Platelet-Agglutinating Protein P37 From a Thrombotic Thrombocytopenic Purpura Plasma Forms a Complex With Human Immunoglobulin G

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We have previously reported the purification of a 37-kd platelet-agglutinating protein (PAP p37) from the plasma of a patient with thrombotic thrombocytopenic purpura (TTP) that was shown to be present in a subset of TTP patients. The platelet agglutination induced by PAP p37 has been shown to be inhibited by IgG from normal adults and the same TTP patient after recovery. To elucidate the mechanism of inhibition of IgG, the interaction between PAP p37 and IgG was studied. The complex formation was demonstrated by the binding of fluid-phase IgG from normal adults and the same TTP patient after recovery to adsorbed PAP by using an enzyme-linked immunosorbent assay. The binding was specific, concentration dependent, and saturable. IgG purified from a 5-month-old baby and the same TTP patient during active disease did not form complex with PAP p37. The IgG covalently cross-linked to PAP p37 became absent when the patient recovered.

THROMBOTIC thrombocytopenic purpura (TTP) is manifested clinically by thrombocytopenia, microangiopathic hemolytic anemia, fluctuating neurological signs, fever, and renal abnormalities. The pathogenesis of this disorder is still not known. Deposition of microthrombi consisting of platelets and fibrin in the small vessels has been believed to be the primary cause of the clinical manifestations in TTP. Clinical studies have shown that some patients with TTP responded successfully to plasma infusion or exchange. The agglutination of autologous and homologous platelets by TTP plasma has been demonstrated in this and other laboratories. This agglutination was not inhibited by hirudin, heparin in the presence of antithrombin III, and other laboratories. This agglutination was not inhibited by human IgG or Fab fragments purified from the normal adult plasma. Recently, we have reported the purification of a 37-kd platelet-agglutinating protein (PAP p37) from a TTP patient's plasma. The platelet agglutination induced by the PAP p37 was not inhibited by any of the aforementioned inhibitors except the IgG from normal adults and the same TTP patient after recovery.

To investigate further the inhibitory mechanism of human IgG on the PAP p37–induced platelet agglutination, we have studied the interaction between PAP p37 and IgG. In this study, we present evidence demonstrating a complex formation between PAP p37 and specific IgG and the inhibition of TTP plasma–induced platelet agglutination by specific IgG.

MATERIALS AND METHODS

Materials. Sephadex G-25, diethyl aminoethyl (DEAE) Sephacel, Concanavalin A (Con A)-Sepharose, protein A–Sepharose CL-4B, and cyanogen bromide–activated Sepharose 4B were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Ultrogel ACA-34 was obtained from LKB Instruments, Inc, Chapel Hill, NC. Enzyme-linked immunosorbent assay (ELISA) reagent used in the iodination study was obtained from Bio-Rad Laboratories (Richmond, CA). Carrier-free sodium (specific activity, 131.9 mCi/mL) was purchased from New England Nuclear, Boston. Molecular weight (mol wt) protein markers (catalase, yeast alcohol dehydrogenase, bovine serum albumin [BSA], ovalbumin, chymotrypsinogen A, and carbonic anhydrase), alkaline phosphatase–conjugated, affinity-purified antihuman IgG (γ chain specific), and p-nitrophenyl phosphate were obtained from Sigma Chemical Co, St. Louis. Human fibrinogen was from Kabi, Stockholm. Microtitration plates were purchased from Dynatech Laboratories, Inc, Alexandria, VA. All other chemicals were of analytic grade.

Protein determination. Protein concentration was determined according to the method of Bradford by using BSA as the standard. For monitoring the column fractions, absorbance at 280 nm was used.

Preparation of washed platelet suspension. Human platelets from normal subjects were isolated from freshly drawn citrated blood by using the albumin density gradient method of Walsh et al as described previously. The washed platelets were suspended in Tris-saline buffer, pH 7.4, containing 133 mmol/L NaCl, 15 mmol/L Tris-HCl, 5 mmol/L KCl, and 1 mmol/L MgCl2 and adjusted to a concentration of 7.5 × 108/mL.

