Autoantibodies Against the Platelet Glycoproteins (GP) IIb/IIIa, Ia/IIa, and IV and Partial Deficiency in GPIV in a Patient With a Bleeding Disorder and a Defective Platelet Collagen Interaction

By Jürg H. Beer, Manuela Rabaglio, Peter Berchtold, Arthur von Felten, Kenneth J. Clemetson, Dimitrios A. Tsakiris, Beate Kehrel, and Sibylle Brandenberger

To evaluate the physiologic importance of the different collagen receptors on platelets, we screened 806 patients admitted to the hospital because of hemorrhagic diathesis for eventual laboratory evidence of a pathologic platelet collagen interaction, and found 5 patients with an isolated deficiency in collagen-induced platelet aggregation. Four of these five patients had a partial defect, one had a complete defect. The structural and functional analysis of the platelets from the patient with a complete defect showed a deficiency in glycoprotein (GP) IV and autoantibodies against GPIIb/IIIa, GPIa/IIa, and GPIV. Patient plasma had only a minimal effect on normal control platelets and NaN02-negative platelets. The analyses of the defect in the patient and of the data in the literature suggest that a single defect may not result in clinical bleeding (GPIV-deficient patients do not bleed), but may become symptomatic in combination with another defect such as the autoantibodies against GPIIb/IIIa, GPIV, and/or GPIIb/IIIa, all of which are involved in platelet collagen interactions (three of four of our immune thrombocytopenic purpura patients with anti-GPIV and anti-GPIIb/IIIa autoantibodies had a bleeding disorder). We hypothesize that it is the synergism of two abnormalities that results in the defective function, a mechanism that is in agreement with earlier studies on platelet collagen interaction that suggests that a double defect in platelet collagen interactions is required to become clinically apparent.

CELLULAR ADHESION to collagen is a critical event in primary hemostasis. Under pathologic conditions, the failure of platelets to adhere on collagen results in a hemorrhagic diathesis. At least three different receptors on platelets have been identified that directly mediate primary platelet collagen interactions, including the glycoproteins (GP) Ia/IIa (VLA-2),13 GPIIb (IIIb),6,8 and GPIV.10 However, patients with a documented deficiency of one of these receptors and a hemorrhagic disorder are exceedingly rare: only two such patients have been reported with deficient GPIa,1,2 one family with an abnormal GPIV,11 and one patient with a GPVI deficiency.9 In addition, indirect platelet collagen interactions are thought to occur between adhesive GP, such as fibrinogen and von Willebrand factor, which adhere to collagen on one hand and their respective platelet receptors on the other.12-14 To evaluate the physiologic importance of this large number of possible interactions, we hypothesized that the analysis of patients with a bleeding disorder and an isolated deficiency in platelet collagen interactions should be helpful in identifying functionally relevant mechanisms.

MATERIALS AND METHODS

Patient screening. We screened the history and laboratory data of 806 patients with hemorrhagic diathesis who were referred to the hematology departments of three Swiss University Hospitals (Zürich, Basel, and Bern). We found five patients with an isolated defect in collagen-induced platelet aggregation. Four of these patients showed a partial and one a complete deficiency. Data pertaining to this latter patient were further analyzed. She was a 24-year-old Vietnamese woman who had lived for several years in Europe. She was healthy until 2 years previous to presentation, during which time she gradually developed a hemorrhagic diathesis with gum and skin bleeding, easy bruising, and heavy menses. Iron deficiency and mild anemia was diagnosed and she was given iron substitution therapy, but she had never required blood transfusions and did not take any known platelet inhibitory drug. The family history had no record of bleeding problems, and she had never been pregnant.

Platelet aggregation. Platelet aggregation studies were performed as described15 in a Payton aggregometer (Payton, Buffalo, NY) using platelet-rich plasma (PRP, 200 × 10^3/L). Blood was anticoagulated with 0.1 volume of 0.1 mol/L Na citrate and PRP was prepared by centrifugation at 200g for 10 minutes at 22°C. Gel-filtered platelets (GFP) were prepared by layering the PRP onto a column of Sepharose 2B and eluting with a modified Tyrode's solution containing no added CaCl2 and 2 mmol/L MgCl2 (138 mmol/L NaCl, 2.7 mmol/L KCl, 0.4 mmol/L NaH2PO4, 12 mmol/L NaHCO3, 2 mmol/L MgCl2, 0.2% bovine serum albumin [BSA], 0.1% glucose, 0.01 mol/L HEPES, pH 7.4).

