von Willebrand's Disease Characterized by Increased Ristocetin Sensitivity and the Presence of All von Willebrand Factor Multimers in Plasma

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In eight members of one family, platelets in platelet-rich plasma aggregated at much lower ristocetin concentrations than normal. Ivy bleeding time was variously prolonged, and von Willebrand factor antigen (vWF:Ag), ristocetin cofactor activity, and factor VIII coagulant activity were decreased. Most of the affected members had had slight to rather severe bleeding symptoms. Platelet-type von Willebrand's disease (vWD) could be ruled out. All multimers of vWF:Ag were found in plasma as well as platelets. Administration of 1-desamino-8-D-arginine vasopressin (DDAVP) to the propositus did not cause thrombocytopenia, and platelet-poor plasma obtained immediately after did not aggregate normal platelets. The molecular defect in this family, inherited as an autosomal dominant, resembles the one in type IIB because of the response to ristocetin but differs from IIB because all vWF:Ag multimers are present in plasma and the response to DDAVP is atypical. We conclude that this family has a new subtype of vWD and propose that structural as well as functional criteria should be used for a proper classification of vWD.

VON WILLEBRAND'S DISEASE (vWD) is one of the most common heritable disorders of hemostasis caused by quantitative or qualitative abnormalities of the von Willebrand factor (vWF). Several distinct subtypes exist for which a much-used classification is based on the electrophoretic demonstration of vWF multimeric patterns in plasma and platelets.[1] Broadly speaking, in type I the amount of protein is reduced, but all the multimers are present.[2-4] In type II the electrophoretic appearance of the vWF is qualitatively different, and the various subtypes (II A to D) are characterized by specific abnormalities in vWF multimeric composition.[5-7] Type IIB differs from the other types by also showing an increased interaction between platelets and the vWF in the presence of ristocetin and by having a normal multimeric pattern in platelets, whereas the largest multimers are lacking in plasma.[8] An increased affinity of the IIB vWF for platelets has also been shown in the absence of ristocetin.[9,10] We describe here another subtype of vWD resembling IIB in its increased sensitivity to ristocetin, but having a plasma vWF multimeric composition consistent with type I.

MATERIALS AND METHODS

Blood collection. Blood was collected in a 3.8% trisodium citrate solution at a ratio of 9:1. Platelet-poor plasma (PPP) was prepared by centrifugation at 2,000 g for 20 minutes and platelet-rich plasma (PRP) by centrifugation at 200 g for ten minutes.

1-Deamino-8-D-arginine vasopressin (DDAVP) administration. DDAVP (Ferring, Malmö, Sweden) was diluted in saline and given intravenously (IV) for ten minutes in a dose of 0.4 μg/kg body weight. Blood samples were collected both before the injection and ten minutes after it was completed.

Platelet count. Platelets were counted visually in a phase microscope or electronically using a Coulter counter, model S-Plus II (Coulter Electronics, Hialeah, Fla).

Washed platelets. The platelets in citrated PRP were gently centrifuged (800 g, 30 minutes) onto a cushion of 35% bovine serum albumin (BSA) in 0.01 mol/L phosphate buffer and 0.1 mol/L NaCl, pH 6.6. The platelet layer was aspirated, the platelets being resuspended in the same buffer and washed three times in the same way. Finally, the platelets were resuspended in Tyrode's buffer at a concentration of 4×10^8/mL.

Platelet lysates. One milliliter of PRP with a known platelet count was layered onto a BSA cushion and centrifuged as just described. The supernatant plasma was siphoned off, the albumin mixed with buffer (0.1 mol/L Tris-HCl and 0.15 mol/L NaCl, pH 7.4), and the platelet button spun down and rewashed. To disintegrate the platelets, 100 μL of buffer was added to the button, and the mixture was frozen and thawed five times, after which 5 μL of Triton X-100 (1:5) was added. The platelet cell fragments were spun down at 30,000 g for ten minutes. The amount of von Willebrand factor antigen (vWF:Ag) in the lysate was determined by immunoradiometric assay (IRMA) and further analyzed by multimeric sizing (see the following sections).

Bleeding time. Bleeding time was measured by a modified Ivy technique using a Simplate-II device (General Diagnostics, Morris Plains, NJ).

