Identification of a New Congenital Defect of Platelet Function Characterized by Severe Impairment of Platelet Responses to Adenosine Diphosphate

By Marco Cattaneo, Anna Lecchi, Anna M. Randi, John L. McGregor, and Pier Mannuccio Mannucci

This study characterizes a congenital hemorrhagic disorder caused by a platelet function defect with the following features: (1) severely impaired platelet aggregation and fibrinogen or von Willebrand factor (vWF) binding induced by adenosine diphosphate (ADP); (2) defective aggregation, release reaction, and fibrinogen or vWF binding induced by other agonists; (3) normal aggregation and release reaction induced by high concentrations of thrombin or collagen; (4) no further inhibition by ADP scavengers of aggregation, release reaction, and fibrinogen or vWF binding, comparable with those observed for normal platelets in the presence of ADP scavengers; (5) normal membrane glycoprotein (GP) composition and normal binding of the anti-GP Ib/IIa monoclonal antibody 10E5; (6) no acceleration by ADP of binding of the anti-GP Ib/IIa monoclonal antibody 7E3; (7) normal platelet-fibrin clot retraction if induced by thrombin or reptilase plus epinephrine, absent if induced by reptilase plus ADP; (8) no inhibition by ADP of the prostaglandin E1-induced increase in platelet cyclic adenosine monophosphate, but normal inhibition by epinephrine; (9) defective mobilization of cytoplasmic Ca2+ by ADP; (10) normal binding of [3H]-ADP to fresh platelets, but defective binding of [2-3H]-ADP to formalin-fixed platelets. This congenital platelet function defect is characterized by selective impairment of platelet responses to ADP, caused by either decreased number of platelet ADP receptors or abnormalities of the signal-transduction pathway of platelet activation by ADP.

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MATERIALS AND METHODS

Patient

The propositus, V.R., is a white man who was born in 1937 in Southern Italy. His parents were first cousins and never suffered from a bleeding tendency. The patient has a lifelong history of mucosal bleeding (especially nose bleeds) and easy bruising. He was transfused with several units of blood products after having suffered from accidental burns to the chest and upper limbs at age 18, and after two episodes of diffuse bleeding from the gastric mucosa at ages 45 and 50. His bleeding diathesis was exacerbated by the ingestion of nonsteroidal anti-inflammatory drugs. In 1978 a defect of the platelet release reaction was diagnosed at another hospital, but was not further characterized. He came to our hospital in 1986. His platelet count, activated partial thromboplastin time, prothrombin time, vWF:antigen, and ristocetin cofactor activity were within normal limits. The template bleeding time (Simplate II; General Diagnostics, Morris Plains, NJ) was 22, 18, and 24 minutes in three different determinations (normal range 2 to 8 minutes). Ristocetin-induced platelet agglutination was normal. The platelet contents of serotonin, ADP, adenosine triphosphate (ATP), β-thromboglobulin, and platelet factor 4 were within the normal range. Serum thromboxane B2 levels were also normal.

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The only relatives of the patient available for study were his brother and sister, who were asymptomatic and had normal bleeding times.

Materials

Carrier-free sodium iodide (125I), 14C-serotonin (5-hydroxtryptamine-3'-14C-creatinine sulphate, 57.5 mcg/mmol), and 4C-ADP (55 Ci/mmol) were obtained from Amersham International (Amer- sham, Buckinghamshire, UK). [2-3H]-adenosine 5'-diphosphate (27.3 and 25.4 Ci/mmol) was from New England Nuclear (Florence, Italy). Apyrase, prepared from potatoes by the method of Molnar and Lorand, was a kind gift of Drs J.P. Mustard and R.L. Kinlough-Rathbone (McMaster University, Hamilton, Ontario, Canada). The activity of the preparation was such that the enzyme (1 μL/mL) converted 0.25 μmol/L ATP to adenosine monophosphate (AMP) within 120 seconds at 37°C. Bovine serum albumin, ATP, ADP, epinephrine, prostaglandin E1 (PGE1), thrombin, the thromboxane/prostaglandin endoperoxide analogue 9,11-dideoxy-11,9-epoxymethano-prostaglandin F2 (U46619), arachidonic acid, creatine phosphate (CP), creatine phosphokinase (CPK), indomethacin, chymotrypsin, imipramine, and collagen were from Sigma (St Louis, MO). Reptilase and adenosine-5'-O-(2-thiodiphosphate) Trilitium salt (ADP-P-S) were from Boehringer (Mannheim, Germany). Hirudin was from Pentapharma (Basel, Switzerland). Fura 2/AM was from Calbiochem (La Jolla, CA). The anti-3P11/IIIa monoclonal antibodies (MoAbs) 10ES and 7E3 were generous gifts of Dr B. Coller (SUNY, Stony Brook, NY). The tissue solubilizer NCS and the nonaqueous count scintillant OCS were from Amersham International. The ACD-buffer (pH 6.5) used in the preparation of formalin-fixed platelets had the following composition: 5.5 mmol/L dextrose, 128 mmol/L NaCl, 4.26 mmol/L Na2HPO4, 7.46 mmol/L Na2HPO4, 4.77 mmol/L Na3 citrate, 2.35 mmol/L citric acid. All chemicals were of reagent grade or better.