The collagen preparation used in this study was a suspension of native collagen fibrils from equine tendons (Collagen-reagent Horm, Hormon Chemie, Munich, Germany). The equine tendon preparation was diluted immediately before use in aggregometry in the acidic buffer supplied by the manufacturer and maintained at 4°C throughout the experiment. The other collagen preparation used in this study was purified collagen type I preparation from human skin, provided by Beate Kehrel, PhD (Department of Medicine, Muenster, Germany).

Mixing experiments. To evaluate the effect of patient plasma on the collagen-induced aggregation of normal platelets, mixing
experiments were performed: one part PRP (600 × 10^9/L) from a normal donor of the same blood group (A, rhesus positive) was mixed with two parts of the patient’s PRP from different time points and incubated for 10 or 60 minutes at 37°C (final platelet concentration 200 × 10^9/L). A sample from PRP with the same control person (200 × 10^9/L) was treated identically and collagen-induced aggregations were performed at several concentrations. Similarly, platelet-poor plasma (PPP) from the patient or a control person was mixed with GFP from a normal donor (1:1, vol/vol) and collagen-induced aggregation was performed. In addition, platelet plasma (two parts) was mixed with PPP (one part, final platelet concentration 200 × 10^9/L) of a NaK+-negative donor or with control-platelets, and collagen-induced aggregations were compared.

Other mixing experiments included the collagen-induced aggregation of a mixture of patient plasma from before treatment with prednisone (see below) with patient platelets from approximately 10^9/L. A sample with PRP from the same blood group (A, rhesus positive) was used at the concentrations indicated in Fig 1A. The aggregation suggested an abnormal platelet collagen interaction. EDTA allows only a qualitative interpretation, it is interesting that the results obtained by fluorometry using a FACScan (Becton Dickinson, San Jose, CA). The fluorescence obtained with a nonspecific mouse IgG was subtracted. The mean fluorescence of platelets from the patient was increased 50% and the patient values were expressed as percentage of normals. The results in Table 1 are the means of two separate experiments.

Analysis of platelet GP by two-dimensional sodium dodecyl sulfitopolyacrylamide gel electrophoresis (SDS-PAGE) The methods used are described in detail in McGregor et al. Briefly, 30 mL blood from the patient and a healthy control were drawn into 1/7 vol/vol EDTA (4 mmol/L). PRP was washed twice with PBS containing 2 mmol/L CaCl₂ and 0.33 mL bead slurry per milliliter collagen solution; Matrex 102; Amicon, Danvers, MA) containing N-hydroxysuccinimide groups. The assay consisted of adding 70 µL patient or control PRP at 22°C into the wells of a microtiter plate, and then adding 5 µL of the collagen-coated beads. The plates were rotated at 270 rpm for 20 minutes at 22°C and the extent of bead aggregation was assessed macroscopically at 30-second to 2-minute intervals with the aid of a magnifying mirror. A delayed or reduced extent of total aggregation suggests an abnormal platelet collagen interaction. EDTA (1.9 mmol/L final concentration) was used to demonstrate the calcium-dependent and -independent interactions. The assay was performed in duplicate on two separate occasions. Although this assay allows only a qualitative interpretation, it is interesting that the visual degree of aggregation correlates inversely with the platelet count in the supernatant and therefore directly reflects the number of platelets incorporated in the agglutinates. Measurement of the content of proteins of the platelet α- and β-granules. The content of the beta-thromboglobulin (BTG) as a measure of the platelet α-granules was determined by radioimmunoassay (RIA) (RIA-Kit; Amersham, Bucks, England). Briefly, PRP was obtained from whole blood using EDTA 4 mmol/L as an anticoagulant in the presence of apyrase (0.15 U/mL, Sigma Chemical Co), adjusted to 20, 10, and 5 × 10⁹ platelets/L, lysed with Triton X-100 2% (1:1, vol/vol), and determined. The content of ADP as a measure of the content of β-granules was determined by reversed-phase high-performance liquid chromatography.