Purification of PAP p37. The PAP p37 was purified from a TTP patient's plasma by aluminum hydroxide adsorption, ammonium sulfate precipitation, DEAE-Sephacel and Con A-Sepharose chromatographies as described previously. The PAP p37 was present in the plasma of the patient during active disease before plasma exchange. After plasmapheresis the amount of PAP p37 was decreased. PAP p37 became absent when the patient recovered.
Iodination of PAP p37 and fibrinogen. Purified PAP p37 and fibrinogen were iodinated by using a lactoperoxidase technique according to the procedure recommended by the manufacturer (Bio-Rad) with modifications. Specific activities of iodinated PAP p37 and fibrinogen were 600,000 and 6,000 cpm/μg of protein, respectively.

Purification of IgG. The IgG was purified from the serum of normal adults, a 5-month-old baby, and the same TTP patient during active disease and after recovery by 50% ammonium sulfate saturation followed by protein A-Sepharose CL-4B chromatography as previously described. Purification of factor VIII/von Willebrand factor. The FVIII/vWF was purified as described previously.

Demonstration of complex formation by enzyme-linked immunosorbent assay. The assay was performed according to the method of Voller et al with modifications. Microtitration plates were coated with purified PAP (0.5 to 2.0 μg/mL), human fibrinogen (2.0 μg/mL), FVIII/vWF (2.0 g/mL), and BSA (IgG free) in Tris-HCl, 0.9% NaCl, 0.05% Tween 20, pH 7.4. Varying amounts of NaHCO3, pH 9.6, 0.02% NaN3) for three hours at 37°C. The wells were washed five times with Tris-saline-Tween buffer (10 mM Tris-HCl, 0.9% NaCl, 0.05% Tween 20, pH 7.4). Varying amounts (0 to 100 μg/mL) of purified IgG in 200 μL from an infant, a normal adult, and the same TTP patient during active disease and after recovery were added to each coated well for one hour at 37°C. The washing procedure was repeated as before to remove the unbound IgG. To each well, 200 μL of alkaline phosphatase–conjugated, affinity-purified goat antihuman IgG (γ chain specific, 1:500 dilution) was added to the Tris-saline buffer, pH 7.4, without the detergent for one hour at 37°C. After washing at least five times with the Tris-saline-Tween buffer, 0.30 mL of 0.006 mol/L p-nitrophenyl phosphate in 0.1 mol/L glycine-HCl buffer, pH 10.4, containing 1 mmol/L each of MgCl and ZnCl (buffer A) was added to each well. After ten minutes, 0.20 mL was removed and diluted to a final volume of 1.0 mL with buffer A. The color intensity was read at 405 nm against a substrate blank without coating protein and IgG. The formation of the complex was expressed as the bound alkaline phosphatase activity at 405 nm/10 min.

Binding of PAP p37 to IgG-linked Sepharose 4B beads. Purified adult human IgG, 2 mg, was covalently coupled to 1.0 mL of cyanogen bromide–activated Sepharose 4B as published before and the instructions provided by Pharmacia Fine Chemicals. Efficiency of coupling was about 90% as determined by measuring protein concentrations of the coupling solutions before and after crosslinking reaction. One hundred microliters of the beads were washed two times with 10 mL of 50 mmol/L Tris-HCl, 0.9% NaCl, 0.05% NP-40, pH 7.4 (buffer B), and incubated in 1.0 mL of buffer B containing 1.0% BSA (IgG free) for two hours at 37°C to saturate nonspecific protein-binding sites. Aliquots containing 25 μL of the beads were removed, washed with 10 mL of Tris-HCl, pH 7.4, and incubated in a total volume of 0.5 mL of 10 mmol/L Tris-HCl, pH 7.4, containing varying amounts of [125I]-PAP (0.88 to 3.5 μg) at 37°C for one hour. The beads were then washed five times with the same buffer containing 0.9% NaCl and 0.05% NP-40. The total bound radioactivity counted in a dual-channel gamma counter. To determine the specificity of reaction, the binding of [125I]-labeled IgG to the IgG–Sepharose 4B beads was measured in a similar manner.

