Abnormal Platelet Function and Arachidonate Metabolism in Chronic Idiopathic Thrombocytopenic Purpura

By Marie J. Stuart, John G. Kelton, and Judith B. Allen

We observed several patients with chronic idiopathic thrombocytopenic purpura (ITP) whose bleeding times were more prolonged than would have been expected from their platelet counts. To investigate this further, we performed in vivo and in vitro platelet function studies, assessed arachidonate metabolism, and measured platelet-associated IgG (PAIgG) in seven patients with chronic ITP. The bleeding times of three of the patients were prolonged for greater than 7 min, and all of these patients had impaired platelet aggregation and abnormal platelet arachidonic acid metabolism as reflected by increased production of the lipoxygenase product HETE and a concomitant decrease in the cyclooxygenase products, TXB2 and HHT (p < 0.001). The abnormalities noted were not due to concomitant drug ingestion, since they were present on repeated evaluation. There was no relationship between the platelet count and the bleeding time; however, there was a significant inverse correlation between the bleeding time and TXB2 production in all patients evaluated (r = 0.81; p<0.05). There was no relationship between the level of platelet-associated IgG and any parameter of platelet aggregation or arachidonate metabolism. The abnormalities noted should be looked for in the individual patient with chronic ITP, since the bleeding tendency is exacerbated by the superimposed impairment of platelet function even at platelet counts of >50,000/μL, levels generally regarded as “safe.”

I N 1972, Harker and Slichter1 reported that 12 patients with idiopathic thrombocytopenic purpura (ITP) had bleeding times that were considerably shorter than would have been predicted by their platelet counts. Other investigators2 have demonstrated that the platelet population from patients with ITP has an increased percentage of larger, more dense (and presumably younger) platelets. Since these platelets have increased reactivity in vitro, it was assumed that this could account for the paradoxically shortened bleeding time. However, Clancy and associates3 reported impaired platelet aggregation in 9 of 11 patients with ITP. We observed several patients with chronic ITP whose platelet counts were greater than 50,000/μL, yet who had purpura and bled after minor trauma and tooth extractions. We therefore assessed various parameters of platelet function in seven consecutive patients with chronic ITP. We focused primarily on the following questions: (1) Since the bleeding time is the most important clinical tool available to assess platelet function, was this test paradoxically decreased in ITP? (2) Could abnormalities in the bleeding time be correlated with changes in platelet aggregation, platelet-associated IgG, or platelet arachidonic acid metabolism? We report that certain patients with ITP have evidence of both in vivo and in vitro platelet dysfunction.

MATERIALS AND METHODS

Patients and Controls

Seven consecutive patients with chronic ITP were studied. All patients had persistent thrombocytopenia for greater than 6 mo. The ages of the 7 patients ranged from 13 to 47 yr (Table I). The diagnosis of chronic ITP was made by the presence of megathrombocytes on peripheral smear, associated with the absence of either microangiopathic or leukoerythroblastic changes. Bone marrow examination demonstrated normal or increased numbers of megakaryocytes without evidence of infiltrate. Physical and serologic examination did not reveal any secondary cause for thrombocytopenia such as systemic lupus erythematosus or hypersplenism. All patients demonstrated increased amounts of platelet-associated IgG (PAIgG). If the patients received corticosteroid therapy, an interval of at least 2 months had elapsed between the administration of the corticosteroids and the platelet function studies. The controls (14 females and 17 males) ranged in age from 12 to 49 yr. No subject, test or control, had ingested any medication known to affect platelet function for at least 2 wk prior to evaluation.

Methods

Bleeding times were performed with the Mielke modification of the Ivy bleeding time.4 Platelet-associated IgG (PAIgG) was determined using an immunoradiometric assay.5 Platelet counts were performed using a Technicon analyser (controls and ITP) and by phase microscopy (ITP).

Blood samples were obtained following informed consent from each subject by a two-syringe technique and anticoagulated using 9 volumes of blood to 1 volume citrate-phosphate-dextrose solution. Platelet-rich plasma (PRP) was obtained by centrifugation of the samples of 200 μL for 20 min. Platelet-poor plasma (PPP) was obtained by centrifugation of the remaining blood for 15 min at 1800 g. Platelet aggregation studies were performed as previously described,6 with the platelet count of PRP adjusted to 150,000-200,000/μL. In the thrombocytopenic patient population, two to four times the control volume of whole blood was used per experiment. Aggregating agents included adenosine diphosphate (ADP) (6 μM) and epinephrine (6 μM).