Measurement of the malondialdehyde (MDA) production. Stimulated MDA production was determined to demonstrate the intactness of the arachidonate pathway as described by Stuart et al. Briefly, 2 mL washed platelets from citrated PRP, 200 × 10^9/L were incubated with N-ethylmaleimide (80 µmol/L) for 1 hour at 36°C; the reaction was terminated by adding an equal volume of 2.3% perchloric acid containing 0.53% thiobarbituric acid. After boiling for 15 minutes, the optical density (OD) at 532 nm of the supernatant was determined and the results expressed in nmol/10⁹ platelets, using the molar absorbance of MDA of 1.37 × 10³.
particles) goat antirabbit antibody (1:100; Aurion, Wageningen, Holland) as a secondary antibody and visualized the bands by silver enhancement.

Detection of platelet-associated and circulating plasma autoantibodies against platelet GP. Autoantibodies against GPIb/IIa, GPIb/IX, GPIa/IIa, and GPIV were detected using the GP-specific immunobead assay as described.²⁰,²¹ Briefly, washed patient platelets or normal platelets incubated with patient plasma were solubilized with Triton X-100. The lysate was mixed with polystyrene beads that had been coated previously with MoAb against GPIb/IIa (2A9, provided by Dr V. Woods, UCLA, San Diego, CA), GPIa/IIa (6F1), and GPIV (OKM5) for the detection of anti-GPIb/IIa, anti-GPla/IIa, and anti-GPIV autoantibodies or against human IgG (HB43). American Type Culture Collection [ATCC], Rockville, MD) for the measurement of anti-GPIb/IX autoantibodies. Any bound immunocomplexes consisting of autoantibodies and GPIlb/IIIa, GPIa/IIa, or GPIV were detected using radiolabeled (Iodine-125) monoclonal anti-human IgG antibody (HB43). Autoantibody GPIb/IX complexes were detected by radiolabeled MoAb against GPIb/IX (6DI). The results were expressed as binding ratio of the patient value, divided by the mean of more than 10 control persons. A binding ratio of more than three standard deviations above the mean of the controls was considered positive (Table 2). Total platelet-associated immunoglobulins were measured by RIA using GFP and monoclonal anti-IgG and -lgM antibodies (ATCC).

A population of 43 immune thrombocytopenic purpura (ITP) patients was analyzed retrospectively for autoantibodies against the above-mentioned GPs as well.

Other methods. The Simplate bleeding time was performed at three different times at least 4 weeks apart. Two to 5 minutes are considered normal, 5 to 8 minutes borderline, and >8 minutes clearly pathological.

After diagnostic evaluation on several occasions, the patient was treated with prednisone for 3 weeks (100 mg daily for 2 weeks and with tapering doses for 1 week). She was reexamined after this period and again after 1 year. The analyses consisted of aggregation studies and the assessment of the autoantibody ratio.

Fig 1. Platelet aggregation studies with citrated PRP or GFP. (A) The aggregation studies with the PRP of the patient (182 × 10⁹ platelets/L) showed normal patterns in response to all agonists tested, including ADP, adrenalin, arachidonic acid, calcium ionophore A23187, PAF, and ristocetin. However, no response could be observed with collagen type I at concentrations up to 20 μg/mL, whereas even 0.5-1 μg/mL resulted in a maximal response with platelets from normals under identical conditions. At concentrations of 10 and 20 μg/mL, a minimal increase in light transmission occurred because of the adhesion of platelets onto collagen fibers in a monolayer pattern without aggregation as confirmed by light microscopy. A shape-change reaction of the platelets, indicated by the initial, transient decrease in light transmission and the thinning of the tracing, could not be detected. (B) Mixing experiments, using two parts PRP of a normal donor of the same blood type with platelet-poor plasma from the patient (final platelet concentration: 200 × 10⁹/L) showed only a minimal inhibitory effect, which could be particularly observed with the lower collagen concentrations used. (A) and (B) show the results of one of two similar experiments 4 weeks apart before the treatment with corticosteroids. (C) The results of the collagen-induced aggregations after a 3-week course of prednisone treatment are shown. Note the reappearance of the shape change at collagen concentrations of 2.5 μg/mL. Full aggregation could be observed in PRP at 5 μg/mL. GFP reacted similarly. The requirement for higher collagen concentrations for maximal aggregation was likely caused by the lower platelet count obtained in GFP (66 × 10⁹/L) than in PRP (226 × 10⁹/L).
PLATELET-COLLAGEN INTERACTIONS

