Factor VIII/von Willebrand Factor Binding to von Willebrand’s Disease Platelets

By Harvey R. Gralnick, Sybil B. Williams, Brenda C. Shafer, and Laurence Corash

A form of von Willebrand’s disease has been described with enhanced ristocetin-induced platelet aggregation and anodal migration of the factor VIII/von Willebrand factor protein (type 1b). We studied two families with this form of von Willebrand’s disease and macrothrombocytopenia. We have found that these platelets bind more of the normal and intermediate-sized multimers of the factor VIII/von Willebrand factor than normal platelets. Analysis of the binding data show an increased affinity of these vWd platelets for the factor VIII/von Willebrand factor. These findings are consistent with an increased number of platelet receptors, which, either by their native topography or migration on the platelet surface, bind factor VIII/von Willebrand factor protein with greater affinity than normal platelets, platelets of other vWd patients, and large platelets of other etiologies.

VON WILLEBRAND’S DISEASE (vWd) is heterogeneous in relation to its inheritance, structure of the factor VIII/von Willebrand factor (fVIII/vWF) protein, response to transfusion, and day-to-day variability of the fVIII/vWF protein activities. A population of vWd patients whose platelet-rich plasma (PRP) showed normal or increased ristocetin-induced platelet aggregation (RIPA) have been described. Ruggeri et al. demonstrated that more of the factor-VIII-related antigen (fVIII R:Ag) of these patients (with increased RIPA) bound to normal and vWd platelets than did the normal antigen and indicated that the enhanced RIPA was related to a plasma fVIII/vWF abnormality. Recently, Takahashi et al. reported that they found the platelets of similar vWd patients bound more fVIII/vWF antigen than did normal platelets.

We report studies on the binding of normal purified fVIII/vWF protein to vWd platelets and normal platelets, and of the purified vWd fVIII/vWF to normal platelets.

MATERIALS AND METHODS

The two families described in this study have clinical, familial, and laboratory findings compatible with vWd. Normal controls consisted of six men and seven women, and the macrothrombocyte controls were three individuals with a compensated immune thrombocytolytic state. Blood was drawn from patients and controls as previously described. Factor VIII R:Ag, ristocetin cofactor assay (vWF), bleeding times, and the crossed antigen–antibody electrophoresis were performed as previously described. RIPA of PRP was performed in either a Chronolog or Payton Dual Channel aggregometer. Identical platelet concentrations of PRP from patients or normals were placed in the aggregometer and 10 μl of ristocetin added (final concentration 0.25–2.00 mg/ml). The initial slope of aggregation was measured.

Platelet counts were performed by (1) paired visual counts, (2) counts from the Coulter S Plus (Coulter Electronics, Hialeah, Fla.) and (3) counts from the Particle Data system. Platelets were isolated from whole blood by a modification of the isosmolar arabinogalactan discontinuous gradient, and the platelets free of other cells were analyzed for platelet volume. Platelet-associated IgG was measured by a solid-phase radioimmunoassay. Platelet survival was performed with autologous 51Cr-labeled platelets.

The fVIII/vWF protein was prepared from normal or vWd cryoprecipitate, concentrated, and characterized as previously described. Radiolabeling of the fVIII/vWF protein with tritiated potassium borohydride and reduction of fVIII/vWF with 0.01% 2-mercaptoethanol (2-ME) were as previously reported.

The platelets for the binding studies were separated from whole blood by either a discontinuous arabinogalactan gradient or by centrifugation and washing. Binding assays were performed using purified normal or vWd fVIII/vWF protein with normal platelets or purified normal fVIII/vWF protein with vWd or normal platelets. The time course of binding was performed in two ways. (1) Normal or vWd fVIII/vWF protein was added to normal platelets followed by the addition of ristocetin. The incubation of 0.4 ml platelets (100,000/μl), 0.05 ml fVIII/vWF protein (10 or 50 μg/ml) and 0.005 ml ristocetin (final concentration 0.55 mg/ml) was allowed to proceed for 30 min. (2) The mixture of 0.4 ml (100,000/μl) of separated normal or vWd platelets, 50 μl of normal fVIII/vWF protein (10 or 50 μg/ml), and 0.005 ml ristocetin (final concentrations 0.25–0.55 mg/ml) was incubated 30 min. In both assays, the incubation mixture was spun at 2500 g for 3 min, the supernatant removed, cell button washed, and both counted. Parallel assays were run with the addition of a 50–100-fold excess of unlabeled fVIII/vWF. The bound fVIII/vWF protein not displaced by the presence of excess amounts of unlabeled protein represented nonspecific binding. Total binding minus the nonspecific binding is defined as specific binding.

