A New von Willebrand Factor (vWF) Defect in a Patient With Factor VIII (FVIII) Deficiency But With Normal Levels and Multimeric Patterns of Both Plasma and Platelet vWF. Characterization of Abnormal vWF/FVIII Interaction

By C. Mazurier, J. Dieval, S. Jorieux, J. Delobel, and M. Goudemand

The patients with inherited bleeding diathesis related to quantitative, structural, and/or functional abnormalities of von Willebrand factor (vWF) are said to have von Willebrand's disease (vWD). We report here the clinical and laboratory features of a 50-year-old woman with a life-long history of excessive bleeding. Her particular laboratory data are factor VIII (FVIII) deficiency, subnormal bleeding time, and the presence of all plasma and platelet vWF multimers in normal amounts. Infused with FVIII/vWF concentrate, she showed a persistent increase in FVIII that led us to discard hemophilia A carrier or "acquired hemophilia" diagnoses. vWF devoid of FVIII purified from normal and patient's plasma by immunoaffinity on anti-vWF monoclonal antibody (MoAb) was immobilized onto polystyrene tubes that were further incubated with purified normal FVIII. The bound FVIII was evidenced using radio labeled anti-FVIII MoAb. The data showed that the patient's vWF, in contrast to vWF purified from normal plasma, was unable to bind FVIII. Furthermore, no inhibitor of FVIII/vWF interaction was evidenced in incubating purified normal vWF with the patient's plasma before the addition of FVIII and anti-FVIII MoAb. These results support the concept that the bleeding diathesis of this patient appears to be due mainly to her abnormal vWF preventing FVIII/vWF interaction. This abnormality, which is not yet described in present classification of vWD, could be considered as a new variant of vWD.

© 1990 by The American Society of Hematology.

THE GLYCOPROTEIN named von Willebrand factor (vWF) is known to have a major role in platelet adhesion to the subendothelium and to be the carrier of factor VIII (FVIII) in plasma.1,2 The term von Willebrand disease (vWD) now defines a heterogeneous bleeding disorder in which the underlying pathogenetic mechanism is related to quantitative and/or qualitative abnormalities of vWF. Both endothelial cells and megakaryocytes synthesize vWF, and recent classifications of vWD are based on quantitative, structural, or functional abnormalities of plasma or platelet vWF.1,2 These abnormalities are sought by the following laboratory tests: (1) measurement of vWF antigen (vWF:Ag) and ristocetin cofactor activity (vWF:RCo) in plasma and platelet; (2) determination of the threshold ristocetin concentration inducing platelet aggregation (RIPA) of platelet-rich plasma (PRP); (3) analysis of the electrophoretic pattern of vWF, including the evaluation of the percentage of high molecular weight (HMW) multimers and the characterization of the different bands of each multimeric unit. As stated recently by Ruggeri1 when proposing a new classification of vWD patients, most patients, named type I vWD, with dominant genetic transmission have reduced amounts but no evident abnormality of plasma vWF. Some so-called severe type I or type III vWD patients have a dramatic quantitative abnormality of plasma vWF characterized by its recessive inheritance. The other patients, who are considered to have variant forms of vWD, have either low vWF:RCo level or enhanced response to ristocetin (RIPA increased); apart from the so-called subtype I New-York,4,5 their plasma vWF either has a reduced amount of HMW forms or none at all. When reinvestigating in depth a patient diagnosed with vWD 18 years ago, we identified a new variant of vWD with no vWF quantitative abnormality, normal RIPA, and all plasma and platelet vWF multimers. The evaluation of the response of this patient to therapeutic FVIII/vWF concentrate infusion and the study of the FVIII/vWF interaction provided evidence that the FVIII deficiency of this patient is related to the inability of her plasma vWF to bind FVIII.

PATIENT AND METHODS

Case report. A 50-year-old French woman originating from Normandy was referred to us in August 1988, before thyroidectomy. Her hemorrhagic history dated from childhood when she had life-threatening bleeding at age 10 after tonsillectomy. At this time she had been diagnosed as having "Rosenthal disease." The diagnosis of vWD was raised later, when at age 32 she had a severe postpartum hemorrhage. Her bleeding symptoms also included epistaxis, hematemesis, and melena. Investigations done in August 1988 showed a prolonged activated partial thromboplastin time (APTT), but normal prothrombin and thrombin times. Her bleeding time (BT) was borderline, and her platelet count and platelet aggregation induced by ADP and collagen were normal. Routine analysis showed no abnormality of vWF and normal or subnormal levels of factors IX, XI, and XII, but provided evidence for an FVIII deficiency.