<table>
<thead>
<tr>
<th>Platelet GP</th>
<th>MoAb</th>
<th>Fluorescence (in % of normal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GP IV</td>
<td>OKM 5</td>
<td>47.3</td>
</tr>
<tr>
<td>GP Ibb/IIIa</td>
<td>10E5</td>
<td>119.9</td>
</tr>
<tr>
<td>GP IIIa</td>
<td>Y2/51</td>
<td>107.4</td>
</tr>
<tr>
<td>GP lb</td>
<td>6D1</td>
<td>99.9</td>
</tr>
<tr>
<td>GP lb</td>
<td>Ib-6</td>
<td>114.5</td>
</tr>
<tr>
<td>GP Ia/IIb</td>
<td>6F1</td>
<td>92.5</td>
</tr>
</tbody>
</table>

Analysis of platelet GP by flow cytometry. PRP was incubated with the MoAbs 10E5, 6D1, 6F1, Y2/51, and OKM5 directed against platelet GP as indicated. Fluorescence was assessed by a secondary, FITC-labeled antibody. The fluorescence obtained with nonspecific mouse IgG was subtracted. The mean fluorescence from five normal donors treated identically in the same series were set as 100% and the patient values were expressed as percentage of normal. The results are the mean of two separate experiments and indicate an important reduction of GPIV and normal values for all the other GP analyzed.

Immunoprecipitation studies were performed as described. Briefly, washed patient and control platelets were biotinylated (10 mmol/L NHS-Biotin, Sigma Chemical Co), lysed and incubated with protein A Sepharose, which had been precleared with a non-biotinylated normal platelet lysate. The beads were washed and incubated with SDS containing sample buffer, boiled, and separated by SDS-PAGE, blotted onto PVDF-blotting paper (Immobilon P, Millipore, Guyancourt, France), and developed with streptavidin alkaline phosphatase and nitrotetrazolium blue plus 5-bromo-4-chloro-3-indolyl phosphate.

RESULTS

The bleeding time was longer than 20 minutes on three separate occasions, whereas the platelet count was only slightly below the normal range (103 to 118 × 10⁹/L).

The routine laboratory data were normal, including liver and kidney function tests. The analysis of plasminogen activation (including von Willebrand factor) was normal (Table 3). A bone marrow aspirate showed increased megakaryopoiesis and erythropoiesis but no signs of infiltration or myelodysplasia. Liver and spleen were normal by clinical examination and ultrasonography. There was no evidence of infectious disease. Chronic immune thrombocytopenia was diagnosed.

Platelet aggregation studies. Platelet aggregation on two separate occasions in citrated PRP showed normal aggregations for ADP, PAF, adrenaline, arachidonic acid, the calcium-ionophore A23187, and ristocetin (Fig 1A). However, collagen-induced aggregation was absent, even at massively increased concentrations of up to 20 μg/mL of both collagen preparations tested. A shape-change reaction of the platelets, indicated by the initial, transient decrease in light transmission and the thinning of the tracing, could not be detected. Under identical conditions, platelets from normal donors showed the normal shape-change reaction at 0.2 μg/mL collagen (Horm) and full aggregation at 1 to 2 μg/mL. In the patient, studies a minimal increase in light transmission occurred at collagen concentrations of 10 to 20 μg/mL, because of the adhesion of platelets onto collagen fibers in a monolayer pattern, without aggregation, as confirmed by phase contrast light microscopy, suggesting at least some adhesion to collagen occurring under these conditions. The mixing experiments with platelets of a normal donor and patient plasma from different time points showed only a minimal inhibitory effect (Fig 1B) regardless of whether the incubation time was 10 or 60 minutes, thus indicating that the potential plasmatic inhibitor had only minimal effect on collagen-induced aggregation with normal platelets. The inhibition on normal and patient platelets was not more prominent if plasma from the time of fully absent collagen-aggregation was used (ie, before the treatment with prednisone) as opposed to plasma from a time with only partially inhibited collagen response (ie, after prednisone treatment), and patient plasma could not inhibit the collagen-induced aggregation of platelets from a Naka*-negative donor more than that of a control person.

No increased sensitivity of ADP-induced aggregation to super low doses of antibody 7E3 could be detected in patient samples compared with control-platelets (data not shown).

To exclude the possibility of platelet activation in vivo and a potential storage pool deficiency, components from the platelet α- and β-granules, BTG, and ADP were determined and found to be within the normal range. The ingestion of aspirin or other drugs affecting the thromboxane generation as well as endogenous defects in the arachidonate pathway were excluded by normal values of MDA production in platelets, in addition to the normal arachidonic acid-induced platelet aggregation on two separate occasions and a negative history of medication. Macrophagocytes could be excluded by the normal platelet size in the blood smear and the normal mean platelet volume.

