Hereditary Abnormality of Platelet Aggregation Attributable to Nucleotide Storage Pool Deficiency

By C. M. Ingerman, J. B. Smith, S. Shapiro, A. Sedar, and M. J. Silver

An abnormality of platelet aggregation has been detected in six family members with mild bleeding tendencies. In citrated platelet-rich plasma, primary aggregation induced by ADP or epinephrine and agglutination in response to ristocetin were present but second wave aggregation and aggregation in response to collagen suspension were absent or greatly reduced. Sodium arachidonate-induced aggregation was normal although aggregation in response to prostaglandin G₂ was reduced and depended entirely on the presence of plasma or ADP. Further tests indicated that the platelets produced prostaglandins but did not release ATP in response to thrombin or sodium arachidonate. Platelets from the patients were found to contain reduced amounts of ADP and 5-hydroxytryptamine and to be unable to retain radioactivity during prolonged incubation at 37°C with radio-labeled 5-hydroxytryptamine. Although electron microscopy revealed an absence of very dense bodies, the platelets appeared otherwise normal. The findings are discussed in relation to previous studies of nucleotide storage pool deficiency and the light they shed on platelet physiology in general.

BLEEDING TENDENCIES of variable severity in some patients have been attributed to impaired release of ADP from platelets. In 1967, two groups of patients with bleeding disorders were described in whom a defect in the release of platelet ADP was associated with decreased platelet aggregation in response to collagen suspension.1,2 Subsequently, Weiss et al.3 detected this defect in six affected members of a family. The platelet defect appeared to have been transmitted as an autosomal dominant, with variable expressivity, through three generations. Later work indicated that platelets from these patients lacked the nonmetabolic pool of adenine nucleotides4,5 thought to be present in specialized intracellular granules specifically extruded from the platelet during the release reaction. The platelets also had reduced levels of 5-hydroxytryptamine, and a decreased number of dense bodies were seen in electron micrographs.6

In this report we describe a second family in whom three generations evidence a mild bleeding disorder attributable to nucleotide storage pool deficiency. Our studies indicate that platelets obtained from six members of this family have properties similar to those in the family described above4,6 and in isolated cases of patients with storage pool deficiency.7,13 We describe additional tests that may be useful in the diagnosis of this disease.

MATERIALS AND METHODS

Patients

III-4, the propositus (Fig. 1), was a 31-yr-old male with a history of bruising, gum bleeding, and nosebleeds. He develops a hematoma at the site of every venipuncture.

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II-1, the father of the propositus, 76 yr of age, had a lifelong history of easy bruising and was subject to rectal bleeding.

IV-5 and IV-6, the sons of the propositus, 9 and 8 yr old, respectively, bruised easily and had occasional nosebleeds. Both have had tooth extractions while under treatment with prednisone and did not bleed excessively.

III-8, a female cousin of the propositus, 54 yr of age, had a history of easy bruising and heavy menstrual flow. She required a transfusion after each of five deliveries and received 8 units of blood during an operation to remove kidney stones at age 27 yr. At age 9 yr she underwent surgery without excessive postoperative bleeding.

Other family members with a history of easy bruising (I-1, II-3, III-5, IV-9) are deceased. III-5 died of hematemesis at age 10 yr; IV-9 died of hemorrhage after an automobile accident at age 18 yr.

III-8 and IV-12 were studied at the Specialized Center for Thrombosis Research, Temple University School of Medicine, Philadelphia, Pa. and have been partially reported elsewhere.

Procedures
Whole blood platelet counts were determined by phase-contrast microscopy and the bleeding time determined simultaneously on two standardized template incisions.

Platelet-rich plasma (PRP) was obtained from venous blood collected into 1/10 volume of 3.8% sodium citrate by centrifugation for 15 min at 220 g at room temperature. Platelet-free plasma was obtained by further centrifugation of PRP for 3 min at 12,000 g.