Preparation of Platelet-rich Plasma (PRP) and Washed Platelet Suspensions

Citrated PRP was prepared as described14; the final platelet count was adjusted to 3 × 10^11/L. For studies of platelet aggregation and binding of 14C-ADP, 125I-labeled fibrinogen, 125I-vWF, 125I-10ES, and 7E3, platelets were washed according to the method described by Mustard et al13 and suspended in Tyrode buffer containing 0.35% albumin, 0.1% dextrose, and apyrase (1 μL/mL). For experiments in which the release reaction was studied, the platelets were labeled in the first washing fluid with 14C-ADP (0.1 μmol/L; imipramine 0.01 μmol/L) and then centrifuged to the method described by Kazal et al17 vWF was purified as described in triplicate and platelet-bound ligand was separated from free ligand by centrifugation through 20% sucrose in Tyrode buffer. For studies of 14C-serotonin (14C-5HT) from prelabeled platelets was measured as described.

Clot Retraction

Glass tubes (45 × 12 mm) were weighed and placed in a water bath at 37°C. Citrated PRP (900 μL) was added to each tube and warmed at 37°C for 5 minutes. Then, 100 μL of thrombin (5 U/mL) or 90 μL of reptilase plus 10 μL of epinephrine (5 μmol/L), ADP (5 μmol/L), or Tyrode was added. At the concentration used in this study, reptilase clotted normal citrated plasma in 24 to 28 seconds. A nickel wire with a terminal loop was placed in each tube, and the stop watch started. After 1 hour of incubation, the tubes were inspected to determine if clot retraction had occurred, then the wire was gently pulled out, bringing the attached clot. After wiping their outer surface, the tubes were weighed, and the amount of serum remaining in the tubes was calculated after subtraction of the weight of the empty tubes. The percent serum remaining was calculated taking as 100% the weight of 900 μL PRP to which 100 μL Tyrode had been added.

125I-Labeled Fibrinogen and vWF Binding

To 165 μL of untreated washed platelet suspension incubated at 37°C, 10 μL of ADP (10 μmol/L), U46619 (1 μmol/L), or thrombin (0.01 and 1 U/mL) was added in the presence of apyrase (40 μU/mL). After 1 minute of incubation at 37°C without stirring, hirudin (0.05 or 5 U/mL) was added to block further effects of thrombin (an equal volume of Tyrode was added to samples stimulated with ADP or U46619). Then, 10 μL of 125I-fibrinogen (50 μg/mL) or 125I-vWF (10 μg/mL) was added. When chymotrypsin-treated platelets were used, 125I-fibrinogen was added without previous stimulation of the platelets. The final volume was adjusted to 200 μL with Tyrode. After a 30-minute incubation at 37°C without stirring, aliquots of 50 μL were sampled in triplicate and platelet-bound ligand was separated from free ligand by centrifugation through 20% sucrose in Tyrode buffer. The radioactivity in the platelet pellet was measured in a gamma counter (LKB 1260, Bromma, Sweden). The specific binding of 125I-fibrinogen and 125I-vWF was calculated after subtracting the radioactivity associated with unstimulated platelets.
Analysis of Platelet Glycoproteins

Crossed immunoelectrophoresis and two-dimensional nonreduced, reduced sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of platelet extracts were performed as previously described.21

Measurement of Platelet Cyclic AMP (cAMP)

Platelet CAMP levels were measured by radioimmunoassay, using a commercially available kit (Amersham). Platelet CAMP was extracted essentially as described by Remuzzi et al.22 One milliliter of 5% trichloroacetic acid (TCA) was added to 1 mL of citrated PRP after 2 minutes of incubation at 37°C with Tyrode, PGE1 (1 μmol/L) plus Tyrode, PGE1 plus ADP (5 and 10 μmol/L), or PGE1 plus epinephrine (5 μmol/L). The samples were snap-frozen in dry ice and methanol, thawed at room temperature, and then shaken at 4°C for 45 minutes. After centrifugation at 10,000g at 4°C for 30 minutes, the supernatant was extracted three times with 5 mL of water-saturated ether, dried under a stream of nitrogen at 60°C, and stored at -20°C. Before assay, the samples were reconstituted with 0.05 mol/L Tris buffer containing 4 mmol/L EDTA.

