A Novel Platelet Aggregating Factor Found in a Patient With Defective Collagen-Induced Platelet Aggregation and Autoimmune Thrombocytopenia

By Tateo Sugiyama, Minoru Okuma, Fumitaka Ushikubi, Shigeki Sensaki, Kenji Kanaji, and Haruto Uchino

We found a novel platelet aggregating factor in a patient with steroid-responsive immune thrombocytopenic purpura that is associated with defective collagen-induced platelet functions. The aggregating factor and platelet functions were analyzed. The patient, a 58-year-old female, had purpura and prolonged bleeding time despite adequate platelet counts (>140,000/μL) after steroid therapy. The patient's platelets responded normally to all agonists except collagen. Platelet adhesion to collagen fibrils was decreased. The patient's plasma induced irreversible aggregation and ATP release in normal platelet-rich plasma (PRP). This platelet aggregating factor was found in F(ab')2 fragments of the patient's IgG, which caused thromboxane B2 synthesis, elevation of cytoplasmic Ca2+ levels, and phosphorylation of 40 kDa protein in normal platelets. Platelet aggregation by the patient's IgG was inhibited by prostacyclin, dibutyryl CMP, diltiazem, disodium ethylenediaminetetraacetate, and antimycin A plus iodoacetate, but ADP scavengers, cyclo-oxygenase inhibitors, and heparin had little or no effect. The aggregating activity of the patient's IgG absorbed to and eluted from normal platelets. The patient's Fab fragments did not induce platelet aggregation in eight of ten normal PRP but specifically inhibited aggregation induced by collagen and by the patient's IgG. The major component of an immunoprecipitate made with the patient's IgG from radiolabeled membrane proteins of normal platelet extract had a 62 kDa mol wt, while no such precipitate appeared in extracts of the patient's platelets. These results indicated that platelet aggregation by the patient's IgG was induced by the reaction of an antibody with a specific antigen on the normal platelet membrane through stimulus-response coupling. This antigen may be a collagen receptor on the platelet, most likely a polypeptide of 62 kDa under reducing condition. The defect of collagen-induced aggregation of the patient's platelets seemed to be due to alteration of the membrane protein related to this putative collagen receptor.

DIOPATHIC thrombocytopenic purpura (ITP) is a common hemorrhagic syndrome characterized by a reduced number of circulating platelets, normal to increased numbers of megakaryocytes, and accelerated platelet destruction.1,2 There is ample evidence that, at least in adults, platelet destruction is caused by the binding of an antiplatelet autoantibody to platelet-specific antigen. Further implication of ITP as an autoimmune disorder is the strong association between ITP and well-defined autoimmune diseases such as Graves' disease,3 Hashimoto's thyroiditis,3 and systemic lupus erythematosus.4 Several reports have been published describing an association between antiplatelet antibodies and various platelet function disorders.5-10 These reports suggest that antiplatelet antibody can contribute to or be responsible for not only quantitative but also qualitative platelet disorders, which can explain why some ITP patients with platelet counts greater than 50,000/μL still present a bleeding tendency. Although these qualitative platelet defects have been attributed to the effect of antiplatelet antibody, the underlying basis for an immune-mediated impairment of platelet functions is still unclear. It may be different from case to case, as recent studies on the antigenic specificity of antiplatelet antibodies in chronic ITP have shown that the target epitope differed among patients.11,12 Accordingly, it should be of great interest to know the nature of the antiplatelet antibody affecting the functions of autologous and/or homologous platelets, especially in relation to the significance of platelet membrane antigen on physiologic platelet responses.

Collagen plays an important role in primary events of hemostasis in vivo.12,13 While it can be assumed that there is a specific molecule on the surface of platelets that interacts with collagen, the identity of a collagen receptor on the platelet remains unclear despite extensive investigations in many laboratories.13-20 In this article we describe a novel platelet aggregating factor found in plasma of an ITP patient who showed a specific defect of collagen-induced platelet aggregation. Analysis of the factor revealed that it has characteristics of an antibody to a putative collagen receptor on the platelet membrane.

