Hereditary Defect in Platelet Function in Rats

By Thomas B. Tschopp and Marjorie B. Zucker

Hemostatic parameters of fawn-hooded (FH) rats with an inherited hemorrhagic diathesis were compared with those of normal rats. Prothrombin time, partial thromboplastin time, plasma factor VIII, plasma fibrinogen, and platelet count and volume were normal. Bleeding time (BT) in FH rats was over 15 min vs. 1-8 min in controls, and platelet retention in glass-bead columns was reduced. Transfusion of platelet concentrates from normal rats corrected the BT of thrombocytopenic FH rats, but FH rat platelets did not shorten the BT of thrombocytopenic controls. ADP-induced aggregation, measured turbidimetrically in heparinized FH platelet-rich plasma, was normal. Connective tissue did not aggregate platelets of FH rats and released a subnormal amount of $^{14}$C-serotonin. In comparison with control platelets washed FH platelets aggregated by thrombin released virtually no ATP and ADP. Platelets, $10^9$ of normal rats contained 4.49 μmoles ATP, 0.98 μmoles ADP, and 0.88 μmoles serotonin compared with only about one-half as much ATP and ADP and one-third as much serotonin in platelets of FH rats. These low values suggest the absence of the platelet, "release pool" rather than a defective release mechanism. The hemorrhagic diathesis seen in the FH rats resembles the platelet defect with storage pool deficiency observed in man.

FEW ANIMAL STRAINS are known to have inherited bleeding disorders due to abnormal platelet function, but animal platelets have been used to study many basic questions of platelet physiology. Broader knowledge of defects in animal platelets may lead to better understanding of human bleeding disorders. The present study was undertaken to determine the cause of the hemorrhagic diathesis in a strain of inbred rats. The platelets exhibited an abnormal release reaction, and the abnormality resembled a disorder observed in man by Holmsen and Weiss.

MATERIALS AND METHODS

Rats with a mild bleeding disorder, the so-called fawn-hooded (FH) strain, were kindly supplied by Dr. Ethel Tobach of the American Museum of Natural History. They resulted from cross-breeding of German-brown rats with white Lashley rats, carried out by Dr. N. R. F. Maier of the Psychological Laboratories, University of Michigan, Ann Arbor, Mich. He detected a bleeding diathesis affecting both sexes during experiments involving brain surgery. Our colony was started with four female and five male FH rats. Long-Evans rats from the American Museum of Natural History and CFN Wistar rats (Carworth Farms, Rockland County, N.Y.) served as controls. The FH and the control rats were bred and kept under identical conditions. They were fed Wayne Lab-Blox (General Offices Allied Mills, Chicago, Ill.) and water ad lib.
Blood Collection and Preparation of Platelet-rich Plasma (PRP)

In the following tests, a number of hemostatic parameters were compared in the FH rats and in the normal controls. The animals were anesthetized with Nembutal (sodium pentobarbital, Abbott Laboratories), 4 mg/100 g body weight, given intraperitoneally. Blood was collected from the aorta by a 19-gauge needle-infusion set (Jelco Laboratories, Raritan, N. J.) in plastic tubes containing one-tenth volume of 129 mM trisodium citrate or 0.15 M saline with 100 U heparin/ml. To obtain PRP, the blood was centrifuged at about 250 g at 4°C for 10 min, since centrifugation at room temperature frequently yielded low platelet counts in the PRP of control rats. Platelet counts were made in duplicate using a model B Coulter Counter (Coulter Electronics, Hialeah, Fla.) for PRP and phase-contrast microscopy4 for whole blood. Hematocrits were determined with heparinized Microhamatocrit tubes (Clay Adams, Division of Becton Dickinson, Parsippany, N. J.)

Platelet Volume Determination

The mean platelet volume was determined by means of Van Allen Thrombocytocrit tubes (A. H. Thomas, Philadelphia, Pa.), shortened above the bulb to fit into the metal bucket of the 4-place swing-out head (HG-4) of a Sorvall RC-2 refrigerated centrifuge. The PRP was labeled with 3H-methoxyinin (New England Nuclear, Boston, Mass.) as described by Born,5 thus allowing measurement of the volume of plasma trapped in the packed platelet column.

Platelet Transfusion

Animals weighing 170–230 g were rendered thrombocytopenic by whole-body x-irradiation with a single 875 R dose,6 kindly performed by Miss Joan Seibert, Physics Department of the New York University Medical Center. Seven to 10 days later, the rats, weighing 20–60 g less, were injected with 2.1 ml of platelet concentrate prepared from 9 ml of citrated PRP pooled from four untreated rats. The platelets in PRP were spun down at 768 g at 4°C for 10 min and resuspended in 2.5 ml of platelet-free plasma by gentle shaking for 5 min. Bleeding times were measured in the rats 2–3 hr after the platelet concentrates were injected into the saphenous vein of the left leg. Whole-blood platelet counts from the saphenous vein of the right leg were taken before and after the transfusion.