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Evaluation of Platelet Arachidonic Acid Metabolism Through the Lipoxygenase and Cyclooxygenase Pathways

To assess platelet conversion of $^{14}$C-arachidonic acid to metabolites by the lipoxygenase and cyclooxygenase enzymes, washed platelets from both controls and patients with chronic ITP were resuspended in HBSS containing 0.5 mM calcium chloride at a concentration of $5 \times 10^9$ platelets/ml. $^{14}$C-arachidonic acid (specific activity 56.5 mCi/mM) made up as a sodium salt in 0.01 M Tris buffer, pH 7.4, was then added to a 1:1 platelet suspension stirred in the aggregometer such that the final concentration of arachidonic acid per sample was 6 $\mu$M. After 6 min, the samples were added to extraction vials containing 10 ml of absolute ethanol. Samples were then diluted, acidified with 1 N HCl to a pH of 3.3, and extracted into diethyl ether. Separation of $^{14}$C-arachidonic acid from thromboxane B$_2$ was studied by thin-layer chromatography of the free acids on silica gel G with diethyl ether:methanol:acetic acid (93:5:3, v/v) used as the eluting solvent. Thromboxane B$_2$ (TX B$_2$) was identified from the patients with chronic ITP (34.1% ± 7.3%) compared to the control population. All patients with chronic ITP demonstrated elevated levels of platelet associated IgG (4–30 fg/platelet). Three of our 7 patients with chronic ITP demonstrated marked prolongations in their bleeding times (nos. 1,4,6). These latter three individuals also exhibited concomitant abnormalities in second wave platelet aggregation to ADP and epinephrine, as did patient no. 3 whose bleeding time was within normal limits.

Platelet Arachidonic Acid Metabolism

When the metabolism of arachidonic acid through the lipoxygenase and cyclooxygenase pathways was evaluated (Tables 1 and 2 and Fig. 1), marked abnormalities were observed in the patient group. Platelets from the patients with chronic ITP synthesized significantly less ($p < 0.001$; Fig. 1C) thromboxane B$_2$ (10.3% ± 3.1%; mean ± SE) compared to platelets from the controls (23.5% ± 1.5%). The amount of HHT synthesized (Fig. 1B) was also decreased ($p < 0.005$) in chronic ITP (23.7% ± 4.9%) compared to the controls (38.3% ± 1.6%). Thus, the overall activity of the cyclooxygenase pathway (TXB$_2$ and HHT) was decreased ($p < 0.001$) in the patients with chronic ITP (34.1% ± 7.3%) when compared to normals.

### Table 1. Hemostatic Evaluation of Controls and Patients with Chronic ITP

<table>
<thead>
<tr>
<th>Group Evaluated</th>
<th>Age</th>
<th>Sex</th>
<th>Platelet Count (x 10$^9$)</th>
<th>PAIgG (fg/plt)</th>
<th>Bleeding Time (min)</th>
<th>TXB$_2$ (%)</th>
<th>HHT (%)</th>
<th>HETE (%)</th>
<th>Aggregations</th>
<th>Bleeding History</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pt. 1</td>
<td>13</td>
<td>F</td>
<td>136</td>
<td>26</td>
<td>14</td>
<td>3.0</td>
<td>8.1</td>
<td>85.4</td>
<td>2nd wave abn</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>15</td>
<td>M</td>
<td>62</td>
<td>5.3</td>
<td>6</td>
<td>20.2</td>
<td>30.3</td>
<td>41.4</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>17</td>
<td>F</td>
<td>90</td>
<td>6.5</td>
<td>7</td>
<td>6.6</td>
<td>35.4</td>
<td>45.5</td>
<td>2nd wave abn</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>19</td>
<td>F</td>
<td>132</td>
<td>4</td>
<td>12</td>
<td>4.3</td>
<td>5.3</td>
<td>89.7</td>
<td>2nd wave abn</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>21</td>
<td>F</td>
<td>105</td>
<td>19</td>
<td>5.5</td>
<td>12.8</td>
<td>32.9</td>
<td>53.1</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>23</td>
<td>M</td>
<td>89</td>
<td>30</td>
<td>10</td>
<td>3.0</td>
<td>19.3</td>
<td>61.9</td>
<td>2nd wave abn</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>46</td>
<td>M</td>
<td>74</td>
<td>27</td>
<td>5</td>
<td>22.3</td>
<td>35.0</td>
<td>39.6</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>Controls (26)</td>
<td></td>
<td></td>
<td>&gt;150</td>
<td>&lt;3</td>
<td>4 ± 0.3</td>
<td>23.5 ± 1.5</td>
<td>38.3 ± 1.6</td>
<td>31.1 ± 1.4</td>
<td>N</td>
<td></td>
</tr>
</tbody>
</table>