Platelet aggregation was studied by conventional photometric techniques at 37°C and the degree of aggregation was expressed as a percentage of maximal change in optical density represented by autologous PFP. Bovine tendon collagen was obtained from Sigma Chemical, St. Louis, Mo. and homogenized and suspended in buffered saline pH 7.4. Ristocetin (Abbott, North Chicago, Ill.), adenosine diphosphate (ADP), and epinephrine were dissolved in saline and added in microliter amounts to PRP. Arachidonic acid (99% pure) was obtained from NuChek, Elysian, Minn. and converted to the sodium salt in 0.1 M sodium carbonate. Prostaglandin G2 (PGG2) in acetone was prepared as described elsewhere. The major product after treatment of PGG2 with triphenylphosphine in ether was PGF2α as judged by TLC. A different peak
corresponding to 15-keto PGF\(_{2\alpha}\) was observed after treatment of PGG\(_2\) with lead tetraacetate and subsequent reduction with triphenylphosphine. PGG\(_2\) was evaporated under nitrogen in a cuvette before the addition of PRP or platelet suspension preheated to 37°C; platelet aggregation was monitored immediately thereafter.

Simultaneous recordings of platelet release of ATP and platelet aggregation were made possible by the generous loan of a Lumis-Aggregometer\(^{18}\) by D. Bierman, Chronolog, Havertown, Pa. Secretion of ATP was followed by measuring luminescence with the firefly luciferase system as described elsewhere.\(^{19}\) The sensitivity was such that as little as 0.2 \(\mu M\) released ATP could have been detected.

Malondialdehyde (MDA) formation in PRP was measured 3 min after the addition of sodium arachidonate or bovine thrombin (Parke Davis, Detroit, Mich.) to stirred PRP at 37°C. MDA was determined by its colorimetric reaction with thiobarbituric acid after precipitation and removal of plasma protein.\(^{20}\)

Uptake and storage of radioactive serotonin was determined by incubating PRP at 37°C with \(^{3}^{3}^{14}\)C 5-hydroxytryptamine creatine sulfate (5-HT), specific activity 57 mCi/m mole (Amersham Searle, Arlington Heights, Ill.) at a final concentration of 0.5 \(\mu M\). Samples of PRP were removed from the incubation mixture at intervals over a 6-hr period and mixed with 0.1 vol 0.1 M EDTA at 0°C. After cooling, the samples were centrifuged at 12,000 g for 1.5 min and the supernatant radioactivity determined by liquid scintillation counting.

Platelet adenine nucleotide content was assayed by Dr. H. Holmsen, Temple University School of Medicine, Philadelphia, Pa.\(^{21}\) Platelet serotonin content was assayed using orthophthalaldehyde for fluorophore production as described elsewhere.\(^{22}\) Platelets were washed by use of an albumin density gradient as described elsewhere.\(^{23}\)

Platelet fixation for electron microscopy\(^{24}\) was initiated by adding 0.1% glutaraldehyde solution (pH 7.3 buffered with sodium cacodylate) to the platelet suspension to give a final concentration of 0.05%. The suspension of platelets was incubated at 37°C for 10 min. The contents were then poured into larger tubes and centrifuged at room temperature for 5 min at 900 g. The supernatant was discarded and the sediment overlayed with 3% glutaraldehyde solution (pH 7.3, buffered as above) overnight. After a 15-min wash in the 0.1 M sodium cacodylate buffer pH 7.3, pieces of the pellet were postfixed in 1% \(\mathrm{Os}\)\(_2\)O\(_4\) (buffered with 0.1 M sodium cacodylate, pH 7.3) for 1 hr. Following this, the blocks were washed for 30 min at 4°C in 0.9% saline and stained for 1 hr in 0.5% magnesium uranyl acetate in 0.9% saline at 4°C with shaking. The specimens were dehydrated in ethanol and embedded in a low-viscosity epoxy resin (Spurr) using a cure period of 13 hr at 70°C. The LKB Ultratome was used to obtain sections of the plastic-embedded platelets. The thin sections on naked copper grids (300 mesh) were stained with lead citrate for 6 min. The sections were examined with a Siemens Elmiskop I electron microscope at 80 kV with a pointed filament, double condenser containing a 200-\(\mu\)m aperture, and a 30-\(\mu\)m silver objective aperture at magnifications of 3,000–25,000 x.