Platelet Retention

Columns containing 1.3 g of glass beads were prepared as described.7 Rats were given 25 U heparin/100 g body weight intravenously, and 1.4 ml of blood from the vena cava was aspirated in 60 sec through a column into a 10 ml syringe by a withdrawal pump (Model 1100, Harvard Apparatus, Dover, Mass.). Platelet retention in per cent was calculated from the platelet count of blood samples added to EDTA7 before and after being passed through the glass-bead column.

Bleeding Time (BT)

BT was measured in two ways in anesthetized animals.

1. The tip of the tail (5 mm) was cut off with a razor blade, and the blood was allowed to flow into 0.15 M saline at 37°C. The elapsed time until blood flow stopped was measured. Since this method allows only one assay per animal at a given time, BT in the transfusion experiments was measured differently.

2. The animal’s shaved, rear leg was tightly cuffed with a rubber band, inducing visible venous distention, to obtain profuse bleeding consistently. A cut, 1 cm long, was made in the caudolateral skin with a razor blade, and the blood was adsorbed on filter paper every 10 sec until bleeding stopped.

Clot Retraction

Clot retraction in duplicate samples was measured by resuspending platelets of six FH and six control rats in plasma of control rats according to the method of Bettex-Galland.8
Platelet Function

Platelet Aggregation
Platelet aggregation induced by adenosine diphosphate (ADP) and connective tissue (CT) was studied turbidimetrically at 37°C using 0.4 ml aliquots of heparinized PRP in glass cuvettes in a Chrono-Log Aggregometer (Chrono-Log, Broomall, Pa.). Platelet counts in PRP were adjusted to about 600,000/μl by dilution with autologous plasma. Velocity of aggregation was measured in mm/min by means of a tangent drawn at the steepest part of the curve. ADP (Sigma Chemical, St. Louis, Mo.) diluted in isotonic, Veronal-buffered saline (pH 7.4)9 was added to the PRP in volumes up to 10 μl.

Connective tissue (CT) was prepared from human subcutaneous fat.10 Release of 14C-serotonin (14C-SHT) from labeled PRP, after aggregation induced by CT, was measured as described.11

Thrombin-induced ATP, ADP, and 14C-SHT Release
PRP from blood collected into one-tenth volume EDTA (54 mM) in 75 mM saline was incubated for 15 min at 37°C with 0.8 μM 14C-SHT (50 μCi/m mole). The platelets were spun down at Gmax = 760 g for 10 min at 4°C and washed in a phosphate-buffered solution,12 spun down again, and resuspended in ice-cold modified Ca2+ and Mg2+-free Tyrode's solution13 containing 3 mM EDTA. A 0.6 ml sample of the platelet suspension, with a platelet count of 600,000/cu mm, was stirred in the aggregometer cuvette at 37°C with 3.3 μl of CaCl2 (1.0 M) and 3 μl of thrombin (100 U/ml) (Parke Davis, Detroit, Mich.). Aggregation was stopped after 3 min by the addition of 50 μl EDTA (57.7 mM) to the cuvette, which was immersed in crushed ice. The samples were centrifuged at Gmax = 26,000 g for 10 min at 4°C. 14C-SHT release was measured in 0.1 ml of the supernatant, and the remainder was used to prepare ethanol extracts for ATP and ADP determinations.14

Platelet Serotonin (5HT) Assays
Platelet 5HT was determined in an Aminco-Bowman spectrophotofluorometer by a modification2 of the method described by Crosti and Lucchelli15 with excitation at 295 nm and emission at 540 nm.

Platelet ATP and ADP Assays
ATP and ADP were measured by the firefly luciferase method described by Holmsen et al.14 using buffered PEP-PK solution. Platelets were lysed at 4°C in neutralized 1% Triton-X-100 (alkylated arylpolyether alcohol, Rohm and Haas, Philadelphia, Pa.) and promptly extracted with an equal volume of ethanol. Calibration curves were obtained by similarly processing solutions containing known amounts of ADP. A preliminary experiment indicated that addition of 1.5 mM EDTA to the Triton-X-100 did not yield higher values for platelet ATP or ADP.