Sucrose density gradient ultracentrifugation. The purified iodinated PAP p37 and human IgG were incubated in Tris-saline buffer, pH 7.4, containing 133 mmol/L NaCl, 15 mmol/L Tris-HCl, 5 mmol/L KCl, and 1 mmol/L MgCl2 for one hour at 37°C. A 0.5-mL portion of the mixture was layered on the top of a 20% to 40% linear sucrose gradient (5 mL) prepared in the same buffer. This was centrifuged at 40,000 rpm for 14 hours at 4°C in a SW65 rotor. The gradient was fractionated by collecting eight drop fractions. The following proteins were used as standards: catalase, sedimentary coefficient (S20,W) = 11.35; IgG, S20,W = 7.05.

GeI filteration studies. The iodinated PAP (3.5 μg) and purified adult human IgG (2.16 mg) were incubated in 1.02 mL of 50 mmol/L Tris-HCl, pH 7.4, containing 100 mmol/L NaCl and 0.02% NaN (buffer C) for one hour at 37°C. The mixture was applied onto a calibrated column of Ultrogel ACA 34 (1.0 x 41.5 cm) equilibrated with the same buffer. The column was eluted with buffer C, and 0.5-mL fractions were collected. The formation of complexes between PAP and human IgG was detected by the elution profile of the iodinated PAP. The column was calibrated under the similar conditions by the following mol wt marker proteins: (a) yeast alcohol dehydrogenase (150,000), (b) BSA (66,000), (c) ovalbumin (45,000), (d) carbonic anhydrase (29,000), and (e) chymotrypsinogen A (25,000). The mol wt of the complexes was determined in a similar manner to the method of Ackers.

Isolation of specific IgG that forms a complex with PAP p37. Purification of IgG in 1.0 mL of cyano gen bromide–activated Sepharose 4B was published and the instructions provided by Pharmacia Fine Chemicals. The column was equilibrated with 50 mmol/L Tris-HCl, pH 7.4, containing 0.2% NaN (buffer D) and used to isolate the specific IgG that forms a complex with PAP p37. All operations were performed at room temperature (24°C). Purified normal adult IgG was dialyzed against buffer D, and 0.4 mL containing 0.58 mg was applied to a PAP p37 affinity column (0.5 x 3.0 cm). After the sample had entered the PAP p37–Sepharose, the column was closed for one hour. Then, the column was washed first with buffer D and then with 0.1 mL of NaCl in buffer D until absorbance at 280 nm became zero. The elution of the bound protein (specific IgG) was achieved with 0.1 mol/L glycine-HCl buffer, pH 2.5, containing 0.02% NaN. The 0.5-mL fractions were collected at a flow rate of 5 mL/h. Fractions under the flow-through peak and the bound peak were pooled respectively, concentrated, and dialyzed against Tris-saline buffer, pH 7.4, for platelet agglutination studies.

Effect of specific IgG (isolated from PAP p37 affinity column) on the TTP plasma–induced platelet agglutination. Platelet agglutination was measured in glass cuvettes with constant stirring in a chronolog aggregometer as described in detail. The IgG in 0.2 mL of Tris-saline buffer that bound to the PAP p37–Sepharose 4B column and the one that did not were incubated separately with 0.2 mL of TTP plasma (from which PAP p37 was purified) for one hour at 37°C. The incubation mixture was centrifuged at 47,800 g for 30 minutes at 4°C. The supernatant was removed and prewarmed at 37°C for five minutes. To this, 0.1 mL of prewarmed platelet suspension was added, and the agglutination was recorded. TTP plasma alone was used as control under similar conditions.

RESULTS

Binding of adult human IgG to the PAP coated on the polystyrene surface. An enzyme-linked immunosorbent assay (ELISA) was used to determine whether PAP forms a complex with IgG. PAP at varying concentrations (0.5 to 2.0 μg/mL) was coated directly on the wells of a plastic microtiration plate. Increasing amounts of purified human IgG was added, followed by the sequential addition of alkaline phosphatase–conjugated, affinity-purified goat antihuman IgG (γ chain specific) and the enzyme substrate. The extent of the complex formation between fluid-phase IgG and adsorbed PAP was detected by the enzymatic activity of the bound alkaline phosphatase (Fig 1). A dose-response relationship of IgG binding to the adsorbed PAP was demonstrated, with maximal binding occurring at about 60 μg/mL.
of IgG. Nonspecific binding of IgG was studied by using varying concentrations of IgG without PAP (Fig 1).