Her two children, a son and a childless daughter, and her parents were said to have no history of bleeding. Her parents, who were orphans, learned they were third cousins.

When she was seen again in September 1988 for gastrointestinal bleeding and in February 1989 for thyroidectomy, her routine laboratory data were confirmed. With her informed consent, she underwent a therapeutic trial. After infusion of 500 mL of Plasmion (Roger Bellon, Neuilly-Paris, France), 400 mL of her plasma were collected by automatic plasmapheresis before infusion of a high purity (HP), solvent/detergent (S/D)-treated FVIII/vWF concentrate6 (CRTS, Lille, France) at a dose of 24 FVIII:C IU/kg body weight (b.w.). Blood samples were collected before and at
different times after infusion. On the basis of FVIII:C data, she was infused 3 days later with the same batch and the same dose of FVIII/vWF just before thyroidectomy. She did not have any bleeding problem either during surgery or during the 10 days postsurgery period that was covered with 2,260 total units of FVIII:C (12 IU/kg bw every 3 days).

Reagents. Carrier-free Na121 was from Amersham International, Amersham, UK. Iodo-gen and Iodo-beads were from Pierce Chemical Co, Rockford, IL. Bovine serum albumin (BSA), RIA grade, was from Sigma Chemical Co, St Louis, MO. Leupeptin, N-ethylmaleimide (NEM), epsilon amino-caproic acid (EACA), benzoquinone, Triton X-100, and urea were purchased from Serva, Heidelberg, FRG. Sodium dodecyl-sulfate was from Biorad, Richmond, CA. Isogel agarose EF was from LKB AB, Bromma, Sweden. Agarose IEF, Sephacryl S-1000, and Mono 75 were purchased from Pharmacia Fine Chemicals AB, Uppsala, Sweden. Ristocetin was from Diagnostica Stago, Asnières, France. Polystyrene tubes (50 x 6 mm) were purchased from Sterilin Limited, Hounslow, UK. Simplate bleed time devices, General Diagnostics, were purchased from Origenon Teknika, Turnhout, Belgium. purified human fibrinogen (protein = 1.8 g/L, fibrinogen = 1.6 g/L, vWF:Ag = 9 IU/L), fibronectin (protein = 2 g/L, fibronectin = 1.5 g/L, vWF:Ag = 300 IU/L), and immunoglobulins (Igs) (protein = 0.95 g/L, Igs = 0.9 g/L, vWF:Ag < 1 IU/L) were from CRTS. All other reagents were of analytical grade from Prolabo, Paris, France, or Merck, Damstadt, FRG.

Antibodies. The rabbit polyclonal antibodies to human vWF used in this study were prepared as already described. A monoclonal antibody (MoAb) (MoAb-239) to human vWF was prepared in collaboration with Immunotech, Marseilles, France. It recognizes all multimeric forms of plasma vWF and has the particular property of binding vWF under physiologic conditions and releasing it under slightly alkaline non-denaturating conditions. MoAb-12G10 to FVIII was prepared in our institute as published by Croissant et al25; it inhibits FVIII:C (-2,500 Bethesda U/mg MoAb) and was shown, by Western blotting, to be directed against the FVIII light chain.10 MoAb-12G10 was labeled with carrier-free Na121 according to the method of Fraker and Speck; its specific radioactivity was 1 to 2.5 µCi/µg.