**MATERIALS AND METHODS**

**Case report.** The patient was a 58-year-old woman in 1986, and her past history revealed that she had given birth to two children without any problem and underwent a cataract operation without excessive bleeding at age 49. There was no family history of bleeding tendencies. In 1982 she was found, during physical examination, to have thrombocytopenia (15,000/μL), but no further examination was undertaken. From September 1983 she suffered from recurrent cutaneous ecchymoses, frequent episodes of epistaxis and gum bleeding, thirst, excessive sweating, palpitation, and weight loss. In December 1983, because of massive nasal bleeding, platelet concentrate prepared from 400-mL blood was transfused at a local hospital, and she was admitted to Kyoto University Hospital with a diagnosis of thrombocytopenic purpura and hyperthyroidism. Physical examination showed multiple ecchymoses and peticiae over the extremities and the trunk as well as diffuse goiter. There was no evidence of...
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A. NOVEL PLATELET AGGREGATING FACTOR IN ITP

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"Acid soluble" collagen (suspension of fine collagen fibnils) was

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pH 7.4)

or in buffer, as indicated in each experiment. Formalin-fixed

mmol/L dextrose), and platelets were pelleted by centrifugation at

5 mmol/L glucose, pH 7.4) and finally resuspended

Platelet adhesion to collagen fibrils.

Platelet aggregation and ATP secretion. Platelet aggregation

experiments were performed nephelometrically 23 as a percentage of the total activity.

Platelet ATP and ADP contents. The platelet contents of ATP and ADP were determined by the firefly-luminescence method as described previously 25.

Release of lactate dehydrogenase. Samples of PRP (0.2 mL) were stirred at 37°C and exposed to the patient's IgG, collagen, or saline. After five minutes, samples were removed and centrifuged at 10,000 g for two minutes. The lactate dehydrogenase (LDH) activity in the supernatant and its total activity after solubilization with 0.5% (wt/vol) Triton X-100 were determined according to the standard assay technique using an LDH kit (Iatron LDH-LQ, Iatron, Tokyo, Japan). LDH release was expressed by the activity in the supernatant as a percentage of the total activity.

Purification of IgG and preparation of F(ab')2 fragment, Fab fragment, and Fc fragment. Purified IgG was prepared from PPP by affinity chromatography with protein-A Sepharose CL-4B (Pharmacia Fine Chemicals, Piscataway, NJ). The IgG fraction was dialyzed against PBS and concentrated to the desired protein concentration on Amicon Ultrafiltration Cell (Amicon, Danvers, MA). F(ab')2 fragments were prepared from purified IgG by pepsin digestion 27 and isolated on a Sephadex G100 column (Pharmacia Fine Chemicals). Residual undigested IgG was removed on a protein-A Sepharose column. Fab fragments and Fc fragments were prepared from purified IgG by papain digestion.24 The digestion products were applied to a Sephadex G100 column to obtain fractions containing Fab fragments and Fc fragments followed by purification of each fragment on a protein-A Sepharose column. Purities of IgG and these fragments were checked by 7.5% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and by Ouchterlony immunodiffusion. Rabbit anti-\alpha, anti-\lambda, anti-Fc (IgG), and polyclonal anti-Ig antiserum were kindly donated by Dr T. Masuda (Institute for Immunology, Faculty of Medicine, Kyoto University).

SDS-PAGE. SDS-PAGE was performed by using slab gels according to the method of Laemmli. 29 The following mol wt markers (Bio-Rad, Richmond, CA) were utilized: myosin (200,000), beta-galactosidase (116,250), phosphorylase B (92,500), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400).

Thromboxane (TX) B2 assay. Platelet counts in the samples were adjusted to 3 × 10^9/L. Aliquots (0.2 mL) of PRP or washed platelet suspension in HEPES-buffered saline were incubated with an agonist or saline in cuvettes at 37°C under continuous stirring on the agitomotor. After five minutes the reaction was stopped with 0.1 mmol/L indomethacin, and the cuvettes were immediately cooled on ice. Samples were centrifuged at 10,000 g for two minutes at 4°C. TXB2 in the supernatant was assayed directly using a TXB2 kit (New England Nuclear, Boston).