Measurement of Cytoplasmic Concentrations of Ionized Calcium

Platelets were washed in 10 mmol/L HEPES Tyrode and incubated with 1 μmol/L of the fluorescent probe Fura 2/AM at 37°C for 45 minutes. Platelets were then washed and resuspended in HEPES Tyrode containing 1 μmol/L apyrase, at a final platelet concentration of 1 x 10^11/L. Fluorescence measurements were performed at 37°C in an Amino-Bowman spectrophotofluorometer (Silver Spring, MD), according to the method of Pollock et al.23

14C-ADP Binding to Washed Platelets

The binding of 14C-ADP to washed platelets was studied by the ultrafiltration technique described by Lips et al,24 with some modifications. Briefly, 80 μL of 14C-ADP (2.34 to 150 μmol/L) was added to 720 μL of washed platelet suspension, and the tubes were incubated at 37°C for 10 minutes. Samples (250 μL) in triplicate were filtered over 0.80 μm Millipore filters by vacuum aspiration. The filters were washed twice with 6 mL of saline and transferred to scintillation vials containing 1 mL of ethylene glycol monomethyl ether (Farmitalia-Carlo Erba, Milan, Italy) to dissolve the filters. After the addition of 5 mL Emulsifier Scintillator (Packard, Downers Grove, IL), the radioactivity was measured in a Beckman LS 1800 scintillation spectrometer (Brea, CA).

[2-3H]ADP Binding to Formalin-Fixed Platelets

Binding studies were performed according to the method described by Jefferson et al16 with slight modifications. [2-3H]ADP was added at a concentration of 10 nmol/L to triplicate samples of formalin-fixed platelets together with increasing concentrations of unlabeled ADP (1 nmol/L to 1 mmol/L). The final volume of the samples was 200 μL and the final platelet count was 2.5 x 10^11/L. After 60 minutes of incubation at room temperature, 100 μL was centrifuged through 400 μL 20% sucrose in Tyrode buffer in an Eppendorf microcentrifuge at 12,000 rpm for 2 minutes to separate platelet-bound ligand from free ligand. The supernatant and sucrose were removed by aspiration and discarded. The tip of the plastic tubes containing the platelet pellet was cut with a razor blade and the platelets were solubilized in a glass scintillation vial containing 0.5 mL NCS at 50°C for 12 hours. After the addition of 10 mL OCS, the radioactivity bound to the pellet was determined.

Data from competition binding experiments were analyzed by computer program (LIGAND).25 Nonspecific binding was treated as a parameter subject to error and was fit simultaneously with other parameters.16

RESULTS

Platelet Aggregation and the Release Reaction

Citrated PRP. ADP, at concentrations ranging from 2 to 100 μmol/L, induced a small and reversible wave of platelet aggregation in patient’s PRP. This aggregation was much lower than that of normal PRP stimulated with the same concentrations of ADP, even when the TxA2-dependent secondary aggregation and the release reaction were inhibited in vitro by 20 μmol/L indomethacin (Fig 1). The aggregation induced by epinephrine (5 μmol/L) was monophasic, and that induced by PAF-acether (0.2 to 2 μmol/L) or U46619 (1 μmol/L) was reversible (Fig 2). Collagen, at a concentration of 1 μg/mL, induced the platelets to aggregate to a small extent, whereas at a concentration of 20 μg/mL it caused extensive aggregation.
(Fig 2). Arachidonic acid induced no platelet aggregation at concentrations lower than 1 mmol/L, whereas at 2 mmol/L it induced reversible platelet aggregation (not shown). The release of 14C-serotonin was impaired with all agonists tested, except for 20 µg/mL collagen (Table 1).