ATP and ADP Disappearance in Heparinized Plasma
Plasma of four FH and eight control rats was rendered virtually free of platelets by centrifugation at Gmax = 26,000 g for 20 min at 4°C. It was then incubated at 37°C for 10 min to permit destruction of adenine nucleotides (AN) liberated during centrifugation. One milliliter of this plasma was incubated at 37°C with 1 μM ATP or 1 μM ADP. At intervals, 0.1 ml EDTA (54 mM) was added, the tubes were immersed in crushed ice, and ethanolic extracts were prepared for assay of ATP and ADP.14

Clotting Tests
The Quick one-stage prothrombin time, kaolin partial thromboplastin time, and fibrinogen concentration were measured as described.16 Factor VIII was measured by the two-stage USPHS Division of Biologics Standards method, using American National Red Cross intermediate-purity AHF concentrate (Lot No. 564) as a standard.17

Statistical Analysis
Results were analyzed by means of the Student's t test and, unless otherwise stated, expressed as means ± SD. Differences with p <0.05 were considered to be significant.
Each rat was injected with 2.1 ml of platelet concentrate 2-3 hr before determination of bleeding time.

<table>
<thead>
<tr>
<th>Test</th>
<th>Fawn-hooded Rats</th>
<th>Control Rats</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prothrombin time (sec)</td>
<td>11.3±2.0 (10)</td>
<td>11.5±1.9 (10)</td>
<td>0.8</td>
</tr>
<tr>
<td>Partial thromboplastin time (sec)</td>
<td>19.5±2.4 (5)</td>
<td>21.1±3.7 (9)</td>
<td>0.4</td>
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<tr>
<td>Plasma factor VIII (U/ml)*</td>
<td>4.6±0.5 (7)</td>
<td>4.5±0.8 (8)</td>
<td>0.8</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>47±6 (14)</td>
<td>48±6 (26)</td>
<td>0.6</td>
</tr>
<tr>
<td>Whole blood (10^9/µl)</td>
<td>820±29 (8)</td>
<td>807±99 (8)</td>
<td>0.7</td>
</tr>
<tr>
<td>Heparinized PRP (10^9/µl)</td>
<td>1149±157 (7)</td>
<td>996±286 (7)</td>
<td>0.3</td>
</tr>
<tr>
<td>Platelet volume (cu µ)</td>
<td>6.0±0.6 (12)</td>
<td>6.4±0.6 (12)</td>
<td>0.2</td>
</tr>
</tbody>
</table>

* One unit is the activity of 1 ml human citrated plasma.
Results are expressed as means ± SD Figures in parentheses indicate number of animals studied.

RESULTS

Clotting Tests, Hematocrit, Platelet Count, and Volume

Prothrombin time, partial thromboplastin time, and factor VIII levels were the same in the FH and control rats (Table 1). Plasma fibrinogen was 3.3 and 2.9 mg/ml in the two FH rats and 2.9 mg/ml in the two controls assayed. Hematocrit, platelet count, and platelet volume were essentially the same in the different strains (Table 1).

Platelet Function In Vivo

All 26 FH rats, regardless of age or sex, had a tail BT of at least 15 min. The bleeding was then stopped by cauterization. The 33 control rats showed a BT of 4.5 ± 2 min.

Platelet retention in glass-bead columns in six FH rats was 18 ± 6%, compared to 72 ± 11% in eight controls (p < 0.001).

Platelet transfusions were carried out to establish the essential role of platelets in this hemorrhagic disorder. A thrombocytopenic FH rat infused with FH platelets showed the expected prolonged BT (Table 2, experiment 1).

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Days After</th>
<th>Rat Strain</th>
<th>Transfused Platelet Concentrate</th>
<th>Platelet Count in Recipient</th>
<th>Posttransfusion Bleeding Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Source of Platelets and Plasma</td>
<td>Platelet Count (10^9/µl)</td>
<td>Pretransfusion (10^9/µl)</td>
<td>2 Hr Posttransfusion (10^9/µl)</td>
<td>Right Leg (min)</td>
<td>Left Leg (min)</td>
</tr>
<tr>
<td>1</td>
<td>10</td>
<td>FH</td>
<td>FH in FH</td>
<td>2336</td>
<td>45.0</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>Con</td>
<td>Con in Con</td>
<td>3154</td>
<td>13.7</td>
</tr>
<tr>
<td>3</td>
<td>7</td>
<td>FH</td>
<td>Con in Con</td>
<td>2894</td>
<td>52.0</td>
</tr>
<tr>
<td>4</td>
<td>8</td>
<td>FH</td>
<td>Con in FH</td>
<td>2454</td>
<td>21.2</td>
</tr>
<tr>
<td>5</td>
<td>9</td>
<td>FH</td>
<td>Con in FH</td>
<td>2562</td>
<td>3.7</td>
</tr>
<tr>
<td>6</td>
<td>7</td>
<td>Con</td>
<td>FH in FH</td>
<td>3321</td>
<td>55.0</td>
</tr>
<tr>
<td>7</td>
<td>8</td>
<td>Con</td>
<td>FH in Con</td>
<td>3377</td>
<td>13.7</td>
</tr>
</tbody>
</table>