Measurement of cytoplasmic free calcium ion concentration ([Ca2+]i). Platelet [Ca2+]i, was determined by using photoprotein aequorin purchased from Dr John Blinks (Mayo Clinic) by a slight modification of the method of Johnson et al. 30 Aequorin-loaded platelets were finally resuspended in HEPES-Tyrode's buffer without Ca2+, and the external calcium concentration was adjusted to 1 mmol/L by the addition of CaCl2 just prior to the measurement; the platelets were equilibrated at 37°C for five minutes. The elevation of

splenomegaly. Her blood counts showed 326 × 10^12 RBCs/μL, 6,100

leukocytes/μL, and 13,000 platelets/μL. Bleeding time by Duke's

method 21 was prolonged (ten minutes; normal < three minutes). A

bone marrow aspirate showed hypercellularity with immature mega-
karyocytes. Prothrombin time, activated partial thromboplastin
time, and plasma fibrinogen concentration were all within normal

limits. Serum triiodothyronine and total thyroxine levels were ele-

vated, and thyroid-stimulating hormone (TSH) level was decreased.

Tests for serum antithyroglobulin and antimicrosome antibodies

were positive. A diagnosis of immune thrombocytopenic purpura

associated with Graves' disease was made, and treatment with prednisolone (45 mg/d) and methimazole (30 mg/d) was initiated. Her platelet count rose rapidly to 168,000/μL, and prednisolone was tapered to 10 mg/d. Abnormal thyroid test results also quickly

her platelet counts remained above 140,000/μL (normal range 130,000 to 350,000/μL), occasional ecchymoses and

occasional

erythrocytopenia were noted. The platelet aggregation

study was repeated with the patient's platelet-rich plasma (PPP, vide infra) obtained after the patient's platelet counts became normal. All tests on platelets were done during treatment with prednisolone (5 to 15 mg/d) and methimazole (5 to 10 mg/d) without any other drugs that are known to interfere with platelet functions.

Normal controls. Healthy adult blood donors who had taken no

drugs for at least 2 weeks were chosen as normal controls. All studies

were done after informed consent was obtained from the patient and

normal subjects.

Platelet preparation. The collection of blood and preparation of

platelet-rich plasma (PRP) as well as PPP were carried out as

previously described. 23 For preparation of washed platelets, PRP was

acidified to approximately pH 6.5 with acid citrate dextrose solution

(38.1 mmol/L citric acid, 74.8 mmol/L trisodium citrate, 122.1

mmol/L dextrose), and platelets were pelleted by centrifugation at

1,100 g for ten minutes. The platelets were washed twice in platelet

washing buffer (10 mmol/L Tris-HCl, 150 mmol/L NaCl, 1

mmol/L EDTA, 5 mmol/L glucose, pH 7.4) and finally resuspended

in HEPES-buffered saline (10 mmol/L HEPES, 145 mmol/L NaCl, 5 mmol/L KCl, 0.5 mmol/L NaHPO4, 6 mmol/L glucose,

pH 7.4) or buffer, as indicated in each experiment. Formalin-fixed

washed platelets (FWP) were prepared according to the method of

Kirby and Mills.24 The FWP were washed twice with platelet

washing buffer and resuspended in 10 mmol/L phosphate-buffered

saline (PBS), pH 7.4. Platelet counts were done by Thrombocounter

(Coulter Diagnostics, Hialeah, FL). All procedures were carried out

at room temperature.

Platelet aggregation and ATP secretion. Platelet aggregation

experiments were performed nephelometrically 23 at a density of 3 × 10^10 platelets/μL on PRP or on washed platelet suspensions using a dual-channel aggregometer (NKK Hematracer, Niko Bioscience, Tokyo, Japan) linked to a pen recorder. Calcium chloride was added to the washed platelet suspension at 1 mmol/L before aggregation experiments. In experiments with platelet-inhibiting agents, PRP was preincubated with the inhibitor for three minutes at 37°C before the addition of IgG (100 μg/mL), except for iodoacetate and antimycin A (Boehringer Mannheim, West Germany) with which PRP was preincubated for ten minutes. For the secretion study ATP release was monitored on Lumaggregometer (Chrono-Log, Havertown, PA), as described previously. 23

Platelet adhesion to collagen fibrils. Platelet adhesion to col-

lagen fibrils was studied according to a modified method of Mant. 29

In short, blood was collected in one tenth volume of 77 mmol/L

EDTA, pH 7.4, and PRP and PPP were prepared as described above.

