Platelet Release Abnormality Associated With a Variant of von Willebrand's Disease

By S. V. Dowling, R. H. Muntz, S. D'Souza, and H. Ekert

A family with a platelet release abnormality (PRA) is described. The only son also showed a reduced rate of platelet aggregation in response to ristocetin, markedly reduced levels of von Willebrand's factor (vWF, ristocetin cofactor), and increased mobility of factor VIII-like antigen, features which were suggestive of von Willebrand's disease (vWD). No inhibition of vWF was found in his plasma. Family studies showed no evidence of vWD in the mother. The father's investigations showed a low rate of ristocetin aggregation on one of the two occasions when it was tested and low vWF on two of four occasions. Despite repeated testing, the findings in the father did not conclusively rule out the possibility of mild vWD, and it was impossible to determine whether the vWD in the son was inherited or arose as a mutation. The findings in this family suggest a possible relationship between abnormalities of the factor VIII complex and defective platelet function.

An association between platelet release abnormality (PRA) and reduced levels of factor VIII procoagulant activity has been well documented. Chesney et al. have described four members of two families in whom there was an association between PRA and reduced levels of factor VIII and where the inheritance of the disorder may have been sex linked. Plasma from these patients contained >200% of factor VIII by immunologic assay, and the patients' procoagulant factor VIII level returned to baseline within 24 hr after plasma or cryoprecipitate infusion. These findings confirmed that in these patients PRA was associated with hemophilia. Crowell and Eisner described five female members of a family who manifested a dominantly inherited bleeding disorder which was characterized as PRA associated with low levels of factor VIII. The dominant inheritance of factor VIII deficiency suggested von Willebrand's disease (vWD), but the study lacked more specific diagnostic parameters of vWD, such as the measure of factor VIII by immunologic methods and the response of platelets to ristocetin.

Recently, Kernoff et al. and Peake et al. have shown that some patients with vWD who have normal or moderately reduced levels of factor VIII and factor VIII-like antigen have a factor VIII-like antigen with abnormal electrophoretic mobility.

In this paper, we describe a family with PRA which in one patient is associated with a constantly reduced response to ristocetin, low von Willebrand's factor (vWF, ristocetin cofactor), and a qualitatively abnormal factor VIII-like antigen.

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CASE REPORT

C.S., aged 18 mo, is the only child of Italian parents. He presented with a 3-day history of bleeding from a lower lip laceration following a fall at home. About 3 mo prior to this, the mother had reported easy bruising of his lower limbs. The only investigation performed at this time was a platelet count, which was normal. In the past, the child had had meningitis at 9 mo. This was followed by moderate anemia. The diagnosis of thalassemia was excluded by hemoglobin electrophoresis. No major operations or circumcision had been performed. The family history obtained by questioning the parents, who, with C.S., are the only members of this family residing in Australia, revealed that the parents, grandparents, and great-grandparents were all born in the same village (Tocco Casauria) in central Italy. Direct questioning revealed no known consanguinity in these three generations. There was no clinical history of bleeding in either father or mother. Investigations were performed on C.S. and his parents, as other members of the family were not available for study. Blood samples were taken from C.S. 2 days (April 9, 1974), 10 days (April 17, 1974), and 16 days (April 23, 1974) after platelet transfusion.

MATERIALS AND METHODS

Definition of von Willebrand’s factor (vWF). The plasma factor(s) necessary for ristocetin-induced platelet aggregation of washed normal platelets that is deficient in vWD.

Platelet rich plasma (PRP) for platelet function studies was prepared from 9 volumes of venous blood anticoagulated with 1 volume of 3.8% trisodium citrate, and centrifuged at 250 g for 10 min at room temperature. Platelet poor plasma (PPP) was prepared from blood collected as for PRP and centrifuged twice at 12,000 g for 20 min at 4°C. Factor VIII procoagulant activity was measured on fresh PPP, while factor VIII-like antigen, vWF, and two-dimensional immunoelectrophoresis were carried out on PPP which had been stored at -20°C. Normal pooled plasma (NPP) for use as a standard for these assays was prepared from equal volumes of citrated PPP from eight healthy members of hospital staff. The PPPs were pooled and frozen at -20°C for up to 1 mo before use. NPP was arbitrarily defined as containing 1 U/ml of factor VIII procoagulant activity, factor VIII-like antigen, and vWF.

