Antivinculin Antibodies in Sera of Patients With Immune Thrombocytopenia and in Sera of Normal Subjects

By Yoshiaki Tomiyama, Riitta Kekomäki, Janice McFarland, and Thomas J. Kunicki

We have characterized a 120-Kd antigen that frequently reacts with serum antibodies from patients with immune thrombocytopenia or normal subjects. Immunoblots made after two-dimensional nonreduced/reduced sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) or two-dimensional isoelectric focusing/SDS-PAGE demonstrated that this 120-Kd protein has the same molecular weight under nonreduced or reduced conditions, is not a surface protein, and has an isoelectric point (pI) of 6.4 to 6.5. From these data, one likely candidate is the intracellular platelet protein, vinculin. Monoclonal antivinculin antibody reacts with this 120-Kd protein, and purified human platelet vinculin is bound by antibodies that recognize the 120-Kd protein. Therefore, we conclude that this 120-Kd protein is identical to vinculin. Data obtained from a sensitive enzyme-linked immunosorbent assay demonstrate the presence of naturally occurring antivinculin antibodies in many normal sera. However, the incidence of antivinculin antibodies in patient sera (67%; 55 of 82 sera) is significantly (P < .01) higher than that in normal sera (40%; 32 of 80 sera), and there is a significant difference (P < .05) between the mean levels of antivinculin antibodies in patient and normal sera. Whereas the levels of these antibodies in patient and normal sera overlap, 2 of 82 sera from patients with thrombocytopenia express unusually high levels of such antibodies. The pathologic significance of these antibodies remains to be determined.

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Immune Thrombocytopenia is a clinical syndrome of different diseases, all of which have in common shortened platelet survival due to antiplatelet antibody or immune complexes.1,2 Platelet destruction results from subsequent complement-mediated lysis and/or phagocytosis. In both acute and chronic autoimmune (idiopathic) thrombocytopenic purpura (ITP), it has been demonstrated that many patients have autoantibodies specific for platelet membrane glycoprotein (GP) Ib-IX and/or IIb-IIIa.3-8 Although circulating autoantibodies are less frequently detected than platelet-associated autoantibodies,4 autoantibodies specific for GPIb-IX and/or GPIIb-IIIa have been demonstrated using newly developed assays in as many as one half of sera from patients with ITP.4,5-6 Autoantibodies reactive with GPIlb and GPIIla can also be detected by an immunoblot assay.4,6,7 However, the sensitivity of this assay may be compromised because certain autoantigenic epitopes on these GP's can be denatured by sodium dodecyl sulfate (SDS).8 On the other hand, the immunoblot assay has proved useful for the detection of protein antigens that are stable to denaturation. Using an immunoblot assay, several putative autoantigens in immune thrombocytopenia have been demonstrated.5,10-17 We previously demonstrated that a 124-Kd protein is the most frequently detected autoantigen in chronic ITP (6 of 40 Japanese patients).10 However, autoantibodies specific for this antigen were also detected in patients with systemic lupus erythematosus (SLE) or aplastic anemia. Therefore, this antigen did not appear to be a specific marker of ITP. Proteins of similar molecular weight have also been demonstrated in both SLE and ITP by others.13,16 but the identity of these proteins has not been determined.

In this study, we analyzed sera from 141 patients with clinically suspected immune thrombocytopenia. Autoantibodies specific for a 120-Kd protein were frequently detected in both patient sera and normal sera. This protein is identical to our previously reported 124-Kd antigen, and is identified as the intracellular platelet protein, vinculin.

Materials and Methods

Antibodies. Blood samples containing antiplatelet antibodies were obtained from patients with clinically suspected immune thrombocytopenia, as determined by the referring physicians. All of the 141 patients selected for this study showed laboratory findings of elevated platelet-associated IgG and/or IgM by nephelometry,18 as well as serum antibodies reactive with platelets by enzyme-linked immunosorbent assay (ELISA).19 Normal sera were obtained from healthy volunteers with no history of pregnancy or blood transfusion. The murine monoclonal antibodies, AP4 (anti-GPIIb heavy chain), AP5 (anti-GPIIIa), and anti–chicken gizzard vinculin (Sigma Chemical Company, St Louis, MO) were used as specific markers of the respective proteins.19 ITP serum (RY) containing autoantibodies specific for both GPIIb and the 124-Kd protein was also used as a marker of these proteins.19 IgG was purified from sera from two patients (PD and MOS) by affinity chromatography on Protein A–Sepharose CL-4B (Pharmacia, Piscataway, NJ). For the preparation of Fab fragments, purified IgG was dialyzed against 0.01 mol/L Na2HPO4, 0.01 mol/L NaH2PO4, and 0.15 mol/L NaCl, pH 7.4 (phosphate-buffered saline); PBS. After adding 10 mmol/L cysteine and 2 mmol/L EDTA, IgG was digested with mercuripapain (Sigma) for 2 hours at 37°C. The reaction was terminated by adding iodoacetamide (Sigma), and the Fab fragments were separated from Fc fragments and undigested IgG by chromatography on Protein A–Sepharose CL-4B. SDS-electrophoresis of the final product under nonreduced conditions showed a single band at 50 Kd by silver staining.

