A New von Willebrand Variant (Type I, New York): Increased Ristocetin-Induced Platelet Aggregation and Plasma von Willebrand Factor Containing the Full Range of Multimers

By Harvey J. Weiss and Ira I. Sussman

We report three members of a family who had reduced levels of plasma von Willebrand factor (vWF) and increased ristocetin-induced platelet aggregation (RIPA) (aggregation of platelet-rich plasma with ristocetin at a concentration of 0.45 mg/mL), as previously reported in type IIB and pseudo-von Willebrand’s disease (vWD). However, in contrast to the latter two disorders in which the larger vWF multimers are absent in plasma, the entire range of vWF multimers was observed in the patients’ plasma after sodium dodecyl sulfate–agarose gel electrophoresis, and all vWF multimers (including the largest) were present in the same proportion as in normal plasma and type I vWD. Thus, despite increased RIPA, the levels and multimeric pattern of vWF in this family’s plasma were indistinguishable from those in type I vWD in which RIPA is usually decreased. Addition of ristocetin to the patients’ platelet-rich plasma resulted in the removal of vWF (and, more selectively, of the large multimers) at lower concentrations of ristocetin than normal, as in type IIB and pseudo-vWD. The defect in the patients was localized to their vWF, which had an enhanced capacity for aggregating washed normal platelets in the presence of low concentrations of ristocetin and for aggregating pseudo-vWD platelets (in the absence of ristocetin). Both glycoproteins (GP) Ib and IIb-IIIa were involved in the enhanced aggregation response. RIPA (at low ristocetin concentrations) in the patients’ platelet-rich plasma was abolished by a monoclonal antibody (AP1) to GP Ib and was markedly reduced by monoclonal antibodies (10E5 and LJ9) that block adenosine diphosphate and thrombin-induced binding of vWF and fibrinogen to GP Ib-IIIa but was unaffected by an antibody (LJP5) that only blocks vWF binding. Partial inhibition of the initial aggregation slope (and complete inhibition of second-phase aggregation) was achieved with creatine phosphate/creatine phosphokinase. EDTA blocked second-phase aggregation but was without effect on the initial slope. The findings in this family combine some features of both type I vWD (normal pattern of vWF multimers in plasma) and type IIB vWD (increased RIPA) and further demonstrate the increasing complexity of the structure-function relationships in vWD.

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MATERIALS AND METHODS

Blood. Venous blood was mixed (9:1) with 3.2% sodium citrate and centrifuged to obtain platelet-rich plasma (PRP) and (platelet-poor) plasma as previously described.

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Bleeding time. The bleeding time was determined with a Simplate-II bleeding time device (General Diagnostics, Morris Plains, NJ).

Standard hematologic studies on plasma. Prothrombin time and partial tromboplastin time (PTT) were determined by standard techniques, and specific coagulation factors (factors VIII and XI) were assayed with reference to pooled normal plasma by a one-stage technique using congenitally deficient plasma as substrates. vWF antigen (previously designated FVIII:Ag) was assayed by the Laurell electroimmunoassay (EIA) as previously described. Ristocetin cofactor in diluted plasma was assayed using washed normal platelets and a ristocetin concentration of 1.25 mg/mL.

Platelet studies. Platelets were counted by phase microscopy. Platelet aggregation induced in PRP by collagen, adenosine diphosphate (ADP), and epinephrine was studied in the aggregometer (Payton Associates, Buffalo) as previously described. Aggregation by graded concentrations of ristocetin was determined by adding 20 μL of ristocetin (Lundbeck, Copenhagen) to 1.0 mL of PRP to achieve a final concentration ranging from 0.3 to 1.5 mg/mL. Results obtained were expressed as the initial slope of the aggregation curve (normalized to a chart speed of 20 mm/min) and as the maximal degree of aggregation. vWF antigen in platelets was measured on lysates of washed platelets by EIA as previously described. Platelet adhesion to rabbit subendothelium was measured by exposing deendothelialized segments of rabbit aorta to platelet-rich plasma prepared from blood anticoagulated with citrate (9:1). The ability of vWF in either plasma, cryoprecipitate, or vWF fractions to support aggregation of washed platelets was studied using platelets from normal subjects that had been separated from citrated PRP by albumin density gradient separation and suspended in calcium-free Tyrode's buffer (pH 7.3) at a final platelet count of 800,000 to 1,200,000/μL as previously described. Adsorption of plasma vWF to platelets in the presence of ristocetin was performed as previously described. For these studies, a suspension of washed platelets was incubated with an equal volume of plasma in the presence of various concentrations of ristocetin for 30 minutes at 37°C, and after centrifugation at 2000 g for ten minutes, the supernatant plasmas were frozen at −70°C until studied. The vWF antigen in the supernatant was assayed by radioimmunoassay (kindly performed by Dr Leon Hoyer, University of Connecticut Health Science Center, Farmington) and vWF multimers were determined as described previously.

