglobulin reagents elicit the same background bands as whole IgG preparations, indicating that the bands do not represent IgG bound to platelet Fc receptors.

Reactions of normal sera with platelet WBs. In addition to antiglobulin background bands, normal sera incubated with platelet blots before development with antiglobulins produced bands with varying frequency at 100 to 110, 90 to 95, 80 to 85, 60 to 75, and 50 to 60 Kd. The 95-Kd band was the most common, elicited by IgG in 85% of normal sera, by
Table 2. Characteristics of WB Bands Considered of Pathologic Significance in Thrombocytopenic Disorders

<table>
<thead>
<tr>
<th>Disorder</th>
<th>No. of Patients</th>
<th>No. (mol wt, Kd)*</th>
<th>Serum Dilution</th>
<th>No. of Controls</th>
<th>No. of Positive (mol wt, Kd)*</th>
<th>Reference</th>
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<tr>
<td>ITP</td>
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<tr>
<td>Chronic</td>
<td>13</td>
<td>9</td>
<td>100†</td>
<td>1/27†</td>
<td>Normal</td>
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<tr>
<td>Acute</td>
<td>8</td>
<td>0</td>
<td>—</td>
<td>1/27†</td>
<td>Donors</td>
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<td>ITP</td>
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<td>Normal</td>
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<td>145</td>
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<td>SLE</td>
<td>9</td>
<td>2</td>
<td>108</td>
<td>1/10</td>
<td>Normal pooled sera</td>
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<td>66</td>
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<tr>
<td>HS with isolated TP</td>
<td>30</td>
<td>29</td>
<td>25</td>
<td>1/100</td>
<td>Non-HS with TP;</td>
<td>7</td>
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<tr>
<td>HS, with AIDS or ARC, without TP</td>
<td>16</td>
<td>15</td>
<td>25</td>
<td>1/100</td>
<td>18 immune, 12 nonimmune</td>
<td>45</td>
</tr>
<tr>
<td>Quinidine purpura</td>
<td>6</td>
<td>6</td>
<td>80†</td>
<td>1/100</td>
<td>Qd-treated patients without TP</td>
<td>10</td>
</tr>
<tr>
<td>Non-Qd drug-TP</td>
<td>3</td>
<td>0</td>
<td>—</td>
<td>1/100</td>
<td></td>
<td>9</td>
</tr>
<tr>
<td>Posttransfusion purpura</td>
<td>1</td>
<td>1</td>
<td>120</td>
<td>1/50</td>
<td>NI</td>
<td>9</td>
</tr>
</tbody>
</table>

Abbreviations: ITP, idiopathic thrombocytopenic purpura; NI, no information; TP, thrombocytopenia; SLE, systemic lupus erythematosus; HS, homosexuals; ARC, AIDS-related complex.

†Distinct from controls.
§Comigrating with GPIIIα, but distinct from PIA1.
‡Eluates used.
∥Mol wts of the most common bands listed.
\[\text{Presumed to be GPV.}\]
††Binding not drug-dependent.
*90 Kd = PIIa; 140 Kd = GPIIb.

IgA in 50%, and by IgM in 30%. Purified IgG prepared commercially from pooled human plasma (when used instead of serum) produced the 95-Kd band, but not the less commonly seen bands.

Of normal sera that contained 95 Kd-reactive IgG (85% of those tested), 65% had titers between 10 and 50. There were no unusual characteristics notable among the normal donors who titered greater than 50 or the few with titers of 640 and 1,280; and titers, whether high or low, did not change significantly on sequential sampling over a period of several years.

Because the 95-Kd bands are obtained with equal intensity when serum is exposed to autologous and homologous blotted platelets, alloantibodies are not responsible for these reactions. Moreover, none of the observed lines produced by normal sera were in the range of the 45-Kd human leukocyte antigen heavy chain GP. The 60 to 65-Kd bands observed with some sera did vary in intensity with WBs prepared from different platelets, but we have not evaluated this reaction in detail.

Because the most common WB pattern produced by normal sera involved IgG binding to a 95-Kd platelet protein, we further characterized this reaction.

The 95-Kd Ig-reactive platelet protein. The 95-Kd protein is associated only with platelets, not with RBCs or WBCs. Approximately 90% of the 95-Kd protein is present in the cytosol of platelet lysates (Fig 4). Attempts to detect the 95-Kd protein by adsorption or immunofluorescence studies indicated that it was not present on the platelet surface (Fig 5). Therefore platelet eluates, often used as sources of antibodies, will not contain all of the normal serum IgG reactive with platelet proteins on WB. Platelet membranes prepared by glycerol loading and hypotonic lysis are associated with approximately 10% to 15% of the 95-Kd protein, suggesting that the protein may be loosely bound to internal membranes or associated with unruptured or resealed vesicles. The protein is not in secretory granules that can be released by thrombin. It is apparent that the age and physical state of platelets (ie, whole, partially lysed, or purified membranes) used for WB preparations will influence the number and intensity of bands obtained with normal or pathologic sera.

In routine WBs, reactions of the 95-Kd protein may be mistaken for GPIIIα. However, the 95-Kd protein differs from GPIIIα in many respects: (1) it is present primarily in
Severe thrombocytopenia occurred with titers less than 10. Several investigators have found that elevated PAIgGs in and mild thrombocytopenia with titers greater than 100. cytosol, not on external membranes; (2) its mol wt is slightly greater than that of GPIIIa; (3) reducing agents do not affect its mol wt (see Fig 6); (4) its mol wt on gels of varying percentages of acrylamide is more constant than that of GPIIIa, possibly reflecting low carbohydrate content of the 95-Kd protein; (5) thrombasthenic (GT type I) platelets contain it in similar amounts to normal platelets (Fig 7); (6) Igs that bind to it do not react with purified GPIIIa on WBs; and (7) it does not react with specific anti-GPIIIa alloantibodies.