Table 1. Release of 14C-SHT From Prelabeled PRP From Patient V.R. and Healthy Volunteers in the Presence or Absence of CP/CPK Plus 1 mmol/L ATP

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Patient V.R.</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Tyrode</td>
</tr>
<tr>
<td>ADP (µmol/L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>1.7 ± 1.5</td>
</tr>
<tr>
<td>20</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>100</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Collagen (µg/mL)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>5</td>
<td>5.1 ± 2.5</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>69</td>
</tr>
<tr>
<td>Arachidonic acid (mmol/L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Epinephrine (µmol/L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>1.3 ± 0.9</td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>PAF-acether (µmol/L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.2</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>1.8 ± 0.6</td>
</tr>
<tr>
<td>U46619 (µmol/L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>3</td>
<td>2.2 ± 0.9</td>
</tr>
</tbody>
</table>

Mean ± SEM. The release of 14C-serotonin was measured in the supernatant of samples obtained by centrifugation 3 minutes after the addition of the agonists. Similar results were obtained when apyrase (40 µL/mL) was used instead of CP/CPK plus ATP. Abbreviation: ND, not determined.

Washed platelet suspensions. The aggregation of patient’s washed platelets induced by 10 to 100 µmol/L ADP in the presence of 0.4 mg/mL fibrinogen was barely detectable (Fig 3). Results similar to those obtained in citrated PRP were obtained in washed platelet suspensions with the other aggregating agents (not shown). Thrombin induced very slight platelet aggregation and release of 14C-serotonin at 0.01 U/mL, whereas aggregation and

![Fig 2. Platelet aggregation induced by epinephrine, PAF-acether, U46619, or collagen in normal and patient's citrated PRP. Numbers beside the aggregation tracings indicate the concentrations of the agonists added.](image)

![Fig 3. ADP-induced aggregation in normal and patient's washed platelets. Fibrinogen (0.4 mg/mL) was added before ADP. Numbers beside the aggregation tracings indicate the concentration of ADP added. No secondary aggregation or release of 14C-SHT was observed because experiments were performed at a physiological concentration of Ca²⁺.](image)
release caused by 1 U/mL were similar to those observed with control platelets (Table 2).

Chymotrypsin-treated platelets from the patient aggregated to the same extent as chymotrypsin-treated platelets from controls on exposure to purified fibrinogen (0.4 mg/mL), in the absence of aggregating agents (not shown).27

Washed normal platelets aggregated to the same extent with all agonists whether they were resuspended in autologous or in patient citrated platelet-poor plasma (PPP). The defect of aggregation of washed platelets from the patient was not corrected by suspending the platelets in citrated PPP from control subjects (not shown).

Effect of apyrase or CP/CPK plus ATP on platelet aggregation and the release reaction. Apyrase (40 µL/mL) or CP (4 mmol/L) CPK (10 U/mL) plus 1 mmol/L ATP inhibited platelet aggregation and the release reaction induced by 10 µmol/L ADP in citrated PRP and suspensions of washed platelets prepared from the blood of healthy subjects. The ADP scavengers partially inhibited platelet aggregation and the release reaction induced by other agonists in

citrated PRP (Fig 4) and washed platelet suspensions from healthy subjects, except when high concentrations of collagen (20 µg/mL) or thrombin (1 U/mL) were used. The extent of aggregation of normal platelets under these experimental conditions was similar to that of patient’s platelets (Fig 4). The percent release of 14C-serotonin from control platelets in the presence of CP/CPK plus ATP or apyrase was similar to that obtained with the patient’s platelets. Apyrase or CP/CPK plus ATP did not further inhibit aggregation or the release reaction from patient’s PRP or washed platelet suspensions. Therefore, in the presence of ADP scavengers, the extent of platelet aggregation and the release reaction of patient’s platelets was similar to that of normal platelets.

Because the defect of patient’s platelets could be mimicked by the in vitro addition of ADP scavengers to normal platelets, we tested the hypothesis that the patient’s platelets or leukocytes (which may contaminate PRP or platelet suspensions) had increased ADPase activity. Two experimental approaches were chosen. In the first series of experiments, 1 mL of unstirred patient’s or normal PRP was incubated with 10 µmol/L ADP and 20 µmol/L indomethacin at 37°C for 5 minutes. The sample was then centrifuged in an Eppendorf centrifuge for 2 minutes to obtain PPP. Patient’s or normal PPP (50 µL) was then added to 200 µL of patient’s or normal PRP, which was stirred in the aggregometer at 1,000 rpm at 37°C. Normal and patient’s PPP induced the same degree of platelet aggregation in normal PRP and only caused platelet shape change in patient’s PRP. Both shape change and aggregation were completely inhibited by CP/CPK plus 1 mmol/L ATP. In a second series of experiments, platelet aggregation was studied in citrated PRP containing 20 µmol/L indomethacin, using ADP-β-S, a structural analogue of ADP that activates platelets, but is not metabolized by ADPases.28 The median (range) increase in light transmission induced by ADP-β-S in the PRP of 10 normal
volunteers was 13% (6.5% to 22%) at 10 μmol/L and 22.8% (13.5% to 27%) at 20 μmol/L. In contrast, the increase in light transmission through the patient’s PRP was 0 at 10 μmol/L and 3.4% at 20 μmol/L. ADP-β-S. Apyrase (40 μL/mL) did not significantly inhibit platelet aggregation induced by ADP-β-S.