Platelet inhibitors. The inhibitory effects of several monoclonal antibodies to platelet GP on RIPA was studied by the addition of these antibodies to PRP. A previously described monoclonal antibody (AP1) against platelet GPIb that blocks ristocetin-induced binding of vWF to platelets was kindly provided by Drs Robert Montgomery and Thomas Kunicki (Blood Center of Southeastern Wisconsin, Milwaukee). A monoclonal antibody (10E5) against GPIIb-IIIa was a gift from Dr Barry Collier (State University of New York, Stony Brook). This antibody has been previously shown to block the binding of fibrinogen to thrombin and ADP (but not ristocetin-stimulated platelets) and, more recently, has been shown to block the binding of vWF, fibronectin, and thrombospondin as well. Other several monovalent Fab fragments of monoclonal antibodies against GPIIb-IIIa were a gift of Dr Zaverio Ruggeri (Scripps Clinic and Research Foundation, La Jolla, Calif). Antibody LJP9 inhibits the thrombin and ADP-induced binding to GPIIb-IIIa of both vWF and fibrinogen. Antibody LJP5 inhibits the binding of vWF but not fibrinogen, and antibody LJP10 binds to GPIIb-IIIa without inhibiting uptake of either vWF or fibrinogen (Dr Z. Ruggeri, personal communication). A possible role for ADP was studied using PRP prepared from blood anticoagulated (9:1) with 3.2% sodium citrate containing 90 mmol/L creatine phosphate and 60 μU/mL creatine phosphokinase (CP/CPK) (Sigma Chemical Co, St Louis). The effect of adding Na2 EDTA was also studied. The studies using CP/CPK, EDTA, and monoclonal antibodies 10E5 and LJP9 were performed at concentrations of these substances that completely abolished platelet aggregation by 20 μmol/L ADP.

Platelet inhibitors. Three members of a family with a mild bleeding history were studied. Patient 1, the propositus, is a 24-year-old male who reported a lifelong history of easy bruising. He bled excessively after a tonsillectomy at age 2½ and received plasma transfusions as treatment for a trauma-induced hematoma in his leg at age 13. However, he did not bleed excessively after a hemorrhhiorrhaphy at age 1½ yrs. His twin sister (patient 2) also bruises easily, bled excessively after a tonsillectomy at age 2½, and at age 16 received blood transfusions for treatment of a large, painful hematoma on the buttocks. A second sister, aged 29, was not studied but is said to bruise easily and had life-threatening hemorrhage after a tonsillectomy at age 4. In addition, she suffered a hemorrhhiorrhaphy of the right ankle at age 23 (for which she received cryoprecipitate) and, in the same year, was treated with fresh-frozen plasma for about ten days after right knee surgery complicated by bleeding and the development of an anterior compartment syndrome. The 48-year-old mother of the propositus (patient 3) bled extensively after tooth extractions and postpartum, had one episode of unexplained gastrointestinal bleeding, and has received both plasma and cryoprecipitate for treatment of bleeding episodes.

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INCREASED RISTOCEIN AGGREGATION: TYPE I, NY

elsewhere) were kindly provided in June 1985 by Dr Judith Ratzan of Miami. The second affected sister of the propositus was not studied but has been diagnosed elsewhere as having vWD (with a normal factor XI level).