Factor VIII coagulant activity (VIII:C) and vWF:Ag. VIII:C was measured with a one-stage assay.[11] vWF:Ag was measured both with a quantitative electroimmunoassay (EIA)[12] and IRMA.[13]

Ristocetin cofactor activity (RcoF). RcoF was determined using formalin-fixed platelets as described by Zuzel et al.[14] Ristocetin was obtained from Lundbeck, Copenhagen.

The reference plasma for all assays was citrated plasma pooled from 20 healthy subjects, the pool having been assayed against the 11th British standard for factor VIII (83/509). The values are expressed as units per deciliter.

Crossed immunoelectrophoresis (CIE). CIE was performed in 1% agarose.[15] Results were expressed as ratios between the migrated distances of the peak of the vWF:Ag arc of a sample and that of normal plasma. A ratio greater than 1.2 was considered abnormal.

Ristocetin-induced platelet aggregation (RIPA). RIPA was measured in PRP as described by Ruggeri et al[8] within 30 minutes after blood collection. The ristocetin concentration necessary to induce aggregation with an initial velocity of 20 mm/min was

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extrapolated from the aggregometer tracings at a number of various concentrations. In normal PRP (n = 7) this concentration ranged from 1.00 to 1.70. Thus, aggregation (20 mm/min) occurring at a ristocetin concentration below 1.00 mg/mL was taken as evidence of increased ristocetin sensitivity.

Platelet aggregation. Platelet aggregation with adenosine diphosphate (ADP), epinephrine, and collagen was performed as previously described.[16] Platelet aggregation induced by post-DDAVP plasma was studied as described earlier.[10]

Multimeric sizing. The multimeric distribution of vWF:Ag in PPP and platelet lysates was analyzed by low- and high-resolution sodium dodecyl sulfate (SDS)–agarose electrophoresis (1.9% and 2.5% agarose concentrations, respectively).[17] The bands corresponding to the multimers were identified in the gels by reaction with a ^{125}I-labeled mouse monoclonal antibody followed by autoradiography. This antibody produces multimeric patterns identical to those obtained with rabbit affinity-purified antibodies.[18] Multimeric sizing was performed on samples from patients included in the pedigree (see the section on patients) and from one patient with type IIB vWD in which the largest multimers are known to be lacking in plasma.[9]

Binding of vWF:Ag to platelets. In mixing experiments, washed platelets (normal or patient) were incubated with normal or patient plasma for 15 minutes at room temperature without stirring. Platelets were separated, and residual vWF:Ag was measured in the supernatant by IRMA and expressed as a percentage of the starting value.

Patients. The pedigree is given in Fig 1. The propositus (II:1) is a 46-year-old woman who was referred to our department for preoperative evaluation (hemorrhoidectomy) because of hemorrhagic diathesis. Her history included easy bruising since childhood, subcutaneous hematomas, and gingival bleeding. She has always had trouble with menorrhagia though her two children had been delivered without complications, and she underwent cholecystectomy without abnormal bleeding. The patient was otherwise healthy and was receiving no regular medication, though she treated herself with tranexamic acid (Cyklokapron, 1.5 g three times a day orally) during menstruation periods, which reduced the bleedings. Her 19-year-old son (III:1) had no symptoms, and her 16-year-old daughter (III:2) had easy bruising but no other symptoms. The children's father (II:4) was healthy without bleeding symptoms. One of the patient's sisters (II:2) has had no abnormal bleedings, whereas the other two sisters (II:3 and II:5) and three of their four children reported easy bruising (III:5 to 7). The patient's father (I:6), who died from cancer, never suffered from abnormal bleedings. Her mother (I:5) had easy bruising and menorrhagia. One aunt (I:4) also bruised easily.

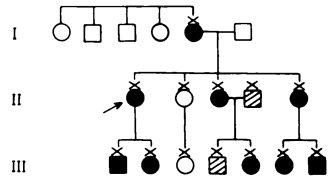

Fig 1. Pedigree of the clinical material. Key: O, female; □, male; ×, laboratory tests performed; ■, increased ristocetin sensitivity; ▨, normal ristocetin sensitivity, low vWF:Ag concentration. The propositus is indicated by an arrow.