"Acid soluble" collagen (suspension of fine collagen fibrils) was

prepared from bovine Achilles tendon collagen (type II, Sigma

Chemical, St Louis). 23 One fifth milliliter of EDTA-anticoagulated

PRP (3 × 10^9 platelets/μL) was continuously stirred in a cuvette at

37°C on the agitomotor, and collagen at a final concentration of

15, 30, or 60 μg/mL, or saline (as a control) was added. After five

minutes, nonadherent platelets in each sample were counted in

Thrombocyto counter C, and platelet adhesion (%) was calculated using the formula: (platelet count of control PRP − platelet count of collagen-treated PRP) × 100/platelet count of control PRP.

Platelet ATP and ADP contents. The platelet contents of ATP

and ADP were determined by the firefly-luminescence method as
described previously 25.
platelet [Ca\(^{2+}\)], induced by IgG was measured on a Platelet Ionized Calcium Aggregometer (Chrono-Log) with simultaneous recording of platelet aggregation. [Ca\(^{2+}\)], was calculated according to Johnson et al.\(^{29}\)

**40 kDa protein phosphorylation.** Washed platelets (2 x 10\(^8\)) suspended in Tris-buffered saline (25 mmol/L Tris-HCl, 145 mmol/L NaCl, pH 7.4), were incubated with 0.4 Ci of \(^{32}P\)-orthophosphoric acid (Radiochemical Centre, Amersham, England) at room temperature for 60 minutes in a total volume of 4 mL. Platelets were then pelleted by centrifugation at 1,100 g for ten minutes and resuspended in assay buffer (25 mmol/L Tris-HCl, 145 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L MgCl\(_2\), 1 mmol/L CaCl\(_2\), pH 7.4) at a density of 5 x 10\(^5\)/\(\mu\)L. After preincubation for 10 min at 37°C, the reaction was started by the addition of IgG. Aliquots (100 \(\mu\)L) were transferred into tubes containing 10 \(\mu\)L of 11% SDS-550 mmol/L dithiothreitol\(^{30}\) and were boiled for two minutes. After the samples were chilled on ice, they were subjected to 10% SDS-PAGE. The gels were stained by Coomassie brilliant blue (Nakarai Chemical, Kyoto, Japan), dried on a filter paper, and autoradiographed by exposure to Fuji X-ray film for 1 to 2 weeks. A band corresponding to a 40 kDa protein was cut into a vial and counted for radioactivity in toluene scintillator (PPO 25 g, dimethyl-POPOP 1.5 g and toluene 5 L).

**Absorption of IgG by FWP.** Various concentrations of FWP in PBS (0 to 4 x 10\(^4\)/\(\mu\)L) were incubated with 2 mg/mL of the patient’s or normal IgG in the presence of apyrase (Sigma) (2 mg/mL) at 37°C for one hour and then at 4°C overnight. The mixtures were centrifuged at 1,100 g for ten minutes at 4°C, and the aggregating activity of each supernatant was tested on normal PRP to assess the absorption of IgG by FWP. The change in light transmittance of normal PRP (200 \(\mu\)L) was measured on a Platelet Ionized Lumiaggregometer, was normal in response to these stimuli and counted for radioactivity in toluene scintillator (PPO 25 g, dimethyl-POPOP 1.5 g and toluene 5 L). The supernatant was transferred to a tube to which a neutralizing reagent for aggregation studies from Hormon-Chemie, Munich, West Germany; arachidonic acid (AA) from Nu-Chek, Elsylia, MN; platelet activating factor (PAF) and A23187 from Calbiochem-Behring; heparin from Kodama, Osaka, Japan. PG\(_{12}\) was a generous gift from Ono. Sepharose 2B was obtained from Pharmacia Fine Chemicals. All other reagents were the same as described previously\(^{30}\) or were obtained from Nakarai, Kyoto, Japan.