Other subjects. Control subjects were normal hospital personnel, aged 21 to 50. Patients with type I vWD included those with decreased levels of vWF in plasma and platelets, which we have previously designated as type I-1 vWD, and those with decreased plasma but normal platelet vWF levels (type I-2 vWD).

RESULTS

Basic hematologic studies. Patients 1 and 2 were studied in our laboratory, and selected studies on patient 3 were done on samples of frozen plasma. Normal values were obtained for hemoglobin, white blood count, prothrombin time, and platelet count. Other studies are shown in Table 1. The bleeding time (performed once) was within the range of normal values. Factor VIII:C and vWF values on the propositus (patient 1) were obtained on four separate occasions. The average value obtained for Factor VIII:C was 26 U/dL; for vWF antigen, 38 U/dL; and for ristocetin cofactor, 41 U/dL. The relatively concordant decrease in these values is characteristic of the findings in type I vWD. The values of factor VIII:C, vWF antigen, and ristocetin cofactor obtained in his twin sister (patient 2) and mother (patient 3) were, in most cases, just at or somewhat below the lower limits of the control values (Table 1). The overall findings in the three affected family members studied suggest a diagnosis of type I vWD with variable degrees of penetrance. Since the vWF level). Other studies.

Table 1. Laboratory Studies

<table>
<thead>
<tr>
<th>Control</th>
<th>Patient 1</th>
<th>Patient 2</th>
<th>Patient 3*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bleeding time (min)</td>
<td>&lt;9</td>
<td>4</td>
<td>7</td>
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<tr>
<td>Plasma values (U/dL)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Factor VIII:C</td>
<td>50–150</td>
<td>26</td>
<td>51</td>
</tr>
<tr>
<td>vWF antigen (EIA)</td>
<td>55–160</td>
<td>38</td>
<td>58</td>
</tr>
<tr>
<td>Ristocetin cofactor</td>
<td>50–150</td>
<td>41</td>
<td>46</td>
</tr>
<tr>
<td>Factor XI</td>
<td>60–150</td>
<td>60</td>
<td>34</td>
</tr>
<tr>
<td>Platelets</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number (×10^3)/µL</td>
<td>180–350</td>
<td>278</td>
<td>232</td>
</tr>
<tr>
<td>vWF antigen (U/10^3)</td>
<td>0.25–0.69</td>
<td>0.46</td>
<td>0.39</td>
</tr>
<tr>
<td>Ristocetin aggregation‡</td>
<td></td>
<td></td>
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<tr>
<td>0.3 mg/mL</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.45 mg/mL</td>
<td>0</td>
<td>60</td>
<td>90</td>
</tr>
<tr>
<td>0.6 mg/mL</td>
<td>0</td>
<td>91</td>
<td>85</td>
</tr>
<tr>
<td>0.9 mg/mL</td>
<td>0–88</td>
<td>93</td>
<td>100</td>
</tr>
<tr>
<td>1.2 mg/mL</td>
<td>5–84</td>
<td>99</td>
<td>100</td>
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</tbody>
</table>

* Assayed on frozen plasmas. (See Note Added in Proof).
‡ Control values obtained on eight to 13 normal subjects.

In patients 1 and 2, average results shown for plasma values and ristocetin aggregation are for studies obtained on four and two separate days, respectively.

Plasma values (U/dL)

<table>
<thead>
<tr>
<th>Patient 1</th>
<th>Patient 2</th>
<th>Patient 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factor VIII:C</td>
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</tr>
<tr>
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<td>38</td>
<td>58</td>
</tr>
<tr>
<td>Ristocetin cofactor</td>
<td>41</td>
<td>46</td>
</tr>
<tr>
<td>Factor XI</td>
<td>60</td>
<td>60</td>
</tr>
</tbody>
</table>

Platelets

| Number (×10^3)/µL | 180–350 | 278 | 232 | normal† |
| vWF antigen (U/10^3) | 0.25–0.69 | 0.46 | 0.39 | — |
| Ristocetin aggregation‡ | | | | |
| 0.3 mg/mL | 0 | 0 | 0 | — |
| 0.45 mg/mL | 0 | 60 | 90 | — |
| 0.6 mg/mL | 0 | 91 | 85 | — |
| 0.9 mg/mL | 0–88 | 93 | 100 | — |
| 1.2 mg/mL | 5–84 | 99 | 100 | — |

†Performed elsewhere.