Platelet counts were performed on whole blood anticoagulated with EDTA, using an electronic counter (Technicon Autocounter, Model 1A). Blood smears were stained with Wright's stain and examined microscopically. Standardized Ivy bleeding times were performed by the template method. The Duke (earlobe) method was used on one occasion.

Platelet aggregation at 37°C was followed turbidometrically using an EEL titrator (Evans Electroselenium Limited) attached to a Hitachi chart recorder. Patients were questioned to ensure that no aspirin or related drugs had been taken for 7 days prior to testing. Aggregating agents used were: adenosine 5'-diphosphate (ADP) (Sigma Chemical Co., St. Louis, Mo.), at final concentrations of 2 or 4 μM; L-epinephrine (Sigma), at final concentrations of 2, 4, or 16 μM; collagen (Sigma), prepared by homogenizing 0.05 g in 20 ml of 0.52 M acetic acid, and stored in aliquots at -20°C. Before use, the collagen was thawed, diluted in 0.1 M acetic acid, and kept on ice. The concentration of collagen used was that which, in our laboratory, constantly produced maximum aggregation in normal subjects; thrombin (Parke Davis, Topical), at a final concentration of 0.5 NIH U/ml; and ristocetin (H. Lundbeck & Co., Denmark), batch 3, at a final concentration of 1.75 mg/ml.

The responses to ADP, epinephrine, and thrombin were studied qualitatively only, and were expressed in terms of the presence of primary and secondary phases of aggregation and/or disaggregation. Collagen responses were compared to those of normal PRP tested at the same time. The response to ristocetin was measured as the rate of aggregation, defined as the slope of the line drawn parallel to the linear part of the aggregation curve, and expressed in arbitrary units per minute.

C14-serotonin uptake and release were performed by the method of Jerushalmy and Zucker. 14C-serotonin (2.5 μCi/ml and 20 μg/ml, Radiochemical Centre, Amersham, Great Britain), 10 μl, was added to 2 ml of PRP and incubated for 30 min at room temperature. Following incubation, 1 ml was removed and tested with 4 μM ADP or collagen (test sample), while the remaining 1 ml was not tested (control sample). Platelets from both samples were separated by centrifugation, and those from the control sample frozen and thawed twice. The radioactivities in the supernatants
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and the control platelet-rich fraction were measured in a liquid scintillation counter (Packard Tri-Carb). The uptake and release of $^{14}$C-serotonin were expressed as percentages.

Washed platelets were prepared by a modification of the method of Walsh.\textsuperscript{7} Freshly collected PRP was mixed with apyrase (Sigma), final concentration 100 $\mu$g/ml, before layering 9 ml of PRP over 1 ml of liquid 40\% bovine albumin (Commonwealth Serum Laboratories, Melbourne) pH 6.5, 300 mOsm/liter, in a 10-ml flat-bottom clear plastic tube. After creating a density gradient between the PRP and the albumin, the tube was stoppered and centrifuged at room temperature at 1500 g for 10 min. The separated platelets were resuspended in 8 ml of Ca\textsuperscript{2+}-free Tyrode's solution, pH 6.5, 300 mOsm/liter, containing apyrase, 100 $\mu$g/ml, and heparin, 25 U/ml. The washing procedure, which was carried out at 37°C, was repeated twice, each time centrifuging at 1150 g for 10 min. The second and third washes did not use heparin. The platelets were then resuspended in a solution of the following composition: NaCl, 7.06 g/liter; KCl, 0.32 g/liter; NaH$_2$PO$_4$·2H$_2$O, 0.17 g/liter; NaHCO$_3$, 2.1 g/liter; MgCl$_2$·6H$_2$O, 0.173 g/liter; glucose, 1 g/liter; CaCl$_2$, 0.176 g/liter; apyrase, 50 mg/liter; pH 7.4; and osmolality, 300 mOsm/liter. The platelets were kept at 37°C till used. When they were to be used for aggregation studies with ADP, epinephrine, and collagen, the apyrase was omitted.

Factor VIII procoagulant activity was measured by a one-stage method based on the partial thromboplastin time of Proctor and Rapaport.\textsuperscript{8} Factor VIII-like antigen was measured by a modification of the method of Laurell.\textsuperscript{9}

Antiserum for use in the factor VIII-like antigen assay was prepared as previously described.\textsuperscript{10} On immunoelectrophoresis, one precipitin line to normal or hemophilic plasma was observed, but none to plasma or cryoprecipitate from a patient with severe vWD. The antiserum was shown to inhibit factor VIII procoagulant activity and vWF.