The binding experiments were performed with various concentrations of radiolabeled fVIII/vWF incubated with normal or vWd platelets and at ristocetin concentrations of 0.25, 0.35, and 0.55 mg/ml. The specific binding was analyzed by Scatchard analysis. Only specific binding was measured. In some studies the binding of the normal fVIII/vWF protein to vWd or normal platelets was performed in the aggregometer. This allowed comparison of the vWF activity from the slope of aggregation and binding of the fVIII/vWF protein.

RESULTS

We have identified seven patients from two families who have vWd with enhanced RIPA, anodal migration.
of their fVIII/vWF antigen, thrombocytopenia, and increased size of their circulating platelets. The coagulation data are summarized in Table 1. The PRP of these patients did not spontaneously aggregate or aggregate after the addition of normal fVIII/vWF protein. However, the RIPA is markedly enhanced at ristocetin concentration of 0.25 mg/ml in 3 of the 6 patients, and in all 6 patients at 0.5 mg/ml. Above 1.25 mg/ml ristocetin (final concentration), the normals and the patients have similar RIPA. In contrast, we have studied patients from 3 other families who have vWD with similar abnormal crossed antigen–antibody electrophoresis patterns and decreased RIPA. None of these patients have thrombocytopenia or increased platelet size. The RIPA of the 3 patients with macrothrombocytopenia was indistinguishable from normal.

The thrombocytopenia has been observed repeatedly in 6 of the 7 patients, while in the 7th patient (B-V), the only platelet count is normal (Table I). The thrombocytopenia varies in its severity from patient to patient, and to some extent varies within the same patient. In 5 of the 7 patients, the mean platelet size is greater than 3 SD of the normal mean. In patient B-II, the platelet size of 8.57 cu μ is greater than 2 SD but less than 3 SD of the normal mean.

Three patients had normal platelet-associated IgG, while one patient (B-III) had a slight elevation of 20.3 fg/platelet (normal < 14.8 fg/platelet). Autologous 51Cr platelet survival in B-I revealed a T½ of 154 hr (normal 120 ± 20 hr). Patient B-III had a bone marrow aspiration performed that showed normal numbers of megakaryocytes and normal morphology.

Platelets from 5 of these patients and 10 normals were separated from whole blood and compared in their ability to bind and to support platelet agglutination with normal radiolabeled fVIII/vWF protein and using varying concentrations of ristocetin. The time course of binding of fVIII/vWF showed more fVIII/vWF protein bound by the platelets of these vWD patients than normal platelets (Fig. I) or platelets from other vWD patients. When the time courses were performed in the aggregometer at different fVIII/vWF protein concentrations and the initial slope of aggregation measured, increased binding of the normal fVIII/vWF protein was associated with increased initial slope of aggregation (vWF activity) using the vWD platelets (Table 2). Greater differences in binding were seen at the lower concentrations of ristocetin. The same findings were found with radiolabeled partially reduced 0.01% 2-ME fVIII/vWF protein (largest multimer present ~ 7.0 × 106) as with the normal intact larger multimer fVIII/vWF protein (largest multimer present > 10 × 106) (Table 2).