**Binding of 125I-Fibrinogen and 125I-vWF**

Patient’s platelets bound very little 125I-fibrinogen or 125I-vWF when stimulated with ADP (10 to 100 μmol/L). They bound much less 125I-fibrinogen or 125I-vWF than normal platelets when stimulated with thrombin (0.01 or 1 U/mL) or 1 μmol/L U46619 (Table 3). Apyrase (40 μL/mL) markedly inhibited the binding of 125I-fibrinogen and 125I-vWF to thrombin- or U46619-stimulated normal platelets, but did not further reduce the binding of 125I-fibrinogen and 125I-vWF to patient’s platelets. In the presence of apyrase, thrombin- or U46619-stimulated platelets from the patient and from control subjects bound similar amounts of 125I-fibrinogen and 125I-vWF. Chymotrypsin-treated platelets from the patient and controls bound similar amounts of 125I-fibrinogen.

**Binding of 125I-Labeled Anti-GPIIb/IIIa MoAbs**

Patient’s platelets had 50,000 binding sites for the 125I-labeled anti-GPIIb/IIIa MoAb 10E5, with an apparent kd of 13 nmol/L; control platelets had 46,000 binding sites per platelet, with an apparent kd of 14 nmol/L. These values are within the described normal range. The anti-GPIIb/IIIa MoAb 7E3 binds at a faster rate to activated platelets than to resting platelets. The rate of 125I-7E3 binding to patient’s platelets was increased by thrombin (1 U/mL) or U46619 (1 μmol/L), but not by ADP (10 μmol/L), whereas the three agonists similarly increased the rate of 125I-7E3 binding to normal platelets (Fig 5).

### Table 3. Specific Binding of 125I-Fibrinogen and 125I-vWF to Patient’s and Control Washed Platelets

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Patient</th>
<th>Control</th>
<th>Patient</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyrode</td>
<td>0.01</td>
<td>0.72</td>
<td>2.1</td>
<td>45.0</td>
</tr>
<tr>
<td>ADP 10 μmol/L</td>
<td>0.12</td>
<td>1.62</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>ADP 100 μmol/L</td>
<td>0.12</td>
<td>1.22</td>
<td>8.5</td>
<td>51.3</td>
</tr>
<tr>
<td>Thrombin 0.01 U/mL</td>
<td>0.10</td>
<td>0.21</td>
<td>10.2</td>
<td>6.4</td>
</tr>
<tr>
<td>Thrombin 1 U/mL</td>
<td>0.68</td>
<td>2.54</td>
<td>46.8</td>
<td>236.6</td>
</tr>
<tr>
<td>Thrombin 0.01 U/mL + apyrase</td>
<td>0.72</td>
<td>0.70</td>
<td>44.6</td>
<td>39.8</td>
</tr>
<tr>
<td>Thrombin 1 U/mL + apyrase</td>
<td>0.15</td>
<td>0.96</td>
<td>9.4</td>
<td>62.8</td>
</tr>
<tr>
<td>Thrombin 1 U/mL + apyrase</td>
<td>0.13</td>
<td>0.09</td>
<td>7.2</td>
<td>8.7</td>
</tr>
<tr>
<td>Chymotrypsin-treated platelet suspension*</td>
<td>1.79</td>
<td>1.76</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Mean values of two experiments.

*Platelets were incubated with 8 U/mL chymotrypsin at 37°C for 30 minutes during the first wash.

**Analysis of Platelet Glycoproteins**

The patient’s major platelet glycoproteins (Ib, IIb, IIIa, IV) were present in normal amounts, compared with controls, when studied on two-dimensional nonreduced, reduced SDS-PAGE that were subsequently silver stained. In addition, CIE experiments using a 125I-labeled anti-GPIIb/IIIa MoAb (LYP2) in the intermediate gel showed normal levels of the GPIIb/IIIa complex in the patient’s platelets. Dissociation of the GP IIb/IIa complex of the patient’s platelets in the presence of EDTA at a basic pH was also normal compared with controls (data not shown).

**Clot Retraction**

When thrombin (5 U/mL) was added to patient’s and normal PRP, fibrin formation occurred, and after a 1-hour incubation at 37°C clot retraction was complete (Table 4). When fibrin formation was induced by reptilase (which does not activate platelets), clot retraction did not occur in normal PRP unless ADP (5 μmol/L) or epinephrine (5 μmol/L) was added.