Two-dimensional immunoelectrophoresis was performed as follows. Ten or 20 $\mu$l of PPP was electrophoresed in the first dimension for 2 hr in a gel 2 cm wide at 10 mA/slide. The buffer used was 0.05 M Tris, 0.05 M Na barbital, pH 9.2, and the distance migrated by albumin was measured using a bromphenol blue marker. Agarose containing 0.3\% of the previously described antibody to factor VIII-like antigen was then added to the slide, and the gel was electrophoresed for 16 hr at right angles to the first dimension at 10 mA/slide. After staining with Coomassie blue, the migration of the factor VIII-like antigen peak from the starting point of the first dimension was measured, and expressed relative to that of albumin.

The vWF was measured by the method of Weiss et al.,\textsuperscript{11} modified by using a final ristocetin concentration of 1.5 mg/ml, a final platelet count of 160,000/µl, and a standard curve constructed by plotting aggregation rate against concentration of vWF in NPP.

To test for platelet aggregation inhibitors, the parents' platelets were washed and diluted to a concentration of 4 x 10$^5$/µl. Then 0.36 ml of platelets were mixed with 0.24 ml of normal PPP, and responses to 2 and 4 $\mu$M ADP, 2, 4, and 16 $\mu$M epinephrine and collagen were measured. Similarly, washed normal platelets, suspended in the parents' PPP, were tested as above.

To test for a vWF inhibitor, the PPP of C.S. was added to an equal volume of NPP, incubated for 1 hr at 37°C with occasional mixing, and the residual vWF measured. Controls were NPP and C.S.'s PPP diluted 1:1 with the buffer used for suspension of washed platelets.

RESULTS

Platelet Counts and Bleeding Times (Table 1)

The platelet counts were normal in both C.S. and mother, but mild thrombocytopenia was noted twice in the father. No giant platelets were seen in smears from any member of the family. The Duke bleeding time was grossly prolonged in C.S., and the Ivy bleeding time marginally prolonged in the father.

Plasma Factors and Platelets in Relation to vWD (Table 2)

The factor VIII procoagulant activity was normal in C.S. and his parents. Factor VIII-like antigen was normal on a number of occasions, except once in C.S., when it was just below the normal range. Two-dimensional immuno-
electrophoresis in C.S. showed that his factor VIII-like antigen had more rapid mobility than normal (Fig. 1). The ratio of mobility of factor VIII-like antigen to mobility of albumin was 0.46. Ten normal controls showed a range of ratios of 0.33–0.39. Electrophoresis of a mixture of normal plasma and C.S.’s plasma showed a humped peak, confirming the greater mobility of his factor VIII-like antigen (Fig. 1). Electrophoretic mobilities of the father’s and the mother’s factor VIII-like antigens were normal. The rate of ristocetin aggregation was below normal twice in C.S. In the father, the rate was below normal once, but normal on a second occasion. In the mother, the rate was always normal. The vWF was grossly diminished in C.S., and in the father it was below normal once, but normal on three other occasions. In the mother it was always normal.

Table 1. Platelet Counts and Bleeding Times

<table>
<thead>
<tr>
<th></th>
<th>Platelet Count</th>
<th>Bleeding Time</th>
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<tbody>
<tr>
<td></td>
<td>per µl of Blood</td>
<td>(min)</td>
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<tr>
<td>C.S.</td>
<td>(4.9.74) 385,000</td>
<td>&gt; 20</td>
</tr>
<tr>
<td></td>
<td>(4.17.74) 345,000</td>
<td>(Duke)</td>
</tr>
<tr>
<td></td>
<td>(4.23.74) 360,000</td>
<td></td>
</tr>
<tr>
<td>Father</td>
<td>(4.23.74) 120,000</td>
<td>8.5</td>
</tr>
<tr>
<td></td>
<td>(4.30.74) 100,000</td>
<td>(Ivy)</td>
</tr>
<tr>
<td></td>
<td>(5.21.74) 150,000</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(7.30.74) 160,000</td>
<td></td>
</tr>
<tr>
<td>Mother</td>
<td>(4.30.74) 150,000</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>(5.21.74) 205,000</td>
<td>(Ivy)</td>
</tr>
<tr>
<td></td>
<td>(7.30.74) 300,000</td>
<td></td>
</tr>
<tr>
<td>Normal range</td>
<td>150,000–400,000</td>
<td>2.5–7</td>
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</table>