**RESULTS**

**Characteristics of the patient's platelets.** Platelet aggregation in the patient’s PRP was normal in response to ADP, epinephrine, AA, ristocetin, A23187, and PAF (Fig 1). Thrombin-induced aggregation was also normal in washed platelet suspension (data not shown). In contrast, collagen (up to 10 \(\mu\)g/mL) induced neither shape change nor aggregation in PRP (Fig 1) or in washed platelet suspension (data not shown). The potentiating effect of epinephrine on collagen-induced aggregation was absent, whereas that on ADP- and AA-induced aggregation was observed, as is normal (data not shown). ATP release of PRP, as determined by Lumiaggregometer, was normal in response to these stimuli other than collagen, which induced no ATP release (data not shown). These results were consistently found over a period of two years. Adhesion of the patient’s platelets to collagen fibrils was 13.7%, 27.5%, and 50.2% at 15, 30, and 60 \(\mu\)g/mL of collagen, respectively. These values were lower than the normal ranges at all collagen concentrations studied (normal

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**Materials.** Pepsin, papain, lactoperoxidase, creatine phosphate (CP), creatine phosphokinase (CPK), and dibutyryl cyclic adenosine monophosphate (dbc AMP) were obtained from Sigma; collagen reagent for aggregation studies from Hormon-Chemie, Munich, West Germany; arachidonic acid (AA) from Nu-Chek, Elsylia, MN; platelet activating factor (PAF) and A23187 from Calbiochem-Behring; heparin from Kodama, Osaka, Japan. PG\(_{12}\), was a generous gift from Ono. Sepharose 2B was obtained from Pharmacia Fine Chemicals. All other reagents were the same as described previously or were obtained from Nakarai, Kyoto, Japan.
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Aggregating agents whose final concentrations are shown in parentheses or at the end of aggregation curves were added as indicated (\(\times\)). Aggregation patterns of control PRP in response to each agonist other than collagen are not shown because they are similar to those of the patient’s PRP. P. patient’s PRP; N, control PRP.

The aggregation response was associated with a transient decrease in light transmission and a lag period, during which ATP release was not observed (Fig 2, lower panels). The minimal concentration of the patient’s IgG required for aggregation of platelets obtained from 22 normal subjects remained within a range of 20 to 40 \(\mu\)g/mL. In the absence of stirring, the patient’s plasma and IgG induced no aggregation, but ATP release was observed on Lumiaggregometer (data not shown). Washed platelets were also aggregated by the patient’s plasma and IgG (data not shown). The patient’s plasma and IgG did not induce agglutination of normal FWP in the aggregometer. LDH release by the patient’s IgG (100 \(\mu\)g/mL), collagen (5 \(\mu\)g/mL), and saline were 3.4%, 3.3%, and 3.0%, respectively (mean of three experiments). Purified F(ab’)2 fragments (50 \(\mu\)g/mL) of the patient’s IgG induced platelet aggregation and ATP release with patterns that were similar to those induced by intact IgG (data not shown). Fc fragments (up to 500 \(\mu\)g/mL) were not effective (data not shown).

The patient’s Fab fragment did not induce aggregation up to a concentration of 1 mg/mL in eight of ten normal PRP, two of which were aggregated, however, by a concentration of 200 \(\mu\)g/mL and 500 \(\mu\)g/mL, respectively. Between these two groups of normal PRP, defined by the presence or absence of platelet aggregation induced by the Fab fragment, there was no obvious difference in the patterns of aggregation induced by collagen and by the patient’s IgG as well as by other agonists, including ADP, epinephrine, and AA (data not shown). When normal PRP, which was not aggregated by the patient’s Fab fragment was used, pre-incubation of the PRP with the Fab fragment for five minutes at 37°C inhibited aggregation induced by the patient’s IgG and by collagen in a concentration-dependent manner (Fig 3). This inhibitory effect of the Fab fragment was complete in all of four normal PRP studied and reached maximum after pre-incubation for five minutes (data not shown). The Fab fragment had no inhibitory effect on aggregation induced by ADP, epinephrine, AA, and ristocetin (data not shown).