Platelet protein preparation. Platelet-rich plasma (PRP) was obtained from normal donors or patients by differential centrifugation of blood anticoagulated with acid-citrate-dextrose (ACD, NIH

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appropriate buffer. After three washes with Ringer's citrate-dextrose (RCD) containing PGE<sub>n</sub>, pH 6.5, the platelet pellet was resuspended in an appropriate buffer.

Washed platelets (10<sup>6</sup>) in 1 mL of PBS were surface labeled with 125<sup>I</sup> using the lactoperoxidase method. The surface-labeled platelets were washed three times with RCD-PGE and resuspended in 0.01 mol/L Tris and 0.15 mol/L NaCl, pH 7.4 (Tris-buffered saline [TBS]) containing 5 mmol/L EDTA. To lyse the platelets, 100 μL of 10% Triton X-100 was added to the 900 μL of the platelet suspension, and the mixture was incubated for 30 minutes on ice. Phenylmethylsulfonyl fluoride (PMSF, Sigma) was added to the PRP to a final concentration of 20 ng/mL.

Reduction in the first dimension was achieved by focusing in the presence of 2-ME (final concentrations), and the mixture was heated at 100°C for 5 minutes. Fifty microliters of this protein preparation were loaded in each first dimension tube gel. Molecular weight standards (Bio-Rad Laboratories, Richmond, CA) were added to each well. The trays were then incubated overnight at 4°C. After three additional washes, 50 μL of diluted antiserum (usually a 1:20 dilution) were added to each well. After incubation at ambient temperature for 1 hour, the wells were washed six times with PBS-Tween, and 50 μL of the substrate (p-nitrophenylphosphate in diethanolamine buffer, pH 9.8) were added to each well, and absorbance at 405 nm was measured using an anti-dsDNA kit assay (Amersham Corp, Arlington Heights, IL).

Anti-DNA antibodies. Anti-dsDNA and anti-ssDNA antibodies were measured using an anti-dsDNA kit assay (Amersham Corp, Arlington Heights, IL).

RESULTS

Antibodies reactive with a 120-Kd antigen. Using an immunoblot assay, we identified platelet proteins that are bound by antiplatelet antibodies in immune thrombocytopenia (Fig 1). The positions of GPIIb and GPIIIa are detected by the binding of a mixture of AP4 and AP5 (lane 1). Aside from the platelet-associated IgG band detected by secondary goat anti-human IgG<sub>s</sub> sera RY<sub>s</sub> (lane 3) and RA<sub>T</sub> (lane 2) contain autoantibodies specific for GPIII and GPIIIa, respectively. However, one of the most frequently detected antigens in this assay has been an unidentified protein having an apparent molecular weight of 120 Kd. Although a faint band at a molecular weight of 120 Kd was detected by many patient sera tested, it was obvious that with 15 of the 141 sera tested, a strong band was detected at this position. Since PD serum (lane 4) and MOS serum (lane 5) contain high levels of this antibody,
they were used as the prototypes for further characterization of this 120-Kd protein. PD serum antibody also reacts with the autologous 120-Kd protein, further substantiating that the antibody(ies) under investigation are autoantibodies (data not shown). Figure 1 also shows that the 120-Kd protein comigrates with the previously described 124-Kd protein detected by antibodies in RY serum (compare RY serum in lane 3 to other sera in lanes 4 and 5). However, antibodies specific for this 120-Kd protein are not restricted to patients with immune thrombocytopenia, because 2 of 17 normal sera (eg, normal serum 1 in lane 6 and normal serum 2, not shown) clearly also contain antibodies specific for the 120-Kd protein.

In the immunoblot assay, normal sera routinely generate weaker bands than those observed with positive patient sera. For example, relative titers, defined as the lowest dilution of serum in which anti-120-Kd antibody is detected by immunoblot assay, are normal serum 1, 1:2,500; normal serum 2, 1:2,500; PD, 1:62,500; and MOS, 1:62,500.