Fig 1. Aggregation of patients’ PRP by low concentration of ristocetin. Ristocetin was added to citrated PRP in a concentration of 0.45 or 0.60 mg/mL. Results are shown for patients 1, 2, and a typical normal subject. For complete dose-response data, see Table 1.
plasma (at a final vWF antigen concentration of 20 U/dL). The enhanced capacity of cryoprecipitate from patient 1 to support RIPA at low concentrations of ristocetin is shown in Fig 3A and 3B. Similar studies using plasma are shown in Fig 3C and demonstrate the enhanced capacity of vWF from patients 1, 2, and 3 to support RIPA when compared with plasmas from normal subjects or from patients with either type I-1 or type I-2 vWD. The experiment was modified by using, instead of a fixed concentration of vWF, increasing concentrations of vWF and a fixed, low concentration (0.6 mg/mL) of ristocetin. The enhanced capacity of vWF from patient 1 to support platelet aggregation by a low concentration of ristocetin is shown in Fig 3D. Finally, the increased reactivity of patient vWF with platelets in the absence of ristocetin was demonstrated using PRP from a subject with pseudo-vWD. We have previously shown that platelets from these patients are aggregated by human vWF without a requirement for ristocetin. The enhanced capacity of vWF from patients 1 and 2 to induce platelet aggregation in a patient with pseudo-vWF is shown in Fig 4.

**Table 2. Densitometric Scan of vWF Multimers in Plasma**

<table>
<thead>
<tr>
<th>Multimer No.</th>
<th>Normal N1</th>
<th>Patients N2</th>
<th>N3</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>I-2</th>
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<td>9</td>
<td>9</td>
<td>9</td>
<td>10</td>
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</table>

*The percentage of the total vWF for bands 1 (the multimer of lowest mol wt) through band 6 and for the vWF accounting for all multimers with mol wt > band 6, determined by densitometric scan of the gels shown in Fig 2.*

**Fig 2.** Multimeric composition of plasma vWF. Autoradiographic pattern of plasma vWF multimers detected by 125I-labeled anti-vWF after electrophoresis in SDS-glyoxyl agarose. The anode is at the bottom. Studies are shown for patients 1, 2, 3, for three normal subjects (N1, N3, and N2, the father of patients 1 and 2), and for three patients with type I (two with I-1 and one with I-2) vWD. The percentage of the total vWF for each of the first six multimers and for the multimers of mol wt greater than band 6 is shown in Table 2.

**Fig 3.** Capacity of vWF to support ristocetin-induced aggregation of washed normal platelets. Platelet aggregation induced by 10 µL of ristocetin was studied in a mixture containing a suspension of albumin density gradient, washed normal platelets and plasma, cryoprecipitate, or fractions prepared from the latter. (1) Top: initial slope (A) or aggregation (B) in a mixture containing 240 µL platelet suspension + 250 µL cryoprecipitate (in Tris-saline, pH 7.3, to a vWF concentration of 70 U/dL) from patient 1 (C) or a control (X) and increasing concentrations of ristocetin. (2) Bottom left (C): 240 µL platelet suspension + 250 µL of pooled normal or patient plasma containing 0.1 units of vWF antigen (achieved, where necessary, by dilution with type III vWD plasma) plus increasing concentrations of ristocetin. The initial slope is shown for normal subjects (X); patients 1 (O), 2 (Δ), and 3 (□); and patients with type I-I (■, ■·) and I-2 (▲) vWD. (3) Bottom right (D): 140 µL washed platelets and 350 µL of a vWF fraction diluted in Tris-saline to contain variable amounts of vWF + ristocetin (final concentration 0.6 mg/mL). The initial slopes obtained with increasing (final) concentrations of vWF antigen are shown for patient 1 (O) and a normal control (X).