Effects of platelet inhibitors on platelet aggregation induced by the patient’s IgG. The effects of platelet inhibi-

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Table 1. TXB2 Production by Platelets

<table>
<thead>
<tr>
<th>Platelets</th>
<th>TXB2 (ng/10^10 platelets)</th>
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<tbody>
<tr>
<td>PRP</td>
<td></td>
</tr>
<tr>
<td>Agonist</td>
<td>Patient</td>
</tr>
<tr>
<td>Collagen (2 (\mu)g/mL)</td>
<td>0.19</td>
</tr>
<tr>
<td>Saline</td>
<td>0.25</td>
</tr>
<tr>
<td>Washed platelets</td>
<td></td>
</tr>
<tr>
<td>Thrombin (0.5 (\mu)g/mL)</td>
<td>208</td>
</tr>
<tr>
<td>Saline</td>
<td>1.2</td>
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</tbody>
</table>

*Mean ± SD; (n).

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Fig 1. Aggregation tracings of the patient’s and control PRP. Aggregating agents whose final concentrations are shown in parentheses or at the end of aggregation curves were added as indicated (\(\times\)). Aggregation patterns of control PRP in response to each agonist other than collagen are not shown because they are similar to those of the patient’s PRP. P. patient’s PRP; N, control PRP.

Fig 2. Dose dependency of aggregation and ATP release of normal platelets induced by the patient’s PPP (plasma) and IgG. Plasma or IgG of the patient was added to normal PRP as indicated (\(\times\)) at various concentrations that were shown at the end of each aggregation curve.
Fig 3. Inhibitory effects of the patient’s Fab fragments on platelet aggregation induced by the patient’s IgG and by collagen. After the five-minute pre-incubation of normal PRP with various concentrations, as indicated, of the patient’s Fab fragments at 37°C, aggregation of the normal PRP was initiated by the stimulation with the patient’s IgG (100 µg/mL) or collagen (5 µg/mL). Percent inhibition of aggregation was calculated by the change in light transmittance seven minutes after the addition of patient’s IgG (0) and by the maximal change in light transmittance induced by collagen (0). Each value represents M ± SD from four experiments.

Table 2. Effects of Platelet Inhibitors on Platelet Aggregation Induced by the Patient’s IgG

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration</th>
<th>% Inhibition</th>
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<tr>
<td>PGI₂</td>
<td>10 nmol/L</td>
<td>100 ± 0*</td>
</tr>
<tr>
<td>dbc AMP</td>
<td>4 mmol/L</td>
<td>100 ± 0</td>
</tr>
<tr>
<td>EDTA</td>
<td>5 mmol/L</td>
<td>74 ± 6</td>
</tr>
<tr>
<td>Diltiazem</td>
<td>1 mmol/L</td>
<td>100 ± 0</td>
</tr>
<tr>
<td>Aspirin</td>
<td>1 mmol/L</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>100 µmol/L</td>
<td>1 ± 1</td>
</tr>
<tr>
<td>Apyrase</td>
<td>2 mg/mL</td>
<td>7 ± 6</td>
</tr>
<tr>
<td>CP/CPK</td>
<td>3 mmol/L/15 U/mL</td>
<td>3 ± 2</td>
</tr>
<tr>
<td>Iodoacetate</td>
<td>0.5 mmol/L</td>
<td>59 ± 8</td>
</tr>
<tr>
<td>Antimycin A</td>
<td>2 µmol/L</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>Iodoacetate/Antimycin A</td>
<td>0.5 mmol/L/2 µmol/L</td>
<td>100 ± 0</td>
</tr>
<tr>
<td>Heparin</td>
<td>2 U/mL</td>
<td>0</td>
</tr>
</tbody>
</table>

*Mean ± SD; n = 3.
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Fig 5. Time course of 40 kDa protein phosphorylation in normal platelets stimulated by IgG. Normal platelets (5 x 10^8/μL) prelabeled with 32P were exposed to the patient’s (O) or normal (△) IgG (100 μg/mL), and aliquots (100 μL) were transferred to the stop solution after varying periods of time as indicated, followed by SDS-PAGE, autoradiography, and the determination of radioactivity as described in Materials and Methods section. Each value represents mean from three experiments.