The specificity of the IgG interaction with the adsorbed PAP was further studied by incubating fluid-phase IgG in separate experiments with the adsorbed fibrinogen, BSA, and FVIII/vWF at the same concentration. Little complex formation was detected between IgG and the surface coated with these proteins (Fig 2).

Complex formation of PAP p37 with the IgG purified from an infant and a TTP patient during active disease and after recovery. The specificity of complex formation between PAP p37 and IgG was studied by using IgG purified from a 5-month-old infant and from the same TTP patient during active disease and after recovery. PAP p37 (2 μg/mL) was coated on the wells of a plastic microtitration plate. Increasing concentrations (0 to 100 μg/mL) of IgG purified from an infant and a TTP patient during active disease and after recovery were added in duplicate. Formation of a complex between PAP p37 and IgG was studied by ELISA as described earlier. As shown in Fig 3, PAP p37 formed a complex with the IgG purified from the TTP patient after recovery, and the complex formation was dependent on the IgG concentration. However, the interaction of PAP p37 with the IgG from the infant and the TTP patient during disease was minimal and was independent of IgG concentrations used.

Complex formation on IgG-linked Sepharose beads. The purified adult human IgG covalently cross-linked to cyanogen bromide–activated Sepharose 4B specifically bound 125I-PAP. The binding of varying amounts of 125I-PAP to the IgG beads is summarized in Table 1. This binding was not reversible because washing of the beads up to five times with Tris-saline buffer, pH 7.4, containing 0.05% NP-40 did not release the bound PAP. To demonstrate further the specificity of this binding, 125I-fibrinogen in over a 30-fold excess concentration than 125I-PAP was incubated with the IgG Sepharose beads under the similar conditions. The beads did not bind 125I-fibrinogen (Table 1).

Demonstration of PAP-IgG complex formation by sucrose density gradient ultracentrifugation. Sucrose density gradient ultracentrifugation was used to determine whether PAP p37 and IgG formed a complex in the fluid phase. Purified 125I-PAP showed a single peak on a 20% to 40% sucrose gradient with a sedimentation coefficient of 3.5S (Fig 4A). When fixed amounts of 125I-PAP were incubated with IgG at molar ratios of 1:30, 1:76 (Fig 4B), and 1:149 (not shown) for one hour at 37°C and the mixture was analyzed on a sucrose gradient, there was an increase in
the shift of the sedimentation value (S\text{20}) of \textsuperscript{125}I-PAP from 3.5S to 19S. Assuming that the partial specific volumes for PAP and the PAP-IgG complex are similar, an approximate estimation of the mol wt of the complex was obtained from the sedimentation coefficients according to Martin and Ames.\textsuperscript{21} The PAP-IgG complex has a mol wt of approximately 468,000.

**Demonstration of PAP-IgG complex formation by gel filtration.** Iodinated PAP p37 was incubated with purified IgG for one hour at 37\textdegree C, and the resulting mixture was chromatographed on a calibrated column of Ultrogel ACA-34. Formation of the complex between PAP and IgG was revealed by a shift in the elution behavior of \textsuperscript{125}I-PAP. As shown in Fig 5, \textsuperscript{125}I-PAP bound with IgG and formed complexes with mol wts ranging from 180,000 to over 350,000. A third radioactive peak eluted after the total bed volume of the column was noticed; this was most likely the free iodine released from the \textsuperscript{125}I-PAP during storage.