When the binding of the normal fVIII/vWF protein to normal platelets and vWD platelets was analyzed by Scatchard plots (at ristocetin concentrations of 0.25, 0.35, and 0.55 mg/ml), an increased affinity of binding of the fVIII/vWF protein to the vWD platelets occurred. The increased affinity to vWD platelets was associated with a lower number of binding sites compared to the normal platelets, especially at low ristocetin concentrations (Table 3, Fig. 2). As the concentration of ristocetin was increased, the difference in binding of the fVIII/vWF protein to the platelets of normal and vWD was not as great. However, a slight increase persisted in the affinity of binding of the fVIII/vWF protein to the vWD platelet receptor. In contrast, the time course and Scatchard analysis of

<table>
<thead>
<tr>
<th>Age</th>
<th>Bleeding Time (min)</th>
<th>C (%)</th>
<th>Ag (%)</th>
<th>vWF (%)</th>
<th>RIPA* (mg/ml)</th>
<th>Platelet Count x 10^7/μl</th>
<th>Platelet Size (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Family A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>32</td>
<td>&gt;30</td>
<td>38</td>
<td>50</td>
<td>12-26</td>
<td>0</td>
<td>17.5</td>
</tr>
<tr>
<td>II</td>
<td>17</td>
<td>&gt;30</td>
<td>47</td>
<td>55</td>
<td>25</td>
<td>0</td>
<td>15</td>
</tr>
<tr>
<td>Family B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>58</td>
<td>17</td>
<td>39</td>
<td>55</td>
<td>30</td>
<td>0</td>
<td>27.5</td>
</tr>
<tr>
<td>II</td>
<td>27</td>
<td>21</td>
<td>48</td>
<td>57</td>
<td>40</td>
<td>0</td>
<td>22.5</td>
</tr>
<tr>
<td>III</td>
<td>68</td>
<td>&gt;30</td>
<td>63</td>
<td>140</td>
<td>44</td>
<td>15.0</td>
<td>25.5</td>
</tr>
<tr>
<td>IV</td>
<td>34</td>
<td>12</td>
<td>50</td>
<td>75</td>
<td>43</td>
<td>10.0</td>
<td>32.5</td>
</tr>
<tr>
<td>V</td>
<td>6</td>
<td>13</td>
<td>43</td>
<td>101</td>
<td>20</td>
<td>ND$</td>
<td>ND</td>
</tr>
<tr>
<td>N1</td>
<td>&lt;9</td>
<td>52-158</td>
<td>58-166</td>
<td>48-152</td>
<td>0</td>
<td>3.0</td>
<td>7.3</td>
</tr>
</tbody>
</table>

*RIPA, ristocetin-induced platelet aggregation of PRP. The values given for RIPA are the initial slope in mm/min.
†Mean of 3 determinations.
‡Mean of 2 determinations.
§Not done.
¶Normal range derived from mean ± 2 SD.
‖Normal range derived from mean ± 3 SD.

Family A-I is aunt of A-II. The father of A-II is known to have thrombocytopenia and vWD. Family B, I and III are brothers, II is a son of I, IV is daughter of III, V is son of IV.
binding of normal fVIII/vWF to the vWd platelets of type IIa were normal (data not shown).

We found that the purified fVIII/vWF protein of one of the patients (B-1) lacked the larger molecular weight polymers and that it was deficient in carbohydrate. When the vWd protein was compared to the normal fVIII/vWF protein (Kd 1.8 nmole, number of molecules per platelet 9 x 10^3) in binding studies with the same normal platelets, it bound with a lower affinity (Kd 14.9 nmole, number of molecules per platelet 48 x 10^3) to the platelet receptor.

**DISCUSSION**

vWd patients with normal RIPA have been recognized by several workers. This was not thought to represent a unique subset of patients. Takahashi et al. described one vWd family with enhanced RIPA and related this abnormality to the vWd platelet binding more fVIII R:Ag than normal, while Ruggeri et al. described vWd individuals with enhanced RIPA and thought that it was a plasma abnormality. In both studies, the investigators measured the supernatant fVIII R:Ag immunologically to estimate the amount of fVIII/vWF bound to the platelet surface.