Analysis of platelet GP by flow cytometry. The mean fluorescence for GPIa/IIa, GPIb, and GPIIb/IIIa showed the same values for the patient as for the five normal controls (Table 1). In the case of GPIb and GPIIIa, the normal values were confirmed by the use of antibodies directed against a different epitope. In contrast, mean fluorescence for GPIV was reduced to 47.3% of normal, thus suggesting a quantitative reduction of GPIV by 50% in the patient.

Analysis of platelet GP by two-dimensional electrophoresis. The fluorography of a two-dimensional slab gel analysis of surface-labeled patient platelets (Fig 2, left) indicated that the band for GPIV was considerably reduced compared with the normal control (Fig 2, right). A quantitative reduction to about 25% of normal GPIV levels could be determined by densitometry. Other GP showed normal values and migration patterns, including GPIb/IIIa, GPIbα, and the other receptors that have been reported to mediate platelet collagen interactions, namely GPIa/IIa and GPVI. Some GP of lower molecular weight (GPIbβ and GPIbδ) showed repeatedly and reproducibly weak labeling, whereas others (GPVI and GPIX) seemed normal. The migration pattern and the amount of proteins as judged by the silverstained gel were normal, which led us to conclude that a reduction in the sialylation or glycosylation of some proteins may account for the weaker labeling. The silver stain of an identically treated gel showed a reduction of GPIV by at least 50% as did the analysis by Western blotting using a polyclonal antibody.
antibody against GPIV (data not shown). Western blot studies using platelet lysates from normal donors and from the patient as antigen and the patient serum as a source of antibodies were negative: in particular, GPIa, GPIb, GPIIa, and GPIV showed no positivity (data not shown).

Taken together, these data indicate a quantitative reduction of GPIV, possibly a heterozygous deficiency, and no qualitative change in the protein structure or in the carbohydrate portion, in which case a different migration pattern but not a reduced protein content would be expected.

Platelet-associated and circulating plasma autoantibodies. As summarized in Table 2, high binding ratios of autoantibodies against GPIIb/IIIa, GPIa/IIa, and GPIV were found. The results indicate strong positivity for platelet-associated antibodies; a weaker positivity could be demonstrated for plasma autoantibodies against these GP as well. However, completely negative results were obtained for autoantibodies against GPIb/IX. Together with the increased megakaryocytes in the bone marrow and the peripheral platelet counts in the subnormal range, the diagnosis of chronic immunthrombocytopenia was established. Further analysis of a large population of patients with active ITP showed that in addition to our patient described in this report, another patient had autoantibodies against GPIa/IIa and 5 of 43 against GPIV. Interestingly, 3 of 4 patients with anti-GPIV and GPIIIb/IIIa antibodies had a bleeding disorder and bleeding times >15 minutes at platelet counts of 90 × 10^9/L or higher. Seven of 39 patients had autoantibodies against the two GP GPIIIb/IIIa and Ib/IX and one patient against three (namely, GPIa/IIa, GPIIb/IIIa, and GPIb/IX).

Immunoprecipitation studies confirmed autoantibodies against GPIIa and suggested the presence of autoantibodies against GPIa and GPIV (data not shown).

Collagen-coated bead agglutination assay. Whereas the normal control preparation reached half-maximal agglutination after 4 minutes and full agglutination after 8 to 15 minutes, the agglutination with the patient platelets was incomplete: half-maximal agglutination was achieved only after 15 minutes and did not proceed to full agglutination. The positive response of normal platelets in the presence of EDTA confirms the presence of divalent cation-dependent and -independent mechanisms of platelet collagen interactions (Fig 3).

Analysis of the patient after the treatment with corticoste-roids. After a 3-week course of prednisone treatment, the following results were obtained: the platelet count increased slightly to ~130 × 10^9/L (Table 3) and at least a partially restored response was obtained in the collagen-induced aggregation with 2.5 μg/mL collagen and a full aggregation could be induced with 3 to 5 μg/mL in PRP. The shape change and a full aggregation response could be obtained with GFP as well. It is remarkable that the collagen concentration required for full aggregation is still 5 to 10 times higher than in normal controls. The requirement for higher collagen concentrations for maximal aggregation is likely because of the much lower platelet count obtained in GFP (66 × 10^9/L) (Fig 1C). The reappearance of the responsiveness to collagen was accompanied by a substantial decrease in the autoantibody ratio against GPIIb/IIIa (from 11.5 to 2.9). Table 2) of platelet-associated autoantibodies and even a reduction of the autoantibodies in plasma to normal levels. However, the partial recovery of laboratory results was not sufficient to reduce the bleeding time nor the bleeding diathesis (bruises, heavy menses). At the follow-up examination after 1 year, the clinical bleeding symptoms persisted and the antibody titers were again high (Table 2), and the collagen aggregation response was partial: concentrations of 2.5 to 5 μg/mL were required for a normal, full response with patient platelets.