RESULTS

The filled and hatched symbols in Fig 1 indicate family members who are affected according to laboratory values.

The laboratory data of the patients are shown in Table 1. The bleeding time was substantially prolonged in the propositus (II:1) and in III:5 and slightly increased or normal in other family members. The propositus had a slightly decreased vWF:Ag level in plasma, measured by IRMA, and somewhat low RcoF. Other members had slightly decreased, borderline, or normal levels of vWF:Ag and RcoF. On CIE the migration of vWF:Ag was normal in all instances (Fig 2).

When DDAVP was given, the levels of vWF:Ag and VIII:C increased. No major change in the platelet count was seen. The bleeding time was not shortened in the propositus but was normalized in two other family members tested. The post-DDAVP plasma did not aggregate normal platelets in normal PRP (not shown).

In the RIPA test (Table 1 and Fig 3) the platelets from the propositus aggregated at much lower ristocetin concentrations than did normal platelets. Seven other family members also showed greater than normal sensitivity to low ristocetin concentrations in this test. Multimeric sizing of plasma vWF

Table 1. Laboratory Data of the Clinical Material

						vWF:Ag					
						EIA	IRMA				
Patient	Sex/Age	Ivy Bleeding Time (Seconds)		Platelet Count (× 10^9/L)		VIII:C (U/dL)	Plasma (U/dL)	Plasma (U/dL)	Platelets ($U/10^{11}$)	RcoF (U/dL)	RIPA test* (mg/mL)
I:5	F/70	600		196		127	114	114	37	108	0.55
II:1	F/47	>1,200	>1,200†	117	131†	46	155†	70	150†	32	117†	22	22†	37	0.73
II:2	F/46	550		169		79	110	114	68	90	1.73
II:3	F/41	740	450†	234	323†	47	193†	50	145†	45	279†	39	51†	51	0.88
II:4	M/42	930		147		83	60	38	10	71	1.10
II:5	F/38	840		185		41	60	37	64	85	0.97
III:1	M/20	840		215		57	64	55	56	46	0.90
III:2	F/16	990		244		74	60	92	60	66	1.12
III:3	F/20	660		286		113	152	152	44	129	1.20
III:4	M/17	630		208		38	34	28	8	38	1.16
III:5	F/15	>1,200	600†	175	160†	83	312†	59	113†	94	17	70	0.90
III:6	F/9	660		201		85	78	80	23	41	0.60
III:7	M/3	780		140		116	100	102	18	117	0.97
Normal controls		360–720		125–340		60–160	50–160	50–175	13–57	50–160	1.00–1.70

*Ristocetin concentration necessary to induce aggregation with an initial velocity of 20 mm/min.

†After DDAVP administration.

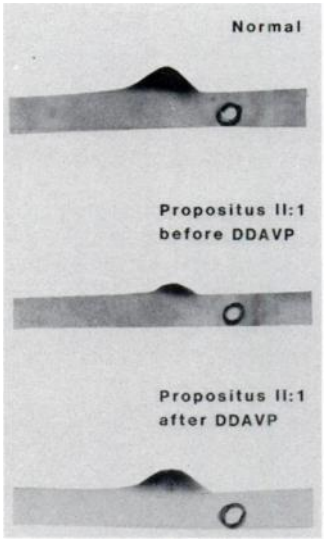

Fig 2. CIE patterns of plasma vWF:Ag from a normal plasma pool and from the propositus (II:1) before and after DDAVP administration. The sample application is indicated by the circled wells. The anode is to the left in the first dimension and to the top in the second dimension.

in the propositus (Fig 4) showed all multimers to be present, though in lower than normal concentrations. All multimers were also invariably found in the other members with a heightened ristocetin response. In family member III:4 a quite different result was obtained with a normal RIPA test despite low levels of vWF:Ag and VIII:C. The father of III:4 (II:4), married to a sister of the propositus, also had a low level of vWF:Ag (IRMA) and a normal RIPA test.

The platelet vWF:Ag concentration was normal in all members except II:4 and III:4 in whom it was slightly low. The pattern of vWF multimers in platelets was normal in all.