**Increased adsorption to platelets of vWF** and of the larger vWF multimers from patient plasma in the presence of ristocetin. In these studies, increasing concentrations of ristocetin were incubated at 37 °C for 30 minutes with a mixture containing equal volumes of washed normal platelets and plasma from either a normal subject or patient 1. The final vWF antigen concentration in the mixture without ristocetin was 20 U/dL, obtained in the case of the normal subject by prior dilution with plasma from a patient with severe (type III) vWD. After centrifugation, the vWF antigen content in the supernatant was measured by radioimmunoassay, and the percentage of vWF antigen remaining (not adsorbed to platelets) was calculated with reference to a control incubation mixture. In addition, the multimer pattern of vWF in each supernatant sample was determined. Figure 5 demonstrates the enhanced ristocetin-induced adsorption of vWF and the larger vWF multimers observed with the plasma of patient 1. In contrast to this abnormality of plasma vWF, the capacity of the patient's platelets to adsorb normal vWF with increasing ristocetin concentrations was the same as in normal platelets (data not shown).
Platelet GP on RIPA. PRP from patient I was used in the study to subendothelium, studied in citrated blood at a shear rate of 2,600 s⁻¹. Several known platelet inhibitors were also studied. EDTA inhibited the initial slope and abolished secondary aggregation. The ADP-removing system CP/CPK was normal in both patients I and 2 (58% and 55%).

Sialic content of vWF. The sialic content of highly purified vWF was the same (98.3 nmol/mg protein) as in a control subject studied in parallel (89.7 nmol/mg protein). Both of these values are somewhat lower than in previous normals studied in the laboratory of Dr Sandor Shapiro (131 ± 16 nmol/mg protein). The reason for this is not clear but could be related to the fact that because of the limited sample only one, rather than two, cryoprecipitations were used in the present study and this could have resulted in a vWF preparation of somewhat lesser purity.

Crossed immunoelectrophoresis. The precipitin arc of vWF obtained by crossed immunoelectrophoresis of plasma from patient I was of the same configuration as that observed in the plasma from a normal subject or from a patient with type I vWD (data not shown).

Effect of platelet inhibitors and monoclonal antibodies to platelet GP on RIPA. PRP from patient I was used in the study, and the results are summarized in Fig 6. Platelet aggregation by 0.6 mg/mL ristocetin was completely blocked by a monoclonal antibody (API) to GPIb. The initial slope of the aggregation tracing was markedly diminished by two different monoclonal antibodies (10E5 and LJP9) that block thrombin and ADP-induced binding of vWF and fibrinogen to GPIIb-IIIa. In contrast, no inhibitory effects on RIPA were observed with either monoclonal antibody LJP10, which binds to GPIIb-IIIa but does not inhibit the binding or any known protein. The effects of several known platelet inhibitors were also studied. EDTA (0.1%) had no effect on (and may have even enhanced) the initial response (slope) to ristocetin, but it abolished secondary aggregation. The ADP-removing system CP/CPK inhibited the initial slope and abolished secondary aggregation.

Platelet adhesion to subendothelium. Platelet adhesion to subendothelium, studied in citrated blood at a shear rate of 2,600 s⁻¹, was normal in both patients I and 2 (58% and 55% control values of 42% ± 5%). To study specifically the properties of patient vWF in supporting adhesion, we added increasing concentrations of normal or patient vWF (in cryoprecipitate) to samples of citrated blood from a patient with severe (type III) vWD and then studied platelet adhesion to subendothelium. When compared at comparable levels of vWF antigen, the ability of patient vWF to correct the adhesion defect in type III vWD was, if anything, somewhat greater than that observed with normal vWF (data not shown).

DISCUSSION

Except for the enhanced platelet aggregation of their platelets by low concentrations of ristocetin, many of the findings in the family reported herein are those of type I vWD. Thus, factor VIII:C and vWF-related properties,
addition, patient vWF was more effective than normal vWF in aggregating the platelets of a patient with pseudo-vWD (Fig 4), demonstrating that the enhanced reactivity of their vWF towards platelets can be demonstrated in the absence of ristocetin. By analogy with type IIB, it might be appropriate to designate these patients as having type IIB vWD, but since this term has already been used for another subtype of type I,\(^3\) we have chosen to designate our family as having vWD type I–New York or, alternatively, type IV vWD.