Subjects were cautioned not to take any medication for 2 wk prior to each visit. Blood from a normal individual was used as a control in each study. The normal range for each test was established using at least 25 normal donors. Human investigations were performed after approval by the Thomas Jefferson University School of Medicine Institutional Review Board; a general assurance is on file with the DHEW.

<table>
<thead>
<tr>
<th>Table 1. Bleeding Times</th>
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<tr>
<td>Subject</td>
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<tr>
<td>----------</td>
</tr>
<tr>
<td>II-2</td>
</tr>
<tr>
<td>III-4</td>
</tr>
<tr>
<td>III-8</td>
</tr>
<tr>
<td>IV-5</td>
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<tr>
<td>IV-6</td>
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<tr>
<td>IV-12</td>
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<tr>
<td>Normal range</td>
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RESULTS

Bleeding times. Each of six family members tested had normal platelet counts and normal or high levels of factor VIII coagulant activity but had very prolonged bleeding times (Table 1).

Platelet aggregation. The results of standard aggregation tests are illustrated for the propositus in Fig. 2 and tabulated for affected family members in Table 2. Epinephrine or ristocetin induced first-phase aggregation or agglutination but did not initiate the second phase of aggregation. The aggregation response to collagen particles was minimal for four of the six subjects and less than 50% of normal for the two others. ADP induced extensive aggregation but disaggregation was evident after a few minutes.

To test for an aspirinlike defect of platelet function, samples of PRP obtained from a volunteer who had ingested aspirin and of PRP obtained from patients II-1, III-4, IV-5, and IV-6 were challenged with sodium arachidonate over the concentration range 0.3–1.0 mM. The patients' platelets, like normal platelets, aggregated irreversibly to sodium arachidonate at the concentration (0.5 mM) used routinely in our laboratory to test cyclooxygenase activity (Fig. 3A), while aspirin-treated normal platelets did not aggregate. The patients' platelets aggregated with as little as 0.4 mM arachidonate. Such responses clearly dis-

Table 2. Results of Aggregation Tests

<table>
<thead>
<tr>
<th>Subject</th>
<th>Collagen (1/20 vol)</th>
<th>Epinephrine (10 μM)</th>
<th>ADP (5 μM)</th>
<th>Ristocetin (1 mg/ml)</th>
<th>Sodium Arachidonate (0.5 mM)</th>
<th>PGG₂ (5 μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>II-2</td>
<td>12</td>
<td>33*</td>
<td>65†</td>
<td>31*</td>
<td>80</td>
<td>80†</td>
</tr>
<tr>
<td>III-4</td>
<td>1</td>
<td>40*</td>
<td>69†</td>
<td>19*</td>
<td>80</td>
<td>80†</td>
</tr>
<tr>
<td>III-8</td>
<td>39</td>
<td>57*</td>
<td>75†</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>IV-5</td>
<td>1</td>
<td>21*</td>
<td>53†</td>
<td>34*</td>
<td>80</td>
<td>80†</td>
</tr>
<tr>
<td>IV-6</td>
<td>1</td>
<td>48*</td>
<td>82†</td>
<td>54*</td>
<td>80</td>
<td>80†</td>
</tr>
<tr>
<td>IV-12</td>
<td>1</td>
<td>12*</td>
<td>20†</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Normal</td>
<td>≥ 57</td>
<td>≥ 80</td>
<td>≥ 65</td>
<td>≥ 83</td>
<td>≥ 80</td>
<td>≥ 80</td>
</tr>
</tbody>
</table>