Fig 6. Autoradiographic patterns of NP-40-solubilized radiolabeled membrane proteins from normal platelets (A and B lanes) and the patient’s platelets (C and D lanes). A and C lanes, whole NP-40 extract (5 μL); B and D lanes, precipitates from the patient’s IgG-treated NP-40 extracts. Note that the patient’s IgG immunoprecipitated five apparent proteins (B lanes) in both reduced and unreduced states from the normal platelet extract but that no such proteins were precipitated from the patient’s own platelet extracts (D lanes). No precipitate was observed in normal IgG-treated normal and the patient’s NP-40 extracts (data not shown).

DISCUSSION

In the present study we described a patient with steroid-responsive ITP associated with Graves’ disease. In spite of adequate platelet counts (>140,000/μL) after steroid therapy, the patient showed a mild bleeding tendency due to platelet dysfunction, which seemed to be derived from the action of antibody. The predominant lesion in the qualitative platelet disorders observed by others in association with antiplatelet antibodies resembled that of storage pool disease; the impairment of platelet functions was detected in response to several agonists, and the patients’ platelets had decreased adenine nucleotide contents. In contrast to those patients, our patient’s platelets showed both normal adenine nucleotide contents and a specific defect only in response to collagen. Whereas other agonists induced normal platelet responses, collagen induced none of the usual platelet responses, including shape change, aggregation, ATP release, and TXB2 synthesis. These results suggested that only collagen failed to trigger the intracellular events that are normally produced or transmitted across the plasma membrane, and that the lesion in the patient’s platelets may exist in a membrane component related to a collagen binding site.

Rao et al reported that a patient with autoimmune thrombocytopenia showed an impairment of only collagen-induced platelet responses, including aggregation and adhesion. However, our patient is clearly distinct from this case since their patient’s plasma inhibited collagen-induced aggregation of normal platelets and since collagen-induced aggregation of their patient’s platelets was restored by combined stimulation with epinephrine. Clancy et al reported the existence of platelet-inhibiting activity in globulin fraction from ITP patients associated with a qualitative platelet disorder. In contrast to their report, however, our patient’s plasma caused aggregation and ATP release in normal PRP, indicating that there was no circulating inhibitor but a platelet-activating factor in the patient’s plasma. This factor was found in the IgG fraction purified by protein-A Sepharose. Aggregation and ATP release induced...
by this factor were not attributable to cell lysis because negligible LDH activity was released by the patient’s IgG. Furthermore, the responses must be independent of complement since the reaction was also observed in a complement-free system containing washed platelets and purified IgG. It has been reported that some MoAbs induced platelet activation in the absence of complement through reaction with specific antigens on the platelet membrane.34,40

Patient’s IgG caused TXB_2 synthesis, [Ca^{2+}], elevation, and 40 kDa protein phosphorylation in normal platelets, suggesting that platelet activation was brought about by the stimulus-response coupling pathway as seen in other physiologic agonists, including collagen and thrombin. Some antiplatelet antibodies agglutinate platelets by a passive antigen-antibody reaction (formation of an antibody-platelet lattice) independently of calcium.40 This possibility can be ruled out for the patient’s IgG because (1) the patient’s IgG failed to agglutinate FWP, which could still absorb it, and (2) the patient’s IgG-induced platelet aggregation was inhibited by an increase of intracellular cyclic AMP and required an intracellular Ca^{2+} flux and metabolic energy, although it was not dependent on released ADP, TXA_2, synthesis, or thrombin participation. The activity could be absorbed by and eluted from platelets, suggesting that the IgG was an antibody to a specific antigen on the platelet membrane. Furthermore, the binding site of the antibody was distinct from the Fc receptor on platelets, which mediates platelet activation by IgG aggregates41 or immune complexes42 because F(ab’)2 fragments prepared from the patient’s IgG-induced platelet aggregation as well as ATP release and because Fab fragments from the patient’s IgG completely abolished the aggregation of normal platelets induced by the patient’s IgG. It is possible that the action of the patient’s IgG may be directed at or near a putative collagen receptor since only collagen-induced aggregation was inhibited by Fab fragments of the patient’s IgG. Thus the specific binding of the patient’s IgG to the specific antigen on the platelet could induce a conformational change of the platelet membrane and/or a direct transmembrane signal, thereby allowing platelet activation.