Table 2. Studies of Plasma Factors and Platelets in Relation to vWF

<table>
<thead>
<tr>
<th></th>
<th>Factor VIII Procoagulant Activity (U/ml)</th>
<th>Factor VIII-like Antigen (U/ml)</th>
<th>Ristocetin 1.75 mg/ml Aggregation Rate (U/min)</th>
<th>vWF* (Ristocetin cofactor) (U/ml)</th>
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<tbody>
<tr>
<td>C.S.</td>
<td>(4.9.74) 0.80</td>
<td>0.48</td>
<td>8</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>(4.17.74) 0.75</td>
<td>0.59</td>
<td>14.5</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>(4.23.74) —</td>
<td>—</td>
<td>—</td>
<td>0.01</td>
</tr>
<tr>
<td>Father</td>
<td>(4.23.74) —</td>
<td>0.77</td>
<td>—</td>
<td>0.38</td>
</tr>
<tr>
<td></td>
<td>(4.30.74) —</td>
<td>0.90</td>
<td>—</td>
<td>0.59</td>
</tr>
<tr>
<td></td>
<td>(5.21.74) —</td>
<td>0.86</td>
<td>10</td>
<td>0.45</td>
</tr>
<tr>
<td></td>
<td>(7.30.74) 0.75</td>
<td>0.67</td>
<td>49.5</td>
<td>1.19</td>
</tr>
<tr>
<td>Mother</td>
<td>(4.30.74) —</td>
<td>1.12</td>
<td>—</td>
<td>0.78</td>
</tr>
<tr>
<td></td>
<td>(5.21.74) —</td>
<td>0.85</td>
<td>55</td>
<td>1.24</td>
</tr>
<tr>
<td></td>
<td>(7.30.74) 1.26</td>
<td>0.80</td>
<td>69.5</td>
<td>1.06</td>
</tr>
<tr>
<td>Normal range</td>
<td>0.55–1.55</td>
<td>0.51–1.55</td>
<td>21–80</td>
<td>0.45</td>
</tr>
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</table>

*(n = 20)*

*vWF* is defined as the plasma factor(s) necessary for ristocetin-induced platelet aggregation of washed platelets which is deficient in vWF.
Fig. 1. Two-dimensional immunoelectrophoresis. (A) Normal plasma. (B) Mixture of normal plasma and the plasma of C.S. (C) Plasma of C.S. The mobility of normal plasma relative to albumin was 0.39, the mobility of C.S.'s plasma was 0.46. The anode is located at the left of the figure in the first dimension, and superiorly in the second dimension, as shown by the plus sign. The bromphenol blue albumin marker is not shown, as it disappears during staining.
Platelet Function Studies

The response to 2 μM ADP was abnormal in all members of the family as shown by disaggregation at 3 min (Fig. 2A). The response to 4 μM ADP was normal. No secondary phase in response to epinephrine in concentrations of 2–16 μM was found (Fig. 2B). Collagen response in all three was markedly reduced compared to normal PRP tested with the same batch of collagen at the same time (Fig. 2C). All platelet function studies were repeated twice at different times and showed similar results.
The response to thrombin at a concentration of 0.5 NIH U/ml was normal in all members of the family and is not shown. ¹⁴C-serotonin uptake was normal in C.S. and his parents, but release in response to 4 μM ADP and to collagen was grossly diminished or absent in all three (Table 3).

**Washed Platelet Studies**

These were performed on the platelets of the parents only, as C.S. was not available for further studies. Washed platelets of the parents in NPP disaggregated with 2 μM ADP and there was a primary phase only with epinephrine, 2-16 μM. The collagen response of the father’s platelets was reduced (Fig. 3), but the mother’s platelets responded normally with the same concentration of collagen. Washed normal platelets in the parents’ PPP responded normally to ADP and epinephrine and collagen.

**Test for vWF Inhibitor**

No inhibitor of vWF could be demonstrated in the plasma of C.S. The residual vWF in a 1:1 mixture of NPP and the plasma of C.S. was 0.68 U/ml.
while that of a 1:1 mixture of NPP and platelet suspension buffer was 0.54 U/ml. A 1:1 mixture of C.S.'s PPP and platelet suspension buffer, incubated for the same length of time, showed a vWf of 0.01 U/ml.