*First wave only.
†Disaggregated.
In PRP both normal platelets and aspirin-treated normal platelets aggregated irreversibly in response to PGG$_2$. On the other hand, PRP obtained from four of the patients showed diminished and reversible aggregation with PGG$_2$ (Fig. 3 B), in contrast to the irreversible aggregation response to arachidonate. The greater effect of arachidonate may be due to the fact that in plasma much more thromboxane A$_2$ is produced from arachidonic acid$^{25}$ than from added PGG$_2$. When platelets from patient III-4 were washed and resuspended in a modified Tyrode's solution,$^{23}$ appreciable aggregation by PGG$_2$ could be produced only after the addition of ADP or plasma (Fig. 4). Platelets from another individual (not a family member) with storage pool deficiency were also tested, with the same result. Arachidonic acid induced shape change but no aggregation in the absence of plasma with platelets from these individuals. Washed normal platelets aggregated in response to PGG$_2$ and arachidonic acid without inclusion of either ADP or plasma.

Platelet secretion. Aggregation of the patient's platelets in response to sodium arachidonate was examined in a Lumi-Aggregometer, which permitted the simultaneous recording of platelet aggregation and ATP secretion. In normal PRP (Fig. 5 A) the time course of ATP secretion (top of figure) was found to correspond to the increase in light transmission (bottom). With PRP obtained
from patients III-4, IV-5, and IV-6 (Fig. 5 B) the same increase in light transmission was seen but there was no release of platelet ATP. No release of ATP from the patients’ platelets was detected in the Lumi-Aggregometer even after the addition of 1 U/ml thrombin, although as little as 0.2 μM ATP could have been detected.
 MALONDIALDEHYDE FORMATION. Malondialdehyde is a byproduct of platelet prostaglandin formation. Table 3 shows that in PRP formation of MDA in response to exogenous sodium arachidonate was normal in the four patients tested but was abnormal in response to thrombin in one of the four.

**Platelet nucleotides and serotonin.** Total platelet ATP and ADP were measured for all six patients (Table 4). Platelet ATP was very low in one subject, slightly reduced in four others, and normal in one. Platelet ADP was very low in all six subjects. Since a much larger percentage of total platelet ADP than ATP is stored in the dense granules, a storage defect would be expected to have a greater effect on platelet ADP content. Platelet serotonin was also very low in the five subjects in whom it was measured. Incubation of the patients' platelets with 14C-serotonin showed a diminished uptake of the radioactive label and a subsequent loss of radioactivity to the plasma during further incubation (Table 4).

**Platelet ultrastructure.** Fixation of normal human platelets in sodium cacodylate-buffered glutaraldehyde and subsequent postfixation in buffered sodium tetroxide was adequate for demonstrating dense bodies. There was approximately one dense body per platelet (Fig. 6).

Platelets from patients IV-5 and IV-6 showed no dense granules in thin sections, correlating with the biochemical findings on these platelets. Other cell organelles observed, such as microtubules, mitochondria, alpha granules (bullseye granules), elements of the canalicular system, and particulate glycogen granules, appeared normal (Fig. 7).

**DISCUSSION**

Although each of the six family members tested had very prolonged bleeding times, normal results were obtained in coagulation tests, which suggested a pos-
Fig. 6. Normal human platelets fixed in sodium cacodylate–buffered glutaraldehyde and OsO₄ showing both alpha granules (bullseye granules) and black dense bodies (arrows). Thin sections stained with uranyl acetate and lead citrate. × 25,200.