Collection of blood and preparation of plasma samples. Venous blood was collected in 1:10 volume of 3.8% trisodium citrate. Platelet poor plasma (PPP) and PRP were prepared as described previously.12 Routine coagulation tests were performed on fresh PPP or PRP. Remaining PPP samples were stored frozen at -25°C until further studied for vWF analysis in the 3 weeks after blood collection. A PPP sample was also prepared from a blood sample taken on sodium citrate with protease inhibitors: 5 mmol/L ethylene diamine tetra-acetic acid (EDTA), 6 mmol/L NEM, and 1 mmol/L leupeptin. For platelet vWF study, blood was collected on EDTA; platelets were prepared and washed during the 2 hours after blood collection and then lysed with Triton X-100 as previously described.12

Routine coagulation tests. Tests for hemostasis were done by the methods in use at the coagulation laboratory of Amiens hospital, France. Most of them have been described elsewhere.11 They included BT performed with the template modification of the Ivy technique14 and RIPA performed in PRP using 1, 0.75, and 0.5 mg/mL ristocetin. FVIII:C was assayed by one-stage (FVIII:C-1)13 and two-stage (FVIII:C-2)14 methods using a pool of citrated plasmas calibrated against the First International Reference preparation of FVIII-related activities in plasma (80/511, NIBSC, London, UK) as standard. Anti-FVIII:C inhibitory activity was sought by the Bethesda method.15 FVIII antigen assay was performed with a solid phase immunoradiometric technique using human antibodies.16

vWF analysis. vWF analysis included the determination of vWF:Ag by an enzyme-linked immunos assay (ELISA) already described,19 and the determination of vWF:RCo by aggregometry using 1 mg/mL ristocetin and fresh-washed platelets.20 The multimeric composition of plasma and platelet vWF was analyzed and quantified by electrophoresis and densitometric scanning as recently described.11 In brief, SDS-electrophoresis was performed using a mixture of 2 commercial agaroses (see Reagents) at 0.8% concentration for the stacking gel and 2.5% concentration for the running gel, as previously published.22 After electrophoresis, gels were fixed, washed, and overlaid with 121I-labeled anti-vWF polyclonal antibodies. The intensity of the bands visualized by subsequent autoradiography was quantified by densitometry with a Helena P24 scanner. The HMW multimers of vWF were assumed to be the bands numbered 6 and above, and their percentage was deduced from computerized areas of scanning profiles. The binding of plasma vWF to normal platelets in the presence of ristocetin was evaluated as published20 by measuring residual vWF:Ag in the supernatant with our usual ELISA assay.19 The binding of plasma vWF to soluble type I and III human collagen, purified as already published21 and immobilized on microplates, was studied as described by Brown and Bosak,22 but using our peroxidase-conjugated anti-vWF polyclonal antibodies.19
Michaelis (M) buffer (25 mmol/L sodium barbital-acetate, 1 mmol/L NaCl buffer, pH 7.35) and washed with M buffer containing 0.3% BSA and 0.1% Tween 20. The amount of immobilized vWF was quantified by counting the radioactivity bound to the A tubes. In the B tubes, 200 μL of purified FVIII (SA > 2,000 IU FVIII:Ag/mL) diluted to 5 IU FVIII:Ag/mL in 20 mmol/L imidazol-HCl, pH 6.8, 20 mmol/L lysine, 150 mmol/L NaCl, 1 mmol/L benzamidine, 1 mmol/L EACA, and 1 mmol/L CaCl₂ were added. After washing, FVIII bound onto immobilized vWF was controlled by adding radiolabeled anti-FVIII MoAb-12G10. The tubes were counted after washing and also after subsequent incubation for 2 hours at room temperature with 0.35 mol/L CaCl₂. In some experiments, the tubes coated with vWF purified from normal plasma were incubated with the patient’s PPP (unheated or previously heated for 1 hour at 56°C) before addition of FVIII.

**RESULTS**

**Routine laboratory evaluation.** The coagulation tests performed on three occasions are summarized in Table 1 and in the Case Report. It is noteworthy that APTT values were prolonged as a function of FVIII:C decrease. FVIII:C-1, FVIII:C-2, and FVIII:Ag levels were similar. Bleeding time was slightly prolonged on one occasion, but strictly normal at two other times. No anti-FVIII:C inhibitory activity was detected in PPP heated for 30 minutes at 56°C. Furthermore, vWF:Ag and vWF:RCo levels and RIPA were always normal.

The availability of a small amount of PPP from the patient’s son allowed us to measure some of his FVIII/vWF-related parameters: vWF:Ag level (0.79 IU/mL) was normal but FVIII appeared decreased (FVIII:C-1 = 0.47 IU/mL, FVIII:C-2 = 0.37 IU/mL, FVIII:Ag = 0.34 IU/mL).