### Table 4. Platelet-Fibrin Clot Retraction

<table>
<thead>
<tr>
<th>Agonist</th>
<th>% Serum Remaining in the Tube</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thrombin 5 U/mL</td>
<td>Tyrode</td>
</tr>
<tr>
<td>Reptilase plus:</td>
<td></td>
</tr>
<tr>
<td>Tyrode</td>
<td>16</td>
</tr>
<tr>
<td>ADP 5 μmol/L</td>
<td>20</td>
</tr>
<tr>
<td>Epinephrine 5 μmol/L</td>
<td>73</td>
</tr>
</tbody>
</table>

Mean of two experiments. Agonists were added to unstirred citrated PRP (final volume 1 mL) at 37°C. After a 60-minute incubation the tubes were inspected against light for clot retraction, then platelet-fibrin clots were removed and the percent serum remaining in the tube was measured (see Materials and Methods for details). Values underlined indicate that no visible clot retraction occurred.
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µmol/L) had been added. In contrast, clot retraction was observed only if epinephrine (5 µmol/L) was added to patient's PRP with reptilase, ADP (5 µmol/L) having no effect.

Platelet Shape Change Induced by ADP and Its Inhibition by ATP

Patient's platelets in citrated PRP, to which 5 mmol/L EDTA had been added to prevent platelet aggregation, underwent shape change when stimulated with ADP (1 µmol/L). However, in experiments with increasing concentrations of ATP (0.01 µmol/L to 1 mmol/L), lower concentrations of ATP were needed to inhibit the ADP-induced shape change of patient's platelets (inhibitory concentration [IC50], 27 and 23 µmol/L in two different experiments) than to inhibit that of platelets from 18 healthy volunteers (IC50, 88 ± 24 mean ± SD, observed range 50 to 155 µmol/L).

Inhibition of PGE1-Induced Increase in Platelet cAMP

The basal levels of cAMP in patient's platelets were similar to those of five healthy volunteers, and they increased to a similar extent as control platelets after stimulation with PGE1 (1 µmol/L) (Table 5). This PGE1-induced increase in platelet cAMP was almost completely prevented in normal platelets by ADP (5 and 10 µmol/L) or epinephrine (5 µmol/L), whereas only epinephrine, but not ADP, prevented the PGE1-induced increase in cAMP concentration of patient's platelets.

Agonist-Stimulated Increase in Platelet Cytoplasmic Ca2+ Concentration ([Ca2+]i)

The [Ca2+]i of resting and stimulated patient's platelets loaded with Fura 2/AM was studied in two separate experiments and compared with those of control platelets from 10 normal volunteers. The [Ca2+]i of resting patient's (123 and 115 nM) and control platelets (112 ± 6, mean ± SEM) were similar. Stimulation with U46619 (1 µmol/L) increased the [Ca2+]i to similar levels in patient's (970 and 1,030 nM) and control platelets (1,083 ± 71). In contrast, the ADP (10 µmol/L)-induced increase in [Ca2+]i was lower in patient's platelets (640 and 413 nM) than in control platelets (1,065 ± 60).

Binding of Radiolabeled ADP

Two binding systems for [3H]-ADP were found in washed platelets, as described by Lips et al24: a saturable system mainly operating at low ADP concentrations (high-affinity system) and a second system that was not saturated at 150 µmol/L. The kd and the number of binding sites in the high affinity system were derived according to Scatchard.21 The patient's platelets had 46,000 and 50,000 binding sites (data from two separate experiments), with an apparent kd of 3.1 and 3.5 µmol/L; the median (range) number of binding sites and kd value of platelets from eight normal volunteers were 65,000 (40,000 to 83,000) and 3.1 (2.7 to 3.6 µmol/L). All these values are within the described normal range.24

However, when [2-3H]-ADP binding to formalin-fixed platelets was studied, it was found that the number of high- and low-affinity binding sites for [2-3H]-ADP was markedly lower in the patient's platelets than in those from eight healthy volunteers (Table 6).

**DISCUSSION**

In this study we describe, for the first time, a new congenital defect of platelet function, characterized by impaired platelet aggregation, release reaction, and fibrinogen and vWF binding in response to several agonists in the absence of quantitative or qualitative abnormalities of the GPIIb/IIIa complex or of increased platelet cAMP concentration. This platelet function defect is probably transmitted as a recessive trait because the parents of the propositus (who could not be studied) were first cousins and his brother and sister were phenotypically normal.