The binding site of the patient’s IgG was examined by immunoprecipitation of radiolabeled, detergent-solubilized platelet membrane proteins and SDS-PAGE. From normal platelet extract, the patient’s IgG precipitated five polypeptides, of which a polypeptide of 62 kDa (reduced state) (57 kDa [unreduced state]) was the main protein, as judged from the intensity of radioactivity. There are two potential explanations for the precipitation of these multiple proteins: (1) the presence of antibodies against several different antigens; (2) the coprecipitation of a multicomponent complex by an antibody directed against only one of the components. The 62 kDa protein, the most prominent precipitate seen in our study, could be similar to the 65 kDa protein, which was isolated by using chick skin collagen immobilized on Sepharose beads,16 or to the 61 kDa protein, which has recently been reported to bind insoluble collagen.20 However, more direct experimental evidence is needed to substantiate the identity of these proteins. Nieuwenhuis et al15 reported a patient with platelets that were totally unresponsive to collagen and did not express surface GPIa, and they suggested that GPIa had collagen receptor activity. Our data, however, do not support this notion because our patient’s platelets had normal GPIa expression in spite of defective collagen-induced aggregation.

Our immunoprecipitation studies could not demonstrate any significant reaction of the patient’s IgG with her own platelet extract, although the electrophoresis of whole platelet extracts showed that the patient’s platelets were iodolabeled in the same way as normal platelets. Our experimental results imply that no additional antigen-antibody reaction could be induced in vitro using the patient’s platelets and that the patient’s platelets may not be activated by her own IgG. Furthermore the absence of the antigens, including the putative collagen receptor, could explain the defect of the patient’s platelet aggregation only in response to collagen. However, the meaning of the absence of such antigens on the patient’s platelet membrane is unknown. One possibility is that these antigens were lost or denatured, leading to destruction of their antibody-specific epitopes. This could occur during the peripheral circulation of the platelets and/or during the maturation of megakaryocytes because antiplatelet antibodies also react with narrow megakaryocytes.3 In a well-defined autoimmune disorder, myasthenia gravis, the acetylcholine receptor on the postsynaptic membrane is denatured and decreased by the action of antiacetylcholine receptor autoantibody through complement-mediated destruction44 or endocytosis.45 By analogy, the antigen possibly responsible for collagen-binding on the platelet membrane may be denatured in the patient’s platelets, preventing their response to collagen.

In our patient platelet adhesion to collagen fibrils was detectable, although it was significantly decreased compared with platelet adhesion in normal subjects. These results suggest that platelet membrane sites responsible for aggregation and adhesion are different, and the results support the concept of Kotite et al18 and Pintigny et al46 that collagen-induced platelet activation consists of a recognition (adhesion) step and an intermediary step (effect of collagen recognition) leading to postreceptor reactions, including the release of granular constituents and fibrinogen binding to platelets. Pintigny et al46 demonstrated that a synthetic octapeptide derived from type III collagen inhibited collagen-induced platelet aggregation without affecting platelet adhesion to collagen, indicating that the octapeptide reacted with the site of the intermediary step but not with the recognition site.46 Similarly, the defect on our patient’s platelets may reside in the site of the intermediary step.

In conclusion, we found a novel antiplatelet antibody in an ITP patient associated with defective collagen-induced platelet aggregation. The antibody induced platelet aggregation and ATP release in PRP as well as in a washed platelet suspension prepared from normal subjects. This antibody showed specificity against antigens, including one that seemed to be related to collagen-binding sites on normal platelets. Thus these studies may serve as a useful tool for further investigation of the platelet membrane constituents involved in collagen-induced platelet activation.
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REFERENCES

A novel platelet aggregating factor found in a patient with defective collagen-induced platelet aggregation and autoimmune thrombocytopenia

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