DISCUSSION

Patients with a bleeding disorder and an isolated defect in platelet collagen interactions that can be detected in routine aggregation studies are rare. This is supported by the paucity of cases published in the literature and the incidence of less than 1% in our selected patient population with a bleeding disorder. Nevertheless, these patients may contribute to the understanding of the clinical importance of platelet collagen interactions observed in vitro.

Platelet collagen interactions seem to be of such vital importance for normal platelet function that a large number of different mechanisms are capable of mediating them. Three GP receptors on platelets have been shown to bind directly collagen of various types, including the GPIa/IIa (VLA-2), GPIV, and probably GPVI. In addition to the primary stimulus, a number of secondary interactions are thought to occur through "bridging molecules," i.e., circulating GP such as fibrinogen, von Willebrand factor, fibronectin, and perhaps thrombospondin. which have all been
shown to bind to collagen and to their respective receptors on platelets (GPIIb/IIIa, GPIb, and GPIc/IIa). Each of these receptors seems to mediate the interaction under certain conditions, ie, in an activation-dependent manner (such as in the case of GPIIb/IIIa), or at a certain shear rate (GPIb), thus bypassing the inhibitory effect of the antibody with secondary positive aggregation response could be obtained.

A murine MoAb (6F1) against GPIIa/IIa induces a complete inhibition of collagen-induced platelet aggregation in the absence of plasma but not in its presence: fibrinogen and other GP adhere to collagen and bridge to their respective receptors on the platelet surface, thus by-passing the concept of more than one defect being located on the platelet.

**Table 2. Autoantibodies Directed Against Platelet GP**

<table>
<thead>
<tr>
<th>Treatment With Corticosteroids</th>
<th>Follow-up</th>
<th>Normal Range</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After 1 Year</td>
</tr>
<tr>
<td>Anti-GP IIb/IIIa, platelet-associated</td>
<td>11.5</td>
<td>2.9</td>
</tr>
<tr>
<td>in plasma</td>
<td>2.6</td>
<td>1.1</td>
</tr>
<tr>
<td>Anti-GP IIb/IX, platelet-associated</td>
<td>0.9</td>
<td>1.2</td>
</tr>
<tr>
<td>in plasma</td>
<td>0.7</td>
<td>1.1</td>
</tr>
<tr>
<td>Anti-GP IV, platelet-associated</td>
<td>—</td>
<td>9.2</td>
</tr>
<tr>
<td>in plasma</td>
<td>—</td>
<td>7.6</td>
</tr>
<tr>
<td>Quantification of platelet-associated immunoglobulins</td>
<td>IgG 118</td>
<td>—</td>
</tr>
<tr>
<td>immunoglobulins</td>
<td>IgM 117</td>
<td>—</td>
</tr>
</tbody>
</table>

Platelet-associated and circulating plasma autoantibodies. The autoantibodies were detected using the GP-specific immunobead assay as described in the text. The results show high binding ratios of autoantibodies against GPIIb/IIIa and normal values against GPIb on the first examination. After treatment with corticosteroids, the platelet-associated autoantibodies (directed against GPIIb/IIIa) were substantially reduced and the autoantibodies in plasma normalized, respectively. They increased after 1 year to levels similar to those initially found. At this point, autoantibodies against GPIIa/IIa and GPIV could also be determined and strong positivities were found. All samples were analyzed in the same series of experiments. Quantification of the platelet-associated immunoglobulins was performed by RIA using GFP and monoclonal anti-IgG and anti-IgM antibodies.