Characterization of the 120-Kd protein. To compare the 120-Kd protein with platelet surface glycoproteins having similar electrophoretic mobilities, we analyzed protein antigens by immunoblot assay after two-dimensional electrophoresis.

Surface-labeled platelet proteins were mixed with unlabeled platelet proteins and used as a source of antigen. The protein mixture was separated by two-dimensional nonreduced/reduced SDS-PAGE and transferred to a nitrocellulose membrane. Immunoblots using PD serum show that the 120-Kd protein has the same electrophoretic mobility under nonreduced or reduced conditions (Fig 2). Autoradiograms of the same nitrocellulose membranes permit one to determine the positions of surface GPs, including GPIa, GPIIa, PECAM-1, GPIc, GPIIbα, GPIIIa, and GPIV (Fig 2A). The 120-Kd protein is not radiolabeled with 125I by Chloramine T method and does not comigrate with any of these surface glycoproteins (Figs 2A and B). In additional experiments (not shown), we determined that the 120-Kd...
protein is also not labeled with 3H using the periodate oxidation method.

Immunoblots after two-dimensional isoelectric focusing/SDS-PAGE demonstrate that the 120-Kd protein has a pl of 6.4 to 6.5 (Fig 3C, indicated by letter a). The immunoblots also show that four other protein components are bound by antibodies in PD serum, that is, a 140-Kd component having a pl of 6.1 to 6.2 (indicated by letter b), a 110-Kd component having a pl of 5.9 to 6.0 (indicated by letter c), a 105-Kd component having a pl of 5.8 to 5.9 (indicated by letter d), and an 80-Kd component having a pl of 6.1 to 6.4 (indicated by letter e). Interestingly, the 80-Kd component is preferentially detected in two-dimensional isoelectric focusing/SDS-PAGE (compare Fig 2B with Fig 3C), and both this component and the 120-Kd protein have a unique saucer shape, which suggests the existence of several isoelectrophoretic forms of either protein. The electrophoretic behavior of the 120-Kd protein in this two-dimensional electrophoresis protocol is reminiscent of that of the intracellular platelet protein, vinculin.25 A murine monoclonal antivinculin antibody reacts with exactly the same five components that were detected by PD serum (Figs 3B and C). In the CBR-stained gel (Fig 3A), three of these five components are visible (the 120-Kd, 110-Kd, and 80-Kd components). Normal serum 1 reacts with only these three components (Fig 3D). The two normally less prominent components (b and d) are probably not detected by normal serum 1 because of its comparatively lower antibody titer. None of these five components are detected with those normal sera that do not contain antibody specific for the 120-Kd protein (data not shown). These data demonstrate that the 120-Kd protein is probably vinculin, and the binding of antivinculin antibody to components other than the 120-Kd protein appears to be specific.

Identification of the 120-Kd protein as vinculin. To confirm that the 120-Kd protein is vinculin, purified human platelet vinculin was used as a source of antigen in an immunoblot assay (Fig 4). SDS gels stained with CBR (Fig 4A, lane 2) show the position of the purified human platelet vinculin, and corresponding immunoblots (Fig 4B) demonstrate that purified human platelet vinculin binds PD (lane 2), MOS (lane 3), and normal serum 1 (lane 4), as well as monoclonal antivinculin antibody (lane 1). Furthermore, we examined whether the 120-Kd antigen in SLE reported by Jouhikainen et al6 is identical to vinculin. Purified human platelet vinculin is also bound by antibody in one prototype serum from that study (data not shown). These data demonstrate that the 120-Kd protein antigen is identical to human platelet vinculin, and that this protein is identical to the previously reported 124-Kd antigen in ITP10 and the 120-Kd antigen in SLE reported by Jouhikainen et al.6 To demonstrate that the binding of IgG to vinculin is immunologically specific, purified human vinculin was incubated with purified IgG (100 μg/mL) or Fab fragments (100 μg/mL) in the immunoblot assay. IgG and Fab fragments were prepared from PD and MOS sera. After washing three times, all strips were incubated with AP-conjugated goat anti-human IgG, F(ab’), specific antibody for 2 hours. Figure 5 shows that the reactivity of purified IgG (100 μg/mL) is essentially the same as that of a 1:100 dilution of antiserum, and that Fab fragments also react with purified vinculin. Fab fragments produced a strong band; however, it is weaker than that produced by purified IgG.