Each rat was injected with 2.1 ml of platelet concentrate 2-3 hr before determination of bleeding time.
and a thrombocytopenic control rat that received normal platelets showed a normal BT (Table 2, experiment 2). Platelet concentrates from control rats corrected the BT of thrombocytopenic FH rats (Table 2, experiments 3–5), whereas FH platelets did not shorten the BT of thrombocytopenic control rats (Table 2, experiments 6 and 7). The BT was not affected by the source of the plasma in which the transfused platelets were suspended (Table 2, experiment 3 vs. experiments 4 and 5, and experiment 6 vs. experiment 7).

In Vitro Characterization of Platelets

Clot Retraction: After 60-min incubation at 37°C, clot retraction with platelets of FH rats was 62.6 ± 1.0%, as compared to 62.1 ± 1.2% in control PRP (p < 0.6). Similar results were obtained after 5-, 15-, and 30-min incubation.

Platelet Aggregation: Addition of ADP to stirred, heparinized rat PRP caused aggregation. It reversed rapidly with low concentrations of ADP (0.1–
I-0

Fig. 3. Mean aggregation velocities in heparinized PRP of four FH-rats (open circles) and eight control rats (black squares) after addition of different amounts of connective tissue. *p < 0.05.

1 μM) and more slowly with 10 μM ADP. Aggregation and disaggregation velocities depended on the concentration of ADP (Fig. 1) and were similar in the two strains, except that in the PRP of the FH rats disaggregation after 1 or 2 μM ADP occurred at a more rapid rate, as well as after a shorter time interval (Fig. 2).

In the PRP of control rats, 1–50 μl connective tissue (CT) induced a change in platelet shape after a lag of about 15 sec, followed by one-phased irreversible aggregation. These effects were either absent (1–10 μl CT) or very much reduced (50 μl CT) in the PRP of FH rats (Fig. 3). Thrombin in the presence of Ca²⁺ aggregated washed platelets of control and FH rats irreversibly, but the extent and velocity of aggregation with FH platelets were significantly less than with control platelets (Table 3).

ATP and ADP Breakdown in Heparinized Plasma: ATP was broken down very rapidly in heparinized plasma of both strains at 37°C, more than 50% having disappeared after 15 sec (Fig. 4). Only trace amounts of ADP appeared. Findings on ADP disappearance were similar.

Platelet Release and Contents: When PRP from animals of both strains was incubated for 15 min at 37°C with 0.8 μM ¹⁴C-5HT (50 mCi/m mole), more than 92% of the radioactivity was bound to the platelets. Control platelets containing ¹⁴C-5HT released 52 ± 3% of their radioactivity during aggregation induced by 50 μl CT, whereas FH platelets released only trace amounts (6 ± 3%). Considerably less ¹⁴C-5HT, ATP, and ADP were detected in the suspension medium of platelets of FH rats than of controls after aggre-

<table>
<thead>
<tr>
<th>Rat Strain</th>
<th>Animals Tested</th>
<th>Samples Tested</th>
<th>Aggregation Velocity (mm/min)</th>
<th>ATP (μmoles/10¹¹ platelets)</th>
<th>ADP (μmoles/10¹¹ platelets)</th>
<th>¹⁴C-5HT (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FH</td>
<td>2</td>
<td>8</td>
<td>90±30</td>
<td>0.08±0.05</td>
<td>0.09±0.05</td>
<td>35±4</td>
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<tr>
<td>Control</td>
<td>2</td>
<td>8</td>
<td>139±49</td>
<td>0.58±0.17</td>
<td>0.57±0.26</td>
<td>67±4</td>
</tr>
<tr>
<td>p &lt;</td>
<td></td>
<td></td>
<td></td>
<td>0.05</td>
<td>0.001</td>
<td>0.001</td>
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</tbody>
</table>
Table 4. Adenine Nucleotides and Serotonin Content of Rat Platelets

<table>
<thead>
<tr>
<th>Rat Strain</th>
<th>ATP (µmoles/10¹¹ platelets)</th>
<th>ADP (µmoles/10¹¹ platelets)</th>
<th>ATP ADP</th>
<th>5-HT (µmoles/10¹¹ platelets)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FH</td>
<td>2.6±0.3 (6)</td>
<td>0.46±0.2 (6)</td>
<td>6.2±2.0</td>
<td>267±47 (10)</td>
</tr>
<tr>
<td>Control</td>
<td>4.5±1.0 (12)</td>
<td>0.95±0.3 (12)</td>
<td>5.2±2.0</td>
<td>689±147 (8)</td>
</tr>
</tbody>
</table>

Results are expressed as means ± SD.
Figures in parenthesis indicate number of animals studied.