Several lines of evidence indicate that the platelet function defect is caused by selective impairment of platelet responses to ADP: (1) platelet aggregation and fibrinogen and vWF binding induced by exogenous ADP is markedly defective; (2) platelet aggregation, the release reaction, and fibrinogen and vWF binding induced by agonists that cause the release of platelet granule contents, including ADP, are partially defective and are not further inhibited by ADP scavengers, which, in contrast, partially inhibit these responses in normal platelets; (3) the extent of platelet aggregation, the release reaction, and fibrinogen or vWF binding induced by release-inducing agents in the presence of ADP scavengers is similar in patient's and normal platelets; (4) the binding of the anti-GPIIb/IIIa MoAb 7E3

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### Table 5. PGE1-Induced Increase in Platelet cAMP and Its Inhibition by ADP or Epinephrine

<table>
<thead>
<tr>
<th>Additions to PRP</th>
<th>Patient</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
<th>Controls* (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyrode</td>
<td>9</td>
<td>10</td>
<td>12 ± 2</td>
<td></td>
</tr>
<tr>
<td>Tyrode + PGE1</td>
<td>39</td>
<td>48</td>
<td>44 ± 2</td>
<td></td>
</tr>
<tr>
<td>ADP 5 µmol/L + PGE1</td>
<td>43</td>
<td>52</td>
<td>13 ± 1</td>
<td></td>
</tr>
<tr>
<td>ADP 10 µmol/L + PGE1</td>
<td>45</td>
<td>49</td>
<td>11 ± 1</td>
<td></td>
</tr>
<tr>
<td>Epinephrine 5 µmol/L + PGE1</td>
<td>10</td>
<td>13</td>
<td>14 ± 1</td>
<td></td>
</tr>
</tbody>
</table>

PGE1 (1 µmol/L) was added with Tyrode or platelet agonists to 1 mL of citrated PRP and incubated at 37°C for 2 minutes. See Materials and Methods for details.

*Mean ± SEM.

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### Table 6. Binding Parameters of [2-3H]ADP to Formalin-Fixed Platelets From Patient V.R. and Eight Healthy Volunteers

<table>
<thead>
<tr>
<th></th>
<th>High-Affinity Binding Sites</th>
<th>Low-Affinity Binding Sites</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>kd (µmol/L)</td>
<td>n (×10^-7)</td>
</tr>
<tr>
<td>Patient V.R.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Experiment 1</td>
<td>0.14</td>
<td>3.2</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>0.17</td>
<td>4.1</td>
</tr>
<tr>
<td>Controls*</td>
<td>0.5 ± 0.25</td>
<td>55.8 ± 14.9</td>
</tr>
</tbody>
</table>

*Mean ± SEM.
is accelerated in a normal manner by thrombin or U46619, but not by ADP; (5) clot retraction is normal with thrombin or reptilase plus epinephrine, but is absent with reptilase plus ADP; (6) the PGE_1-induced increase in platelet cAMP is inhibited by epinephrine, but not by ADP; (7) the cytoplasmic Ca^{2+} levels are normally increased by U46619, but not by ADP.

The abnormality described in this study differs from Glanzmann thrombasthenia in that platelets from patients with Glanzmann thrombasthenia have quantitative or qualitative defects of GPIIb/IIIa, fail to aggregate or bind fibrinogen whatever the type and concentration of the agonist, and do not support the retraction of clots induced by thrombin or reptilase plus epinephrine. Although no abnormalities of the platelet GPIIb/IIIa complex were detected in the patient's platelets by crossed immunoelectrophoresis, two-dimension nonreduced/reduced SDS-PAGE, or studies of binding of 125I-labeled anti-GPIIb/IIIa MoAbs, the presence of an abnormality of an epitope on the GPIIb/IIIa complex selectively exposed by ADP cannot be formally ruled out. However, the finding that a GPIIb/IIIa-independent platelet response to ADP, such as the inhibition of adenylate cyclase, is also defective suggests that the abnormality does not reside in the GPIIb/IIIa complex.