**Further analysis of vWF.** Platelet vWF assays performed on a blood sample collected on EDTA showed normal level of vWF:Ag (0.35 IU/10⁹ platelets as compared with normal range: mean ± SD, = 0.45 ± 0.11) and vWF:RCo (0.34 IU/10⁹ platelets as compared with normal range: mean ± SD = 0.44 ± 0.18). Furthermore, the multimeric pattern of vWF in the lysate of patient’s platelets was normal (data not shown).

The patterns of vWF obtained after SDS-agarose electrophoresis of the patient’s PPP were also normal (Fig 1). The percentages of HMW multimers (numbered 6 and above) of vWF in two different samples of the patient’s PPP were 56.6% and 53.8%, respectively, as compared with 56.8% and 52.8% for a pool of normal citrated plasmas analyzed in the same electrophoretic runs. The multimeric unit of plasma vWF, displaying the characteristic “quintuplet” pattern composed of two subbands flanking each side of the major bands, appeared normal in both patients’ PPP samples (Fig 1). The multimeric pattern of the plasma vWF from the blood sample taken in the presence of protease inhibitors was similar (Fig 1A). In addition, the binding of the patients’ plasma vWF to normal platelets in the presence of ristocetin and to collagen were also strictly normal (data not shown).
VARIANT OF vWD WITH NO VWF-FVIII INTERACTION

1. **Effect of FVIII/vWF concentrate infusion.**
   
   When the patient was infused in September 1988 with S/D-treated FVIII concentrate for gastrointestinal bleeding, her 1-hour postinfusion data were: FVIII:C-1 = 1 IU/mL and vWF:Ag = 2.4 IU/mL. One day later her FVIII:C level was still high (FVIII:C = 1 IU/mL) whereas her vWF:Ag had dropped to 0.8 IU/mL.

   Before her thyroidectomy, the response of the patient to the infusion of two vials (24 FVIII:C and 69 vWF:Ag IU/kg b.w.) of an HP, S/D-treated FVIII/vWF concentrate was carefully examined. FVIII and vWF levels of pre- and postinfusion PPP samples are reported in Fig 2B. For comparison, the data of a hemophilia A patient infused with three vials (30 FVIII:C IU/kg b.w.) of the same product are reported in Fig 2A. The response obtained in the two cases was quite different. In contrast to the hemophilia A patient, our patient showed a delayed and sustained rise in FVIII:C. Her FVIII:C recoveries, calculated on expected values based on a 43.3 mL/kg b.w. plasma volume and 1-hour and 2-hour postinfusion FVIII:C-1 levels, were 139% and 334%, respectively, in comparison with 90% and 81% values in the hemophilia A patient. Furthermore, FVIII:C half-life determined according to Allain was around 30 hours, as compared with 13 hours in the hemophilia A patient.

2. **Purification of plasma vWF.**
   
   More than 95% of vWF from normal and patients' plasma was bound on MoAb-immunoadsorbents. In elution fractions, devoid of FVIII, 44% to 56% of vWF was recovered. The SA of the dialyzed preparations obtained after ammonium sulfate precipitation of eluted fractions prepared from various normal and patients' PPP samples ranged between 17 and 35 vWF:Ag IU/mg. The only contaminants detected and identified by electrophoresis and radial immunodiffusion were immunoglobulins G and M, fibronectin, and fibrinogen.

3. **FVIII binding to purified plasma vWF.**
   
   Purified vWF, prepared as described above from either normal or patients' plasma, was coated onto polystyrene tubes. The results obtained when using purified labeled vWF as tracer indicated that the vWF of the two samples bound in a similar dose-dependent manner to polystyrene tubes; in both cases 0.3 to 5 \( \times \) \( 10^{-2} \) IU vWF:Ag were immobilized. The dose-response curves obtained when controlling the subsequent FVIII binding to coated tubes by reaction with radiolabeled anti-FVIII MoAb are shown in Fig 3. The percentages of bound radioactivity (B/T) \( \leq 5% \) were assumed to represent nonspecific FVIII binding because such values were obtained when the tubes were coated with fibronectin, fibronectin, or immunoglobulins instead of vWF, or after removing bound FVIII with 0.35 mol/L CaCl\(_2\). In fact 4.7 \( \times \) \( 10^{-3} \) IU vWF:Ag of immobilized normal vWF was sufficient to evidence FVIII binding (B/T = 6.7%), whereas 4.5 \( \times \) \( 10^{-2} \) IU vWF:Ag of immobilized patients' vWF was necessary to obtain a similar FVIII binding. Furthermore, the FVIII binding to vWF prepared from normal plasma was dependent on the amount of vWF immobilized, and a similar
dose-dependent curve was obtained when vWF (SA = 120 vWF:Ag IU/mg) purified from therapeutic cryoprecipitate was tested (data not shown).