PAIgG, platelet-associated IgG; TXB$_2$, thromboxane B$_2$; HHT, 12 l-hydroxy-5,8,10, heptadecatrienoic acid; HETE, 12-l-hydroxy-5,8,10,14 eicosa-tetraenoic acid; N, normal; abn, abnormal. (Results of control bleeding time, TXB$_2$, HHT, and HETE expressed as mean ± 1 SE.)

### Table 2. Quantitative Radio-TLC Analyses of $^{14}$C-Arachidonic Acid Conversion to Products of the Cyclooxygenase and Lipoxygenase Pathways in Chronic ITP

<table>
<thead>
<tr>
<th>Patient Evaluated</th>
<th>Total (cpm)</th>
<th>TXB$_2$ (cpm)</th>
<th>HHT (cpm)</th>
<th>HETE (cpm)</th>
<th>AA (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>76,131</td>
<td>2,284</td>
<td>6,167</td>
<td>65,016</td>
<td>2,664</td>
</tr>
<tr>
<td>2</td>
<td>92,276</td>
<td>18,640</td>
<td>27,960</td>
<td>38,202</td>
<td>7,474</td>
</tr>
<tr>
<td>3</td>
<td>73,269</td>
<td>4,836</td>
<td>25,937</td>
<td>33,373</td>
<td>9,159</td>
</tr>
<tr>
<td>4</td>
<td>145,390</td>
<td>6,252</td>
<td>7,705</td>
<td>130,415</td>
<td>1,018</td>
</tr>
<tr>
<td>5</td>
<td>82,353</td>
<td>10,541</td>
<td>27,094</td>
<td>43,729</td>
<td>989</td>
</tr>
<tr>
<td>6</td>
<td>94,158</td>
<td>2,825</td>
<td>18,172</td>
<td>58,284</td>
<td>14,877</td>
</tr>
<tr>
<td>7</td>
<td>155,324</td>
<td>34,637</td>
<td>54,363</td>
<td>61,508</td>
<td>4,816</td>
</tr>
</tbody>
</table>

*The phospholipid peak at the origin of the free acid plate was excluded when calculating the products formed. However, in all experiments this area was scraped and counted and found to be insignificant (<3%).

RESULTS

Bleeding Times, PAIgG Values and Platelet Aggregation Studies

Table 1 depicts the results of the hemostatic evaluations on the 7 patients with chronic ITP when compared to the control population. All patients with chronic ITP demonstrated elevated levels of platelet associated IgG (4–30 fg/platelet). Three of our 7 patients with chronic ITP demonstrated marked prolongations in their bleeding times (nos. 1,4,6). These latter three individuals also exhibited concomitant abnormalities in second wave platelet aggregation to ADP and epinephrine, as did patient no. 3 whose bleeding time was within normal limits.
Fig. 1. The prostaglandin metabolites (A) HETE, (B) HHT, and (C) TXB₂ formation in control platelets and in patients with chronic ITP. Mean ± 1 SE.

(61.8% ± 1.8%). In contrast, the production of HETE through the lipoxygenase pathway (Fig. 1A) was significantly increased ($p < 0.001$) in chronic ITP when compared to the adult controls (59.5% ± 7.8% versus 31.1% ± 1.4%).

Relationship Between Bleeding Time, the Platelet Count and Platelet TXB₂ Formation

There was no correlation between the platelet count and the bleeding time. In contrast, there was a significant inverse relationship between the bleeding time and platelet TXB₂ formation in the patients with chronic ITP ($r = 0.81; p < 0.05$).