In the two families we have studied, 6 of the 7 patients have thrombocytopenia. In family B we studied 8 other family members and none had thrombocytopenia or vWd alone. The thrombocytopenia that has been found in these two families is suggestive of a thrombocytolytic state, since the mean platelet volume is increased. However, platelet-associated immunoglobulin is normal in members of both families and an autologous ^51Cr platelet survival was normal in one family, B. A therapeutic platelet transfusion showed a normal increment and survival in one member of the other family, A. Thus, the etiology of the thrombocytopenia does not seem to be due to peripheral destruction.

We have demonstrated that the platelets in these patients are responsible for increased RIPA. These
Table 2. Binding of fVIII/vWF Protein and vWF Activity
With vWd Platelets*

<table>
<thead>
<tr>
<th>Ristocetin concentration (mg/ml)</th>
<th>Intact</th>
<th>Reduced†</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25</td>
<td>8.80</td>
<td>5.80</td>
</tr>
<tr>
<td>0.35</td>
<td>3.40</td>
<td>4.20</td>
</tr>
<tr>
<td>0.55</td>
<td>0.92</td>
<td>4.40</td>
</tr>
</tbody>
</table>

*All values are expressed as percent increase in binding or vWF activity of the vWd platelets compared to normal platelets with intact or partially reduced normal fVIII/vWF protein.
†The reduced fVIII/vWF protein had 50% of the initial slope of aggregation with normal platelets as the intact protein.
§The increase of binding is calculated from the comparison of binding of 2.5 µg of intact or reduced radiolabeled fVIII/vWF protein to 4 x 10^9 normal or vWd platelets at the ristocetin final concentrations noted.

vWd platelets bind more normal and intermediate-sized fVIII/vWF, aggregate or agglutinate faster to the same concentration of fVIII/vWF protein (i.e., increased levels of vWF activity), and bind fVIII/vWF at lower concentrations of ristocetin than normal. The Scatchard analysis shows their platelets have a greater affinity than normal platelets at both low and "normal" ristocetin concentrations. This is unique to these type IIb vWd patients, since the platelets from patients with type IIa vWd have normal time courses and binding kinetics, and the three patients with macrothrombocytes also had normal RIPA and binding kinetics. The vWd fVIII/vWF protein from type IIb binds to normal platelets with decreased affinity and cannot support normal platelet agglutination or aggregation with ristocetin. The genetic basis for this plasma protein and platelet abnormality in vWd is at present unknown.

The time course of binding of the fVIII/vWF protein implies that the vWd platelets have more receptors per platelet than normal. However, this is not borne out in the analysis of concentration-dependent binding. We think that this apparent discrepancy is related to the increased affinity of binding of the fVIII/vWF protein to these vWd platelets. In the calculations of binding
data, we have made the assumption that one molecule of fVIII/vWf protein binds to one platelet receptor; but the data are more compatible with one fVIII/vWf protein molecule binding to multiple receptors. We hypothesize that the increased affinity is due to multiple receptor attachments to the fVIII/vWf protein. We suggest that these vWd platelets are unique in their affinity to bind larger and intermediate multimers of vWF because they have more receptors per platelet than do normal platelets or that the topography of these receptors or their migration on the platelet surface is such that they bind fVIII/vWf protein more avidly. This platelet abnormality may act as a compensatory mechanism to counteract the vWd plasma defect in primary hemostasis. The ability of the vWd platelets to bind normal or intermediate size fVIII/vWf more avidly either to other platelets or possibly platelets to the vascular subendothelium could partially compensate for the plasma fVIII/vWf defects.

REFERENCES

Factor VIII/von Willebrand factor binding to von Willebrand's disease platelets

HR Gralnick, SB Williams, BC Shafer and L Corash

Updated information and services can be found at:
http://www.bloodjournal.org/content/60/2/328.full.html

Articles on similar topics can be found in the following Blood collections

Information about reproducing this article in parts or in its entirety may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#repub_requests

Information about ordering reprints may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#reprints

Information about subscriptions and ASH membership may be found online at:
http://www.bloodjournal.org/site/subscriptions/index.xhtml