**Table 3. General Coagulation and Platelet Data**

<table>
<thead>
<tr>
<th>Treatment With Corticosteroids</th>
<th>Normal Range</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Before</td>
</tr>
<tr>
<td>Bleeding time (Simplate)</td>
<td>&gt;20 min</td>
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<tr>
<td>Partial thromboplastin time</td>
<td>36</td>
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<tr>
<td>Prothrombin time (Quick)</td>
<td>11.8</td>
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<tr>
<td>Fibrinogen (Clauss)</td>
<td>2.3</td>
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<tr>
<td>Thrombin time</td>
<td>16</td>
</tr>
<tr>
<td>Ethanol gelation test</td>
<td>neg</td>
</tr>
<tr>
<td>Factor VIII coagulant activity</td>
<td>100</td>
</tr>
<tr>
<td>von Willebrand factor (antigen)</td>
<td>110</td>
</tr>
<tr>
<td>Ristocetin cofactor</td>
<td>116</td>
</tr>
<tr>
<td>Factor IX</td>
<td>89</td>
</tr>
<tr>
<td>Factor XI</td>
<td>95</td>
</tr>
<tr>
<td>Factor XIII A</td>
<td>Normal</td>
</tr>
<tr>
<td>Platelet count</td>
<td>113-118</td>
</tr>
<tr>
<td>Mean platelet volume</td>
<td>9.4</td>
</tr>
<tr>
<td>Clot retraction</td>
<td>Normal</td>
</tr>
<tr>
<td>Beta-thromboglobulin (P-content)</td>
<td>42.1</td>
</tr>
<tr>
<td>ADP (P-content)</td>
<td>42.8</td>
</tr>
<tr>
<td>MDA production</td>
<td>12.6</td>
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</table>

The general coagulation and platelet data include a massively prolonged bleeding time at platelet counts in the subnormal range; all the other values, especially von Willebrand factor, the platelet volume, the platelet-granule contents, and the production of MDA are in the normal range, thus excluding macrothrombocytes, a storage pool deficiency, or a defect of the aspirin-type.
one of two similar experiments. Full agglutination was achieved
by PRP (220 X 10^9/L) was performed as de-
tated at 270 rpm at 22°C and the extent of bead agglutination was
assessed macroscopically over 20 minutes. The assay was per-
formed in duplicate on two separate occasions. The results show
one of two similar experiments. Full agglutination was achieved
with control platelets after 8 to 15 minutes. With the patient’s
platelets, the initial phase is delayed and only half-maximal aggluti-
ation is reached; a similar degree of agglutination can be observed
with normal platelets in the presence of 1.9 mmol/L EDTA, except
for the initial lag phase.

Another monoclonal antibody directed against GPIIa
(LY22) reduces but does not abolish collagen-induced
shape change and aggregation of washed platelets; the
antibody inhibits thrombin- and arachidonic acid-induced
aggregation as well.

A third MoAb against GPIIb (PM1-1) inhibits platelet
adhesion to collagen by 80%. The fact that platelets un-
dergo the release of ~50% of their granule content in this
assay supports the concept of a secondary amplification
mechanism for collagen platelet interactions, mediated by
GPIIb/IIIa and, in this case, inhibited by antibody PM1-1.

A family with a bleeding disorder and a qualitative defect
of GPIV that is thought to be caused by an altered glycosyla-
tion of GPIV has been reported. In addition, these plate-
lets were unusually large, suggesting a second defect in a
structural platelet GP. The amount of GPIb was normal.
The role of GPIV, a major platelet GP, as a functionally
relevant collagen receptor is controversial: important stud-
ies have shown that it can mediate platelet collagen interac-
tions. However, laboratory evidence indicates that
GPIV can mediate platelet collagen interactions: GPIV-deficient platelets have a defective initial stage of ad-
hesion to fibrillar collagen. It seems possible that certain
interactions are restricted to specific types of collagen. In-
deed, GPIV-deficient platelets do not aggregate with type V
collagen. Anti-GPIV antibodies (Fab fragments) inhib-
ited collagen-induced aggregation of normal platelets in one
study but not in another. It seems possible that GPIV can
interact with collagen and transduce a signal that is ampli-
ﬁed by other receptors such as GPIa/IIa or GPIIb/IIIa. In
fact, GPIV has been shown to be physically associated with
tyrosine kinases, which points to its possible role in signal
transduction. Studies with monocytes, which also express
GPIV, have conﬁrmed this role of signal transduction. Recently,
Deckmyn et al reported a patient with a pro-
longed bleeding time and an antibody against a 90-Kd GP
comigrating with but apparently not identical to GPIV.
However, the inhibition was incomplete: a shape change
could be obtained with 1 μg/mL of collagen type I (Horm)
and a primary aggregation with 2 μg/mL, which is 10 times
less than the 20 μg/mL that gave no response with the plate-
lets from our patient on the first two examinations.