DISCUSSION

The bleeding diathesis of the FH rats was originally observed during surgery. Despite a very prolonged BT, spontaneous hemorrhages occurred only occasionally in the caged animals from the eyes. The defect persists at a constant level throughout life, as shown by repeated prolonged BT on the same rat. The mode of inheritance of the disorder is not clear. FH rats have not been crossed with normal animals to determine whether the defect is dominant or recessive.

Autosomal inheritance, a prolonged BT, and low platelet retention in glass-bead columns are common findings in von Willebrand's disease, but FH rats do not have this disease as shown by their normal levels of factor VIII. In fact, a significant defect of the extrinsic or intrinsic pathway of coagulation is unlikely because of the normal prothrombin times and partial thromboplastin times. The kaolin partial thromboplastin time is much shorter in rats than in man, perhaps because of the high plasma levels of factor VIII observed by us and others.¹⁸

The BT of irradiated, thrombocytopenic animals was prolonged in both strains. Using this model, we showed that normal platelets infused into FH
rats normalized the BT, whereas FH platelets infused into control rats did not correct the BT. Thus, platelets were responsible for the hemostatic defect.

Platelet aggregation was studied in heparinized PRP, since citrated rat PRP required much higher concentrations of aggregating agents probably because of the low, diffusible Ca²⁺ concentration in rat plasma. ADP-induced aggregation was normal but aggregation induced by 1–10 μl CT was virtually absent in FH rats. This did not result from accelerated destruction of ADP released into plasma by CT, since the plasma ATP- and ADPase activities of FH and control rats were equal.

Platelet count and hematocrit values agreed with those reported by others. Since the platelet count and volume for the FH and control rats were essentially the same, we were not analyzing a selected platelet population and could express the values for ATP, ADP, and 5HT per number of platelets. ATP and ADP levels in control rats agreed with those reported by Mills and Thomas, considering that values are 30% lower after ethanol extraction than after perchloric acid extraction, a difference attributed to AN bound to the contractile protein in the platelets. ATP, ADP, and 5HT levels were low in platelets of FH rats; therefore, the poor aggregation induced by CT, and the concomitant absent release of ¹⁴C-5HT, is most likely attributable to diminished release. The observation that low amounts of ATP, ADP, and ¹⁴C-5HT are released from washed FH platelets by thrombin supports this hypothesis.

The low retention of FH platelets in glass-bead columns is probably also related to the low amounts of platelet AN. Attempts were made to measure platelet factor 3 (PF-3) availability in heparinized PRP aggregated by ADP. However, after neutralizing the heparin with Polybrene, this PRP had an inconsistent effect on the clotting time of recalcified citrated plasma. With citrated PRP, CT induced equal PF-3 activity in both strains. However, the aggregation defect in the FH rats was not demonstrated under these conditions, since even in the control rats the aggregates were small.

The hemostatic defect seen in the FH rats is similar to the platelet disorders described in patients by several authors (Refs. 7–13 and 26 as listed in Ref. 25 of this paper). Some of the patients were albinos. The patients had normal platelet counts, coagulation factors and ADP-induced aggregation, prolonged BT, and defective platelet aggregation with CT or collagen, as well as with thrombin and epinephrine when this was tested. Results of measurements of platelet volume or size, platelet adhesiveness, and platelet factor 3 availability varied, and the mode of inheritance was not clear.

Weiss et al. found reduced amounts of ATP and ADP in the platelets of one group of patients studied. CT and kaolin released subnormal amounts of ADP from these platelets. Platelets of three out of four patients contained subnormal amounts of 5HT. Holmsen and Weiss, using platelets labeled with ¹⁴C-adenine, concluded that the abnormal platelets lacked the storage pool of nonmetabolic AN and 5HT that is normally released by CT. Its absence would account for the failure of CT to induce aggregation. The findings in these patients and in the FH platelets suggest a defect in the osmiophilic dense granules that store ATP, 5HT, and calcium.
ACKNOWLEDGMENT

We gratefully acknowledge the skillful assistance of Miss Dale Hansen, who carried out the measurements of platelet retention and the early studies on bleeding time and AHF.

REFERENCES


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