Isolation of specific IgG that formed complexes with PAP p37 and its effect on the TTP plasma-induced platelet agglutination. The specific IgG molecules that formed complexes with PAP p37 were isolated by PAP p37 affinity chromatography, and its effect on the platelet agglutination

<table>
<thead>
<tr>
<th>Protein in the Reaction Mixture (µg)</th>
<th>\textsuperscript{125}I-Protein Bound (µg)</th>
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<tbody>
<tr>
<td>\textsuperscript{125}I-fibrinogen (control)</td>
<td>0.0</td>
</tr>
<tr>
<td>56.5</td>
<td>0.0</td>
</tr>
<tr>
<td>113.0</td>
<td>0.0</td>
</tr>
<tr>
<td>\textsuperscript{125}I-PAP p37</td>
<td>0.12</td>
</tr>
<tr>
<td>0.88</td>
<td>0.19</td>
</tr>
<tr>
<td>1.75</td>
<td>0.26</td>
</tr>
<tr>
<td>2.63</td>
<td>0.29</td>
</tr>
<tr>
<td>3.50</td>
<td>0.29</td>
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IgG-linked Sepharose beads (25 µL) were incubated with varying amounts of \textsuperscript{125}I-fibrinogen (specific activity, 6,000 cpm/µg) or \textsuperscript{125}I-PAP p37 (specific activity, 600,000 cpm/µg) in a final volume of 0.5 mL, for one hour at 37\textdegree C, washed five times in 10 mmol/L Tris-HCl, pH 7.4, containing 0.9% NaCl and 0.05% NP-40, and then counted in a dual-channel gamma counter.

Fig 4. Sucrose density gradient ultracentrifugation of PAP p37 and fibrinogen. \textsuperscript{125}I-PAP either alone (A) or together with purified human IgG at molar ratios of 1:30 (open circles) or 1:76 (solid dots) (B) was incubated in Tris-saline buffer, pH 7.4, for one hour at 37\textdegree C. A 0.5 mL portion of the mixture was layered on separate 20% to 40% linear sucrose gradients (5 ml each) in the same buffer and sedimented at 40,000 rpm in a Beckman SW65 rotor for 14 hours at 4\textdegree C. Fractions of 0.16 mL were collected and the radioactivity counted in a gamma counter. Standards used were (a) IgG (7.0S) and (b) catalase (11.3S). The S\text{20}W of these proteins are given in parentheses and are plotted in the inset to the figure from which the S\text{20}W of the PAP and complex formed between PAP and IgG was determined.

Fig 5. Gel filtration of \textsuperscript{125}I-PAP p37 after interaction with purified human IgG. Iodinated PAP p37 (3.5 µg) and adult human IgG (2.16 mg) were incubated in 1.02 mL of 50 mmol/L Tris-HCl, pH 7.4, containing 100 mmol/L NaCl and 0.02% NaN\textsubscript{3} (buffer C) for one hour at 37\textdegree C. The mixture was analyzed on a calibrated column of Ultrogel ACA 34 (1.0 × 41.5 cm) equilibrated with buffer C. The formation of complexes between the two proteins was detected by the elution profile of \textsuperscript{125}I-PAP as shown by a solid line. The arrows indicate the elution of the following mol wt markers: (1) yeast alcohol dehydrogenase (150,000), (2) BSA (66,000), (3) ovalbumin (45,000), (4) carbonic anhydrase (29,000), and (5) chymotrypsinogen A (26,000). The elution profile of PAP alone is shown by a dotted line. The last radioactive peak eluted after the total bed volume of the column was most likely the free iodine released during the storage of \textsuperscript{125}I-PAP.
TABLE 2. EFFECT OF PAP p37 AFFINITY COLUMN–PURIFIED IgG ON THE TTP PLASMA–INDUCED PLATELET AGGREGATION.