To study whether the increased platelet aggregation at low ristocetin concentrations was due to an abnormality of the vWF or of the platelets, mixing experiments were performed (see Materials and Methods). Figure 5 demonstrates

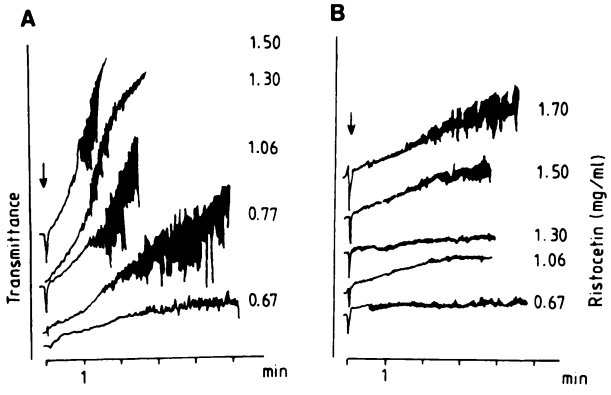

Fig 3. (A) Ristocetin-induced platelet aggregation in PRP from the propositus (II:1). (B) A sample of normal PRP prepared at the same time as the patient's PRP was tested in each experiment.

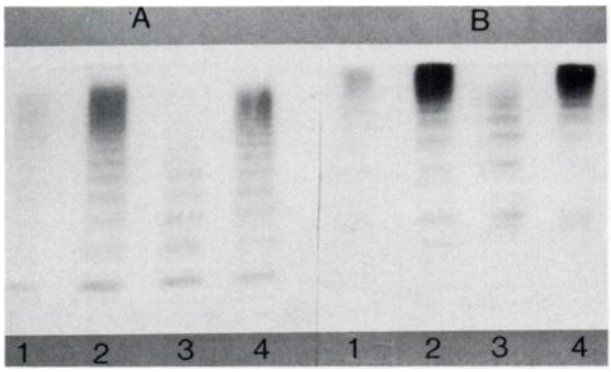

Fig 4. vWF:Ag multimers in plasma analyzed by SDS–agarose gel electrophoresis and identified by reaction with ^{125}I-labeled antibodies followed by autoradiography. Electrophoresis was performed both in 1.9% (A) and in 2.5% (B) agarose gels. Plasma samples from the propositus (II:1) were analyzed before (1) and after (2) DDAVP administration. Control samples from a patient with type IIB vWD (3) and from a normal plasma pool (4) are also shown.

increased binding of patient vWF:Ag to normal and patient platelets at low ristocetin concentrations. Normal vWF did not show an increased binding to the patient's platelets. Moreover, normal platelet aggregation was found in the propositus when performed with ADP, epinephrine, or collagen. Neither normal plasma nor a factor VIII concentrate (fraction I-0) containing vWF induced aggregation of the patient's platelets.

DISCUSSION

In the family described here the disease differs from all variants of vWD hitherto described. The most conspicuous laboratory finding in the propositus was that platelet aggregation in PRP occurred at much lower ristocetin concentrations than in normal PRP. This indicates an increased interaction between the platelets and the vWF. It was further

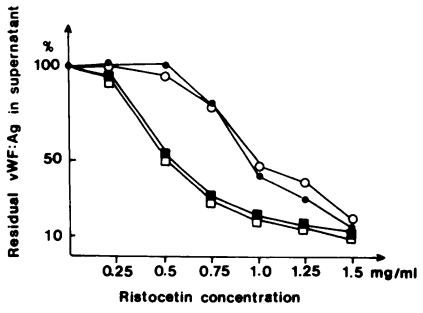

Fig 5. Binding of vWF:Ag to platelets in the presence of ristocetin as determined by mixing experiments (see Materials and Methods). Washed platelets (normal or patient) were incubated with normal or patient plasma for 15 minutes. Platelets were separated and residual vWF:Ag measured in supernatant and expressed as a percentage of the starting value. Key: ●—●, normal platelets + normal plasma; ○—○, patient (II:1) platelets + normal plasma; ■—■, normal platelets + patient plasma; □—□, patient platelets + patient plasma.

demonstrated that the patient's platelets interacted normally with normal vWF and that the abnormality resided in her vWF. An increased interaction between platelets and vWF because of an abnormality in the latter is typical of type IIB vWF[8] and can also be demonstrated in the absence of ristocetin.[9,10,19]