DISCUSSION

This study indicates that certain patients with chronic ITP have defective in vitro and in vivo platelet function. In vitro platelet function was assessed by platelet aggregation studies and measurement of arachidonate metabolism. In vivo platelet function was assessed by the bleeding time. In 1972, Harker and Slichter reported that the bleeding time was paradoxically shortened in all of their 12 patients with chronic ITP.¹ These investigators found that the bleeding time was normal if the platelet count was above 50,000/$\mu l$. All of our patients had platelet counts of greater than 50,000/$\mu l$ when tested. 3 patients had a prolonged bleeding time. The two patients with the longest bleeding time had platelet counts of greater than 130,000/$\mu l$. This prolongation of the bleeding time is even greater than would be expected in aregenerative thrombocytopenic disorders. Thus, several of our patients with chronic ITP not only did not have normal or "supranormal" platelet function but had dysfunctional platelets. To investigate this observation, platelet aggregation studies were performed. Four of the seven patients had abnormal (absent or decreased) secondary wave aggregation following stimulation with either ADP or epinephrine. These four patients also manifested minimal aggregation followed by disaggregation to 6 $\mu M$ arachidonate in the washed platelet system.

A significant decrease in the products of the cyclooxygenase pathway was demonstrable when the metabolism of exogenous arachidonate by platelets of patients with chronic ITP were compared to normal controls under the experimental conditions utilized in our study. The platelets also produced significantly
more HETE, a metabolite of the lipoxygenase pathway, than did the controls. There was no relationship between the platelet count and the bleeding time. In contrast, there was a significant inverse relationship between the bleeding time and platelet thromboxane B₂ formation (r = 0.81; p<0.05). In those patients in whom TXB₂ formation was markedly decreased to 3%–4%, the bleeding time was significantly prolonged and there were abnormalities in platelet aggregation and a clinical history of impaired hemostasis. In contrast, those patients with normal to low normal levels of TXB₂ were clinically asymptomatic and demonstrated normal platelet aggregation. From the data on patient no. 3, it appears that TXB₂ formation of approximately 25% normal is enough to ensure a normal bleeding time (Table 1).

This report demonstrates certain patients can have impaired platelet function and are at hemostatic risk despite having a platelet count considerably above 50,000/μl. It should be noted that the incidence of this abnormality of platelet function in chronic ITP is unknown. Although we did not select our patients for inclusion in this study on the basis of clinical history of bleeding, patients with recurrent bleeding symptoms are more likely to be referred to our center and consequently investigated. The underlying mechanism responsible for the abnormalities in platelet arachidonate metabolism is unknown. The concomitant decrease in products of cyclooxygenase metabolism with increase in lipoxygenase products is consistent with impairment in the function of the platelet cyclooxygenase enzyme. Although there was no correlation between the level of IgG on the platelet surface and the observed inhibition of platelet function, it is possible that PAIgG serves to block the action of the cyclooxygenase enzyme or that certain types of anti-platelet antibodies (or immune complexes) sterically inhibit an agonist binding site and inhibit aggregation. The possibility of an impairment in cyclooxygenase activity could be further investigated by evaluating the production of platelet thromboxane in the presence of labeled endoperoxides, thus bypassing the cyclooxygenase step in the arachidonate pathway. Exact localization of the PAIgG in those patients with ITP who manifest the abnormality in platelet arachidonic acid metabolism versus those who demonstrate no such abnormality may also provide some insight into the mechanisms involved.

Defective platelet aggregation, reduced levels of storage pool adenine nucleotides, and the circulation of so-called “exhausted” platelets has been reported in renal allograft rejection, hemolytic uremic syndrome, thrombotic thrombocytopenic purpura, and in systemic lupus erythematosus. Most recently, Weiss and coworkers observed acquired storage pool deficiency in a patient with chronic ITP. Platelets in chronic ITP may thus develop their abnormalities following in vivo exposure to inducers of the release reaction, such as antiplatelet antibody. Whatever the mechanism involved, platelet dysfunction and impaired thromboxane B₂ formation occurs in chronic ITP. This association should be investigated in the individual patient, since the bleeding tendency in these patients is exacerbated by the superimposed impairment of platelet function.

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REFERENCES

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