<table>
<thead>
<tr>
<th>Reaction Mixture*</th>
<th>IgG Concentration (µg/mL)</th>
<th>Platelet Agglutination (%)</th>
<th>Inhibitor (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTP plasma plus buffer</td>
<td>0 77</td>
<td>27.5 24.7</td>
<td>0 10</td>
</tr>
<tr>
<td>TTP plasma plus IgG that flowed through p37 affinity column</td>
<td>77</td>
<td>24.7</td>
<td>10</td>
</tr>
<tr>
<td>TTP plasma plus IgG that bound to p37 affinity column</td>
<td>77</td>
<td>7.3</td>
<td>73</td>
</tr>
</tbody>
</table>

*A mixture of 0.2 mL of TTP plasma (that contains PAP p37) and 0.2 mL of Tris-saline buffer with or without IgG was incubated for one hour at 37°C. The mixture was spun at 47,800 g for 30 minutes. To the supernatant 0.1 mL of platelet suspension (7.5 × 10⁵/mL) was added, and the percentage of platelet agglutination was recorded. Platelet agglutination induced by TTP plasma in the absence of IgG was used as control.

We demonstrated that the TTP plasma–induced platelet agglutination was inhibited by the IgG purified from seven normal adult donors and the same TTP patient after recovery but not from infants under 4 years of age and the patient during acute disease. The IgG that did not form a complex with the PAP p37 came off in the flow-through peak. At 77 µg/mL it inhibited only 10% of TTP plasma–induced platelet agglutination. However, the specific IgG that formed a complex with PAP p37 was eluted with 0.1 mol/L glycine-HCl, pH 2.5; at the same concentration (77 µg/mL), it inhibited over 70% of TTP plasma–induced platelet agglutination.

DISCUSSION

We demonstrated that the TTP plasma–induced platelet agglutination was inhibited by the IgG purified from seven normal adult donors and the same TTP patient after recovery but not from infants under 4 years of age and the patient during acute disease. The IgG that inhibited the agglutination was not restricted to any specific subclass. The inhibitory activity could be found in IgG1, IgG2, and IgG3 separated by protein A chromatography. Prior incubation of IgG with TTP plasma caused a significantly greater reduction of platelet agglutination than that of IgG and platelet suspension, which suggested that IgG inhibits TTP plasma–induced platelet agglutination through a specific direct interaction with the platelet-agglutinating protein present in the TTP plasma rather than that on the platelet membrane.

Recently, we purified a 37-kd platelet-agglutinating protein from a TTP patient’s plasma. It was shown that PAP p37 was present in the plasma of three of five TTP patients. The PAP p37–induced platelet agglutination was inhibited by the IgG from normal adults and the same patient after recovery. To better understand the inhibitory action of IgG, we studied the interaction between purified PAP p37 and IgG. In this study, using four independent methods we presented direct evidence that PAP p37 forms a complex with the purified IgG from normal adults and the same TTP recovery patient. As shown in the ELISA assay, the binding of IgG to PAP coated on microtiter wells was specific, concentration dependent, and saturable. In the sucrose density gradient ultracentrifugation there was an increased shift of [125I]PAP from 3.5S to 19S in the sedimentation value (S_{wo}) as the molar ratio of IgG to PAP raised from 30:1 to 76:1. Even at the 149:1 molar ratio of IgG to PAP, there was still a lot of free PAP left, which suggested that only certain specific IgG molecules interact with PAP. To confirm this, we isolated those specific IgG molecules that formed a complex with PAP by PAP affinity chromatography and showed that this IgG inhibited TTP plasma–induced platelet agglutination. Because the complex between IgG and PAP is so specific, the inhibition of the agglutinating activity of PAP by IgG is therefore most likely through the formation of complex.

As we previously described, the IgG from infants and patients during acute disease does not inhibit TTP plasma–induced platelet agglutination, but that from the same patient after recovery does. In this study, we further confirmed that the IgG purified from an infant and the same TTP patient during active disease did not form a complex with the PAP p37. However, the IgG purified from the same patient after recovery formed a complex with PAP p37. Therefore, it is probable that the specific inhibitory IgG is acquired and induced by PAP p37 or PAP p37–like substances. Because PAP p37 is not present in normal plasma or patients with autoimmune thrombocytopenia or disseminated intravascular coagulation, it is likely that PAP p37 is a foreign substance and possibly derived from infectious agents. This possibility is currently under investigation.

REFERENCES

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Platelet-agglutinating protein P37 from a thrombotic thrombocytopenic purpura plasma forms a complex with human immunoglobulin G

FA Siddiqui and EC Lian