The biochemical analysis of our patient showed two ab-
normalities, namely, a quantitative reduction of GPIV to
50% of normal, determined by flow cytometry, and to less
than 50%, determined by two-dimensional electrophoresis,
whereas normal levels for GPIa/IIa, GPIV, GPIb, and
GPIIb/IIIa were found. The second abnormality was the
presence of autoantibodies directed against GPIIIb/IIIa,
GPIa/IIa and GPIV. Other reasons for common and un-
usual etiologies of hemorrhagic diathesis (defective arachi-
donic acid metabolism, an inherited or acquired storage
pool deficiency, altered or deficient important platelet re-
ceptors) could be virtually excluded with the normal results
of the experiments performed. The results of the two-di-
mensional electrophoresis suggest the possibility of a re-
duced sialylation/glycosylation of some proteins, such as
GPIbα and GPIIbβ, which are surface labeled at a much
lower intensity compared with the normal controls and the
results from the silverstained gel. A reduced glycosylation
may also account for the difference in GPIV of the patient
as measured by two-dimensional electrophoresis (~25% of
normal) and by flow cytometry (~50%). A reduced carbo-
hydrate content may favor the exposure of antigen sites not
normally exposed.

The functional analysis showed an isolated defect in plate-
let collagen interactions, which was complete in the platelet
aggregation studies (with partial recovery after corticoste-
roid treatment) and partial in the bead-agglutination assay.

The clinical aspect was an acquired hemorrhagic diathesis
and a bleeding time of more than 20 minutes.

In an attempt to analyze separately the effect of plasma
and platelets of the patient, we performed mixing experi-
ments in the aggregometer: plasma from the patient mixed
with normal control platelets or platelets of a NaK-negative
donor showed the same response to collagen at concentra-
tions of 2.5 μg/mL or higher. At 0.5 μg/mL, a small inhibi-
tory effect was observed (Fig 1B). Furthermore, because pa-
tient plasma obtained before the treatment with prednisone did not have a more pronounced inhibitory effect on normal and patient platelets than patient plasma after prednisone, the plasma autoantibodies do not seem to be sufficient to reproduce the effect with normal platelets. It is likely that the platelet-bound antibodies differ in their binding specificities and their effects from the plasma antibodies, as has been suggested by a recent study on ITP patients. The eluate from patient platelets could not be tested because it was negative for anti-GPIIb/IIIa activity in the immuno-bead assay, possibly because of inactivation of the antibody during elution with extreme pH buffers (pH 2.5 or 11.2, data not shown). The GPIV deficiency by itself does not seem to be responsible for bleeding (as demonstrated by the Japanese Nak*-negative population) or for an impaired response to collagen type I.

In conclusion, we believe that this patient has possibly a heterozygous GPIV deficiency, but it was not until she acquired autoantibodies against several GP involved in platelet collagen interaction that she developed the hemorrhagic diathesis. Indirect evidence for the relevance of these autoantibodies may be the functional recovery of the collagen aggregation after prednisone-induced reduction of the antibodies. However, the inverse was not the case: the increasing titer of anti-GPIIb/IIIa antibodies was not associated with a disappearance of the partial collagen response. This patient fits well in our hypothesis of the requirement for a double partial defect: the reduced primary signal caused by a biochemical reduction of GPIV and possibly a functional defect (autoantibodies against GPIV and/or GPIa/IIa) will be amplified to a lesser extent because of an antibody against GPIIb/IIIa. Our finding that three of four of our ITP patients with anti-GPIV and anti-GPIIb/IIIa antibodies had a bleeding disorder seems to support the hypothesis. Alternatively, the combination of autoantibodies against GPIa/IIa and GPIV together with a reduction of total GPIV may be sufficient to result in an impaired platelet collagen interaction. The latter explanation is in accord with our finding, that the patient's platelets activated by ADP are not more sensitive to super low doses of an inhibitory MoAb against GPIII/IIa than normals. Adhesion to collagen is mediated by several receptors. A single partial defect can remain clinically silent, but may put the carrier at an increased risk for the development of a hemorrhagic diathesis, because the combination of two or more such “subthreshold disorders” may result in a clinically manifest bleeding diathesis, as in this case.

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Autoantibodies against the platelet glycoproteins (GP) IIb/IIIa, Ia/IIa, and IV and partial deficiency in GPIV in a patient with a bleeding disorder and a defective platelet collagen interaction

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