Direct antigen ELISA using intact vinculin. Because it is difficult in the immunoblot assay to evaluate a weak positive reaction of serum antibody with vinculin, a direct antigen ELISA was used to examine more carefully the frequency of antivinculin antibodies in patients and normal individuals. Eighty-two patient sera and 80 normal sera were reexamined (Fig 6). The mean absorbance value obtained using 11 normal sera that did not react with vinculin in the immunoblot assay was .019 ± .021 (mean ± SD), and an absorbance value of greater than .082 was arbitrarily considered positive (> 3 SD above control mean). Fifty-five of 82 patient sera (67%) and 32 of 80 normal sera (40%)
Titers of PD and MOS in the antigen ELISA are 1:10,240 and 1:1,280, respectively. These titers are 64-fold and eightfold higher than that given by any normal serum (Fig 7). However, the titer of RY (1:160) is essentially the same as that of the strongest normal sera.

Clinical analysis of patients with suspected immune thrombocytopenia. As shown in Table 1, in 34 of 82 patients whose sera were examined by ELISA as described, clinical
Fig 6. Reaction of antibodies in sera of patients with immune thrombocytopenia and in sera of normal subjects with purified human platelet vinculin as determined by ELISA. Microtiter wells coated with purified human platelet vinculin were incubated with 50 µL of a 1:20 dilution of serum antibodies for 1 hour, and the extent of binding of IgG antibodies was measured by addition of AP-conjugated goat anti-human IgG and appropriate color development. Absorbance at 405 nm is indicated on the ordinate. The positions of absorbance values obtained with PD antiserum, MOS antiserum, RY antiserum, and normal serum 1 are indicated. Bars indicate mean values.

histories were available. These clinical correlations show that the incidence of antivinculin antibody in patients with chronic ITP is similar to that in patients with nonimmune thrombocytopenia. It is noteworthy that PD is a patient with chronic myelomonocytic leukemia suffering from immune thrombocytopenia after tumor lysis syndrome, and MOS is a patient with acute ITP associated with herpes zoster infection. These data suggest that the induction of antivinculin antibody occurs more often secondary to cell destruction rather than as a result of a primary autoimmune mechanism.

To examine whether there is a relationship between the occurrence of antivinculin and anti-DNA antibodies, nine patient sera, including PD and MOS, that were found to contain antivinculin antibodies in the immunoblot assay were examined for the presence of anti-DNA antibodies.

Table 1. Clinical Analysis of Patients With Suspected Immune Thrombocytopenia

<table>
<thead>
<tr>
<th>Antivinculin Antibody</th>
<th>Total</th>
<th>Positive</th>
<th>Negative</th>
<th>Positive %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient sera examined by ELISA</td>
<td>82</td>
<td>55*</td>
<td>27</td>
<td>67</td>
</tr>
<tr>
<td>With clinical follow-up</td>
<td>34</td>
<td>22</td>
<td>12</td>
<td>65</td>
</tr>
<tr>
<td>Primary ITP</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Chronic</td>
<td>19</td>
<td>11</td>
<td>8</td>
<td>58</td>
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<td>Acute</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Secondary ITP</td>
<td>5</td>
<td>4</td>
<td>1</td>
<td>80</td>
</tr>
<tr>
<td>Nonimmune TP</td>
<td>9</td>
<td>6</td>
<td>3</td>
<td>67</td>
</tr>
<tr>
<td>Normal sera</td>
<td>80</td>
<td>32</td>
<td>48</td>
<td>40</td>
</tr>
</tbody>
</table>

*An absorbance value of > .082 in ELISA is arbitrarily considered positive.

None of these sera were found to contain antibodies against dsDNA or ssDNA.