GPIII, which has a mol wt of 97 Kd that is unaltered by reducing agents, could be confused with the 95-Kd protein on the basis of mol wt measurements alone, but it is only present on external platelet membranes.

GPV, identified by some investigators as a target antigen in various thrombocytopenic disorders (see Table 2), has a reported mol wt of 82 to 89 Kd. It is an external membrane protein that is lost from platelets within 72 hours of venipuncture. Findings indicating that the 95-Kd protein is not GPV are: (1) washed normal platelets stored in saline for 72 hours contain approximately 80% to 90% of the quantity of 95-Kd protein found in fresh platelets; (2) thrombin treatment that removes 100% of GPV from platelets by hydrolysis does not diminish the 95-Kd protein; and (3) BS platelets deficient in GPV contain a normal amount of 95-Kd protein per platelet.

**Characteristics of platelet-reactive Igs in normal serum.** Highly purified F(ab')2 fragments prepared from 95 Kd-reactive IgG produce a 95-Kd band. An approximate sixfold higher molar concentration of F(ab')2 than parent IgG required for reaction may reflect alteration or partial removal of the affinity site by purification. No platelet Fc receptor at 95 Kd or similar mol wt has been identified, and purified Fc does not bind to the 95-Kd protein. Thus, binding of certain normal serum IgGs to the 95-Kd receptor occurs, at least in part, via the F(ab')2 portion of the molecule.

GPIIIa has been reported to bind IgG and F(ab')2 on the platelet surface in relatively small amounts compared with the ability of GPIIIa to bind anti-PIA1. This has been postulated to be due to an alteration of GPIIIa resulting in a senescent antigen. However, there are no reports of normal Igs reacting in WB with GPIIIa.

Titers of 95 Kd-reactive IgG, IgA, and IgM in individual sera were not necessarily similar (data not shown). For example, sera containing a reactive IgG could have higher- or lower-titered or unmeasurable reactive IgA or IgM.

**Reactions of pathologic sera.** Sera from 86% of a group of patients with various thrombocytopenic disorders produced 95-Kd bands on platelet WBs. Identical results using thrombasthenic and reduced platelets indicate that GPIIIa was not involved in these reactions. Titers of 95 Kd-specific IgG were >50 in 57% of these sera, compared with 17% of normal donors. However, there is no correlation between titer of 95 Kd-reactive IgG and severity of thrombocytopenia. Severe thrombocytopenia occurred with titers less than 10 and mild thrombocytopenia with titers greater than 100. Several investigators have found that elevated PAIgGs in various thrombocytopenic disorders are at least partially due to nonspecific or nonpathologic IgG, IgA, and/or IgM. Conceivably, the internal Ig-binding proteins may foster accumulation of normal platelet Igs in injured platelets, but we found no correlation between serum titers of 95 Kd-reactive IgG and PAIgG values of platelets from the same samples. We and others find that PAIgG is increased in stored platelets, but it remains to be determined whether binding of Igs by internal proteins is responsible.

A large percentage of autoantibodies identified previously by WB in sera from thrombocytopenic patients (Table 2) react with platelet protein(s) in the 80 to 95 Kd and 120 Kd mol wt range, similar to the mol wt ranges of common reactions with normal Igs. For example, serum autoantibodies directed against GPV (80 Kd) at titers >100 have been described in all of six patients tested with quinidine purpura, and an autoantibody directed against a 120-Kd protein was described in the acute serum of one patient with PTP. However, we found that 4 of 5 patients with quinidine/ quinine purpura reacted only with the 95-Kd protein, and none of sera from five different acute phase PTP patients had a 120-Kd band stronger than background bands commonly seen.

Controls in most studies usually involved serum dilutions of 1/50 or 1/100, which would decrease the frequency of positive 95-Kd reactions. However, in one study where a 1/10 dilution of pooled normal AB serum was used for a control, an 86-Kd band was observed that appeared to be due to a cytoplasmic platelet protein, and may be identical to the 95-Kd band we have described.

**Significance of Ig-binding platelet proteins.** The fact that titers of 95 Kd-reactive IgGs can vary widely among normals from less than 10 to greater than 1,280, and tend to remain unchanged within a particular individual over a period of years makes a physiologic role for these proteins seem unlikely. However, identification of intracellular Ig-binding proteins in a cell that secretes but does not produce Igs suggests possible relevance of these proteins to incorporation of Igs into α granules and other PAIg phenomena.

The 95-Kd Ig-binding protein is of special interest in that an analogy can be drawn between it and bacterial proteins such as Staphylococcal Protein A (SPA) and Streptococcal Protein G (SPG) that react nonspecifically with human Igs of varied isotype (SPA) and subclass (SPG), as well as with Igs from several species of animals (both SPA and SPG). The bacterial proteins bind primarily to the Fc portion of Igs, but have also been clearly shown to have some reactivity with F(ab')2 fractions of Igs. Whether the 95-Kd platelet protein reacts nonspecifically with more restricted isotypes and subclasses of Igs than does SPA or SPG, or whether the reaction is immunospecific, remains to be determined.

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Western blot identification of platelet proteins that bind normal serum immunoglobulins. Characteristics of a 95-Kd reactive protein

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