sible defect in platelet function. Bleeding tendencies of variable severity attributable to platelet dysfunction may result from deficiencies of platelet adhesion as in von Willebrand disease,²⁷ prostaglandin synthesis as in cyclooxygenase deficiency,²⁶ or secretion of adenine nucleotides as in storage pool disease.⁴ Only first-phase aggregation was observed in response to ristocetin or epinephrine,
suggesting that the patients had either aspirinlike cyclooxygenase deficiency or storage pool disease. The normal platelet aggregation and malondialdehyde formation in response to sodium arachidonate indicated a functioning cyclooxygenase system. Therefore we investigated the possibility that the patients had platelet storage pool deficiency. By using a Lumi-Aggregometer it was possible
Mobile dispersed repeated DNA elements in the \textit{Drosophila} genome$^{1,2}$

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washed human platelets in the absence of plasma proteins and our own findings\textsuperscript{32} with normal platelets and arachidonic acid. In the present studies, PGG\textsubscript{2} did not cause aggregation of the washed storage pool-deficient platelets from patient III-4 in the absence of plasma. This suggests that washed normal platelets aggregate in response to PGG\textsubscript{2} because some stored substance is being secreted from the platelets that acts in concert with the endoperoxide. Irreversible aggregation of washed storage pool-deficient platelets could be produced by adding ADP prior to PGG\textsubscript{2}. These results suggest that the secretion of stored ADP is necessary for the aggregation of washed normal platelets by PGG\textsubscript{2} in the absence of plasma proteins.

In summary, by using platelets from these patients we were able to show that primary aggregation by PGG\textsubscript{2} is similar to that induced by ADP in that it occurs in the absence of secretion and depends on the presence of plasma proteins. In contrast to ADP, however, PGG\textsubscript{2} can cause almost immediate secretion of stored ADP.\textsuperscript{30} The concerted action of ADP and PGG\textsubscript{2} provides a mechanism for platelet aggregation that is independent of plasma proteins.

Electron microscopy of platelets obtained from patients IV-5 and IV-6 showed an absence of very dense granules as was reported by others for storage pool disease.\textsuperscript{6,10} It now appears that a major portion of platelet Ca\textsuperscript{2+} is present in dense granules\textsuperscript{13} and that this cation is largely responsible for the electron density.\textsuperscript{13} Storage pool-deficient platelets have approximately 40\% of the Ca\textsuperscript{2+} content of normal platelets, and there is a strong correlation with their deficiency in ADP.\textsuperscript{13} It is presently believed by many that the movement of intracellular calcium is necessary to couple the effect of a stimulus on the platelet membrane to an intracellular contractile response. However, it seems unlikely that the calcium stored in dense granules is responsible for stimulus-response coupling,\textsuperscript{34} since storage pool-deficient platelets aggregate normally in response to arachidonic acid.

The propositus in the study by Weiss et al.\textsuperscript{3} had a lifelong history of bruising but did not bleed after an appendectomy. Similarly, two members of our family who bruised easily did not bleed after tooth extraction. This illustrates that other mechanisms of hemostasis exist that are not dependent on the secretion of platelet ADP. Obviously, one such mechanism is the coagulation system. Another mechanism that could potentially be important is the synthesis of prostaglandin endoperoxides and thromboxane A\textsubscript{2} by the storage pool-deficient platelets. Not only can these substances cause platelet aggregation independent of ADP secretion\textsuperscript{30} but they are also potent vasoconstrictors.\textsuperscript{17} Hemophiliacs are warned not to take aspirin because of its known adverse effect on hemostasis in the absence of coagulation. Aspirin inhibits platelet prostaglandin synthesis and therefore could also be hazardous when ingested by patients with nucleotide storage pool deficiency.

On the other hand, all of our patients had markedly prolonged bleeding times in spite of normal coagulation and normal platelet cyclooxygenase systems, suggesting that platelet ADP is an important factor in the bleeding time test.

ACKNOWLEDGMENT

We thank the members of the Specialized Center for Thrombosis Research, Philadelphia, Pa., for supplying data on two family members.
STORAGE POOL DEFICIENCY AND AGGREGATION

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