The basis for the enhanced capacity of the vWF in patients with vWD type I–New York to support platelet aggregation by low concentrations of ristocetin remains to be determined. As in type IIB vWD,\(^3\) this enhanced aggregation was associated with increased adsorption to platelets of vWF and, more specifically, of the larger vWF multimers (Fig 5), which may bind to GPIIb with the highest affinity.\(^1\) Not surprisingly, platelet aggregation by low concentrations of ristocetin was completely blocked by a monoclonal antibody to GPIIb since aggregation by ristocetin requires, initially, the binding of vWF to this platelet membrane GP \(\alpha\) (5–8,40–42).\(^\) Aggregation by low concentrations of ristocetin was also greatly diminished by monoclonal antibodies to GPIIb-IIIa (10E5 and LJP9) that block ADP- and thrombin-induced binding of vWF and fibrinogen to this membrane GP,\(^33,33\) but was not inhibited by antibody LJP5, which only blocks vWF binding.\(^3\) These findings together with the inhibitory effect of the ADP-consuming system CP/CPK suggest that the binding to GPIIb-IIIa of fibrinogen (but not vWF) through endogenous platelet ADP may also contribute to the enhanced RIPA in vWD type I–New York. These conclusions are consistent with recent studies that have demonstrated that the binding of fibrinogen to GPIIb-IIIa plays a role in the aggregation of normal platelets that is initiated by binding of vWF to GPIb.\(^40,44\) The enhanced initial response to low concentrations of ristocetin in the presence of EDTA (which blocks binding of proteins to GPIIb-IIIa)\(^4\) may be related to the fact that calcium is not required for the initial binding of vWF to GPIb\(^b\) and suggests that this binding may even be enhanced in vWD type I–New York.

Several aspects of this disorder remain to be clarified. For one, it is not clear why, as in type IIB vWD, the larger multimers are more selectively removed from plasma by low concentrations of ristocetin, yet (unlike type IIB) the native plasma contains the full range of multimers (and the same proportion of larger multimers as in normal plasma). In theory, an increased clearance of larger multimers from plasma, as in type IIB vWD,\(^2\) could be compensated for by increased synthesis, but we have no evidence to support this possibility. The relationship of the abnormal vWF in vWD type I–New York to the bleeding disorder in this family is also not entirely clear. Platelet adhesion (in citrated blood) to subendothelium was not diminished in these patients under conditions that have demonstrated decreased adhesion in patients with severe vWD,\(^2\) although we have not systematically studied patients with milder forms of type I vWD. However, we found no evidence to suggest that the vWF in type I–New York is deficient in its capacity to support platelet adhesion, and in fact, where this was specifically examined, vWF type I–New York appeared to correct the
platelet adhesion defect in severe vWD to a greater extent than normal vWF. An interpretation of the findings in this study is further complicated by the somewhat decreased factor XI values in two members of the family, although this was normal in one other clinically affected family member (patient 2) and was said to be normal in a second severely affected sister of the propositus who was not studied by us. In all likelihood, the bleeding disorder in the family is probably the result of low to marginal levels of vWF and factor VIII:C, and in some cases, factor XI, which are unable to support normal hemostasis under some circumstances. The findings in the present study demonstrate further the evolving complexity of the clinical and laboratory findings in vWD and demonstrate the problems in attempting to define the disorder in terms of either the plasma multimer patterns or functional studies such as those involving ristocetin aggregation. The family designated as having vWD type I–New York in the present study has features of both type I vWD (multimer patterns) and type IIb vWD (enhanced ristocetin aggregation) and underscores the desirability of assigning, ultimately, subtypes on the basis of the molecular defects in the vWF protein.

NOTE ADDED IN PROOF
We recently studied patient 3 in our laboratory and, on freshly drawn blood specimens, obtained values as follows: platelet aggregation 61% at a ristocetin concentration of 0.6 mg/mL. Factor VIII: C = 64 U/dL, Factor XI = 65 U/dL, vWF antigen = 52 U/dL, and ristocetin cofactor = 50 U/dL. Platelets, 305,000/μL; bleeding time, 7 min.

ACKNOWLEDGMENT
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