**Results of Platelet Transfusion**

Bleeding from a lower lip laceration followed an injury on April 3, 1974, and persisted for 4 days. It ceased within 3 hr of platelet transfusion given on April 7, and did not recur. Platelet function studies were performed on C.S. 2 days later (April 9). Responses to ADP, epinephrine, and thrombin were normal. The response obtained with collagen was reduced compared with normal but was greater than that obtained when C.S. was tested 10 days (April 17) and 16 days (April 23) after platelet transfusion. The ristocetin-induced platelet aggregation rate was reduced. The vWf was not measured after platelet transfusion.

**DISCUSSION**

The clinical and investigative features of PRA have been well documented by studies of individuals and families and reviewed in a number of publications. There can be no doubt that C.S. and his parents showed features of PRA, as the response of their platelets to ADP, epinephrine, and collagen was abnormal both in PRP and in the parents' washed platelets suspended in NPP. No inhibitors were found in the parents' PPP. The release of $^{14}$C-serotonin in response to ADP and collagen was absent or severely reduced. Response to high concentrations of ADP was normal. Uptake of $^{14}$C-serotonin at 30 min was normal, and this suggested that storage was not defective. Pareti et al. showed that although $^{14}$C-serotonin uptake in six patients with storage pool disease was within the normal range in the first 5 min, it was grossly diminished at 30 min, while two patients with the release defect had normal $^{14}$C-serotonin uptake at 30 min. In our patients, the failure to release $^{14}$C-serotonin despite its normal uptake suggested that the PRA was due to a defect in the release mechanism rather than an abnormal storage pool.

Mild thrombocytopenia was noted in the father, a finding that has been documented in at least one other patient with a platelet release defect. Another explanation may be that low platelet counts have been observed in some southern European people. The absence of giant platelets in Wright's-stained smears in all members of the family, and normal ristocetin responses in the mother, and in the father on one occasion, excluded a diagnosis of Bernard-Soulier syndrome.

The investigations of factor VIII procoagulant activity, factor VIII-like antigen, and response to ristocetin clearly showed that C.S. had some of the abnormalities that are usually associated with vWd. Although factor VIII procoagulant activity was normal, and factor VIII-like antigen was below normal only once, the rate of ristocetin aggregation in PRP was low and the vWf levels were severely reduced on both occasions that they were tested. Mixing experiments showed that a vWf inhibitor was not present in the PPP of C.S. In addition, two-dimensional immunoelectrophoresis showed that the factor VIII-like antigen in C.S. had abnormal mobility, a finding that has been documented in a few other vWd patients with normal or near normal levels of antigen.
The bleeding time in C.S. was markedly prolonged when compared to the parents. Similarly, despite the clear laboratory findings of PRA, neither parent had a clinical bleeding tendency; this is in contrast to C.S., who suffered severe hemorrhage after a minor injury. These findings may be explained by the association of vWF deficiency and PRA in C.S. A platelet transfusion stopped the bleeding from a small wound in C.S., and 2 days later platelet function studies were still normal although there was no evidence of correction of ristocetin-induced platelet aggregation. This transfusion may have been clinically effective by correcting the platelet defect, thus eliminating one of the hemostatic abnormalities. The amount of vWF infused in the 2 units of platelets was not measured, and the changes of vWF levels after transfusion were also not measured. It is possible that factor VIII-like antigen associated with the platelet surface and contained within the platelets may have had vWF activity and contributed to the arrest of bleeding.

The father’s hemostatic defects are more difficult to interpret, as his laboratory findings neither confirm nor exclude the possibility that he has mild vWD. Meyer et al. have documented variable penetrance of vWD in one family, and this may explain the severe vWF deficiency in C.S., if it has been inherited from the father. However, there was abnormal mobility on two-dimensional immunoelectrophoresis of the factor VIII-like antigen in C.S. and normal mobility in his father. This constitutes evidence against the inheritance of the severe vWF deficiency. Peake et al. have reported one family with vWD where abnormal mobility of factor VIII-like antigen was transmitted from one generation to another, and we have confirmed these findings in another family (unpublished observations). It is thus impossible to determine whether the vWF deficiency in C.S. is inherited or constitutes a new mutation.

Recently Weiss has described a group of patients with platelet release reaction abnormalities and abnormal response to ristocetin. One of these patients showed decreased levels of factor VIII typical of vWD and our patient had somewhat similar findings. The relationship between abnormalities of the factor VIII complex and defects of platelet aggregation are not clear at the present time, but the term “von Willebrand’s syndrome,” as suggested by Weiss, may be appropriate for these patients.

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