DISCUSSION

Using an immunoblot assay, several putative autoantigens in immune thrombocytopenia have been demonstrated. Recently, it has also been demonstrated that certain platelet proteins bind normal serum IgG in an immunoblot assay. In this article, we characterize a 120-Kd antigen, one of the most frequently detected autoantigens in immune thrombocytopenia, as identical to our previously reported 124-Kd antigen and vinculin. This 120-Kd protein has the same molecular weight under nonreduced or reduced conditions, is not a surface protein, and has a pI of 6.4 to 6.5. From these data, one likely candidate for this protein is the intracellular platelet protein, vinculin. Indeed, monoclonal antivinculin antibody reacts with this 120-Kd protein, and purified human plate-
let vinculin is bound by antibody that binds to the 120-Kd protein. Additional evidence confirms that the 120-Kd antigen seen in SLE and reported by Jouhikainen et al. is also identical to vinculin, because a prototype serum used in that study behaves, in our hands, exactly like those sera used in this study. Immunoblots after two-dimensional isoelectric focusing/SDS-PAGE demonstrate four other components detected by antivinculin antibody. The 140-Kd component is probably metavinculin, a protein of higher molecular weight that is immunologically related to vinculin. It is noteworthy that the 80-Kd component is preferentially detected in two-dimensional isoelectric focusing/SDS-PAGE. Rosenfeld et al. reported that the concentration of an 85- to 90-Kd degradation product of vinculin increases in high salt extracts of platelets. Given the high salt concentrations used in isoelectric focusing gels, it is probable that the 80-Kd component is the same major degradation product of vinculin observed by Rosenfeld et al.

The IgG binding to vinculin appears to be immunologically specific because (1) immunoblots after two-dimensional isoelectric focusing/SDS-PAGE demonstrate that a murine monoclonal antivinculin antibody reacts with exactly the same five components that are detected by PD serum; (2) none of these five components are detected with those normal sera that do not contain antibody specific for vinculin; and (3) Fab fragments of IgG from certain patient sera react with purified vinculin. Although Fab fragments produced a strong band, it is weaker compared with that produced by IgG. However, it is likely the result of differences in the reaction of secondary antibodies rather than a reflection of real differences in specificity.

Using a sensitive direct antigen ELISA, we demonstrated that 55 of 82 patient sera (67%) contain IgG antibodies that bind to vinculin. However, we also demonstrated an unexpectedly high incidence of antivinculin IgG antibodies in normal sera: 32 of 80 normal sera also contain antivinculin antibodies (40%). Because these normal sera were obtained from healthy subjects with no history of pregnancy or blood transfusion, our data suggest that naturally occurring IgG antibodies that bind to vinculin are present in a high percentage of many normal sera. Recently, it has become apparent that the normal immune system has the capacity to produce antibodies to a variety of self-antigens, including cytoplasmic and cytoskeletal proteins, soluble plasma constituents, and nucleic acids. Such naturally occurring (auto)antibodies constitute a substantial part of normal serum circulating immunoglobulins. We demonstrate here the existence of naturally occurring antibodies specific for vinculin, a finding that is consistent with the recent study of Pfue ll er et al.

It is noteworthy that there are, albeit infrequent, discrepancies between the results of the immunoblot assay and the direct antigen ELISA. As one example, normal serum 2 reacts with denatured vinculin in the immunoblot assay but is negative in ELISA using purified vinculin. Antibodies in normal serum 2 may thus distinguish cryptic antigens expressed only on denatured vinculin. Whereas patient sera PD and MOS showed a similar reactivity in the immunoblot assay, PD had an eightfold stronger reactivity than MOS when tested against purified vinculin in the ELISA. These data suggest, not surprisingly, that the epitope(s) detected on vinculin are heterogeneous. One might define at least two classes of epitopes: one class that is expressed on purified vinculin as well as denatured vinculin, and a second class composed of cryptic antigens that are exposed by SDS. Both classes of epitopes are recognized by antivinculin antibodies in normal sera as well as patient sera.

Our data also demonstrate differences between antivinculin antibodies in patient sera and those in normal sera. The incidence of anti-vinculin antibodies in patient sera (67%) is significantly ($P < .01$) higher than that of naturally occurring antivinculin antibodies (40%); the titers of antibodies in some patient sera, for example, PD and MOS, are significantly higher than those in any normal serum; and there is a significant difference ($P < .05$) between the mean levels of antivinculin antibodies in patient and normal sera. At this time, it is still difficult to differentiate patients from normal subjects based solely on the strength or titer of circulating antivinculin antibodies. The levels of most antivinculin antibodies in patient and normal sera overlap, and the titration curves of antivinculin antibodies in many patient sera are similar to those observed with some normal sera.

Taken together, our findings suggest that antivinculin antibodies are present both in patient sera and sera of certain normal subjects, and that only modest quantitative differences of antibody levels differentiate patient and normal sera. Although the role of these antibodies remains to be determined, it is unlikely that antivinculin antibodies play a role in the etiology of immune thrombocytopenia. Indeed clinical analysis suggests that cell destruction may contribute to the induction of antivinculin antibody. In patients already affected by immune thrombocytopenia, antivinculin antibodies may be generated in a secondary immune process subsequent to removal of damaged platelets and could have important pathologic effects.

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