Several findings, however, differentiate the disease described here from type IIB. In IIB, preferentially the large-molecular weight multimers are bound to platelets and thus depleted from plasma, leaving behind the intermediate and low-molecular weight multimers. This is in contrast to the present cases. The normal multimeric pattern of plasma vWF indicates that all molecular forms share the same increased affinity for platelet receptors.[10]

It has recently been shown that patients with type IIB vWD respond to an infusion of DDAVP with thrombocytopenia. This is due to the release by DDAVP of an abnormal vWF with platelet-aggregating properties causing platelet aggregation in vivo.[9] Plasma obtained from IIB patients after DDAVP administration also aggregates platelets in vitro.[9] None of these phenomena occurred in the patients described here. DDAVP infusion did not cause thrombocytopenia, even though the infusion caused a marked increase of vWF:Ag levels; and post-DDAVP plasma did not aggregate platelets in vitro. Thus an interaction between vWF and platelets different from that in type IIB can be postulated also from these findings. Recently some patients have been described demonstrating enhanced RIPA at low ristocetin concentrations, spontaneous platelet aggregation, and thrombocytopenia.[20,21] These patients lacked the high-molecular weight multimers in plasma as in type IIB and may represent one extreme of this variant. They are obviously different from the patients described here.

Type I vWD is characterized by a more or less severe reduction of the vWF in plasma. Typical is the presence in plasma of all vWF multimers, although in reduced amounts, when analyzed by thin-layer SDS-agarose electrophoresis. Different subtypes of type I can be distinguished depending on the relative concentrations of high-molecular weight multimers to lower-molecular weight forms[3] or on the presence in platelets of normal or reduced amounts of vWF[22] or even a qualitatively abnormal platelet vWF.[4] The variant of vWD present in the propositus and other members of her family would be consistent with a type I variant with a normal plasma multimeric pattern and normal platelet vWF. Weiss and Sussman[23] have reported three members of a family who had reduced levels of plasma vWF, with all multimers present, and increased RIPA. Probably our and their families represent a similar vWF trait.

The inheritance of the abnormal ristocetin response in this family obviously follows an autosomal dominant pattern. There was a fairly large scatter in ristocetin sensitivity even among affected members, indicating a variable expression of the genetic trait. This is also reflected in the variable prolongation of the bleeding time from borderline normal to excessively long, in the variability of the clinical picture from rather severe bleeding symptoms to no symptoms at all, and in the variation in factor VIII-associated variables. In addition, it would seem that another abnormal vWF gene is also present in the family. The son (III:4) of one of the propositus's sisters (II:3) had a reduced vWF:Ag level in plasma and platelets but a normal ristocetin sensitivity in the RIPA test. The same was also seen in his father (II:4). The abnormality in these two subjects is consistent with mild type I vWD.

Classification of the various types of vWD has become very intriguing, which is easily understandable in view of the great complexity of the protein involved, the vWF. A large number of mutations of the gene coding for the vWF can be foreseen, leading either to reduced synthesis of the protein, abnormal processing, or structural abnormalities in the amino acid sequence. Thin-layer agarose electrophoresis in SDS[5] has been an invaluable tool in the classification of the various structural abnormalities of vWF. The variant described here shows that this method alone is inadequate for the analysis and classification of vWD. According to multimeric sizing alone, the disease in this family would have been classified as type I vWD, which is unsatisfactory in view of the unequivocal evidence of a functional abnormality of the vWF resembling that in type IIB. Thus both structural and functional criteria are necessary for proper classification. The RIPA test should be used as a regular test for distinguishing variants with an increased vWF-platelet interaction from those with a normal or decreased interaction.

Within the group of increased interaction further subgroups can now be delineated: first, original type IIB patients who respond with platelet aggregation and thrombocytopenia after challenge, eg, with DDAVP and in whom preferentially the large-molecular weight multimers interact with the platelets; second, patients who have spontaneous platelet aggregation, thrombocytopenia, and binding of the high-molecular weight multimers to platelets; and third, patients described here in whom all multimers seem to have the same increased affinity for platelet receptors and in whom thrombocytopenia or platelet aggregation cannot be provoked.

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