The defect may be caused by increased platelet and/or leukocyte ADPase activity, or to defects in the ADP receptor(s) or to abnormalities in the signal-transduction pathway of platelet activation by ADP. The results of experiments in which patient's and normal PRP were incubated with ADP for 5 minutes suggest that the patient's platelets or leukocytes do not have increased ADPase activity because the same ADP-dependent aggregating activity remained in patient's and normal PPP after incubation of PRP with ADP. Moreover, the patient's platelets were unresponsive also to ADP-B-S, a structural analogue of ADP that activates platelets but is not metabolized by ADPases. The experiments in which the binding of radiolabeled ADP to the patient's platelets was examined do not provide conclusive evidence of defects in the platelet ADP receptor(s) because they gave conflicting results. 14C-ADP bound to the same extent to control platelets and platelets from the patient. In contrast to these findings with fresh platelets, the number of high- and low-affinity binding sites for [2-3H]ADP in formalin-fixed platelets of the patient was lower than for formalin-fixed platelets from controls. The difference can be explained by the different techniques used, both of which have drawbacks. The technique described by Lips et al uses fresh platelets; therefore, the results may be affected by the rapid metabolism by platelets of added ADP, or by other metabolic changes that occur during platelet activation. These metabolic conversions of ADP render equilibrium binding studies invalid. On the other hand, the technique described by Jefferson et al uses formalin-fixed platelets, which do not metabolize added ADP, but are unresponsive to platelet agonists. Therefore, this technique does not allow us to establish directly that the binding sites for ADP constitute the receptors that are actually implicated in platelet activation. In addition, chemical changes in the binding sites induced by the fixative might affect the accessibility of the ligand. Although the question of whether patient's platelets are deficient in ADP receptor(s) remains open until a more reliable method is available, all the functional abnormalities found in these platelets are compatible with the hypothesis that the platelets from the patient have a defect in the ADP receptor(s). The finding that patient's platelets undergo normal shape change on activation with ADP is compatible with a partial defect in ADP receptors because occupancy of few receptors by ADP may be sufficient to induce platelet shape change. This interpretation is supported by the findings that lower concentrations of ADP are required to induce shape change than to induce platelet aggregation. Moreover, the IC_50 of ATP for inhibition of ADP-induced shape change of patient's platelets was lower than normal, suggesting that fewer receptors were occupied by ADP in the platelets of the patient than in control platelets.

Looking for abnormalities in intracellular messengers, we studied agonist-induced shifts in [Ca^{2+}]_i, which is an important stimulus-response coupling agent in platelets. ADP caused a lower than normal increase in [Ca^{2+}]_i (approximately 0.5 μmol/L) in patient's platelets, consistent with the demonstration that 0.5 μmol/L [Ca^{2+}]_i is sufficient to induce platelet shape change, but not platelet aggregation. This finding may suggest the presence of some abnormality in the signal-transduction pathway of platelet activation by ADP; however, it is also compatible with a defect of ADP receptor(s), since metabolic responses to agonists cannot occur if the interaction of the agonist with its receptor is inhibited.

The defect has some similarities with previously described abnormalities of platelet function. The aggregation and secretion response of patient's platelets to agonists other than ADP is similar to that of platelets with defects of the release reaction or primary response to weak agonists. The aggregation response to exogenous ADP, in contrast, is different, because platelets with defects of the release reaction show a normal primary wave of aggregation in response to ADP. However, it is possible that milder defects of sensitivity to ADP than that found in this patient may allow a more prominent primary wave of ADP-induced aggregation, resembling that of patients with platelet secretion defects. Therefore, some defects of platelet secretion with normal content of dense granule constituents and normal arachidonic acid metabolism may be caused by mild impairment of platelet sensitivity to ADP. An easy way to discriminate between this condition and platelet secretion defects would be to challenge platelets with high concentrations of ADP (eg, 20 μmol/L), which elicit extensive primary wave of aggregation only in platelets normally sensitive to ADP (see Fig 1). Another condition characterized by abnormalities of platelet function similar to that found in this patient has been described and interpreted as a variant of Glanzmann thrombasthenia (Thrombasthenia Christchurch), despite the fact that no quantitative or qualitative abnormalities of the GPIIb/IIIa
complex were found. Studies of platelet-fibrin clot retraction with thrombin and with reptilase plus ADP or epinephrine would be a simple and helpful test for differential diagnosis between variant Glanzmann thrombasthenia and selective impairment of platelet responses to ADP. Finally, the defect of patient’s platelets is very similar to that induced by the antiplatelet drug ticlopidine in normal subjects. We can safely rule out the possibility that the patient is surreptitiously taking this drug for several reasons: (1) his platelet aggregation defect was documented for the first time in 1978, when the drug was unknown; (2) platelets from subjects taking ticlopidine have a normal number of ADP binding sites after formalin fixation; (3) the aggregation defect was documented in our laboratory also at the end of a 12-day hospitalization of the patient for severe gastric bleeding, when he could take no drug orally.

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Identification of a new congenital defect of platelet function characterized by severe impairment of platelet responses to adenosine diphosphate

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