The abnormality of FVIII binding to patients' vWF was confirmed using four purified vWF samples obtained from patient's PPP collected in September 1988 and February 1989, and four purified vWF samples prepared from four different normal individual plasmas.

To see if the dramatic decrease in binding of the vWF prepared from patients' plasma could be due to the presence of an inhibitor, coated normal vWF was incubated for 1 hour at 37°C with either unheated or heated patients' PPP before addition of FVIII. No significant change in FVIII binding to normal vWF was noticed in either case (data not shown).

**DISCUSSION**

In light of the biologic results, the hemorrhagic syndrome of the patient described here appears to be essentially related to a decreased level of FVIII:C. The bleeding time is normal or borderline, which is in accord with the fact that no quantitative or qualitative plasma or platelet vWF defect could be demonstrated by the conventional tests. Such results, together with the fact that FVIII:Ag and FVIII:C levels are identical, are usually found in hemophilia A carriers in whom “Lyonization” accentuates the FVIII deficiency and is responsible for an FVIII:vWF ratio of less than 0.75.31 They could also be compatible with the presence of anti-FVIII:C antibodies. Nevertheless, these diagnoses could be discarded given the impossibility of demonstrating anti-FVIII:C activity in the patients' PPP and the patient's response to the infusion of FVIII concentrates. In fact, the persistence of a high FVIII:C level 24 and even 48 hours after infusion proves that the biologic abnormalities are due not to a defect in the synthesis of functional FVIII (as in hemophiliacs or hemophilia carriers in whom the exogenous FVIII has a half-life of around 8 to 12 hrs23) nor to the presence of an inhibitor causing low FVIII recovery and half-life. On the contrary, this response, which also shows a very high FVIII:C recovery levels with a peak only two hours after infusion, is an example of the sustained paradoxal rise in FVIII usually experienced with vWD patients. It is generally interpreted as being due to an effect of the exogenous vWF on the protection or the synthesis of the patient's FVIII.1-32 Therefore, these results led us to hypothesize that an abnormality of the patient's vWF, and not of the FVIII itself, was the explanation for the FVIII deficiency. Using plasma vWF purified approximately 1,300 times by immunoaffinity, we were able to show that the patient's vWF was unable to bind FVIII normally. In contrast, vWF purified from normal plasma bound FVIII and this binding was demonstrated to be dependent on vWF: it did not occur on other purified plasma proteins and was reversible under the conditions known to dissociate FVIII/vWF complex.13 Furthermore, no inhibitor of FVIII/vWF association was evidenced in either unheated or heated patient's plasma. This led us to conclude that patient's vWF failed to bind normal FVIII. To our knowledge, such a vWF defect has not yet been demonstrated in this way. Moreover, the patient's vWF binds normally to platelets and collagen, and the patient shows no signs of a primary hemostasis disorder: normal BT and no excessive bruising, gingival bleeding or menorrhagia, which are the most common symptoms, together with epistaxis in vWD women.1-2 Therefore this patient has an abnormality of her plasma vWF leading to a hemorrhagic syndrome comparable with that of patients with an isolated FVIII deficiency (mild hemophilia A, hemophilia A carriers, acquired FVIII inhibitors). Nevertheless, considering the fact that her hemorrhagic diathesis results from a qualitative abnormality of her plasma vWF, this patient should be classified under type II vWD if the vWF qualitative defect is no longer restricted to a lack of HMW multimers.3 Furthermore, as this variant illustrates a new physiopathologic mechanism of FVIII deficiency (ie, qualitative, without quantitative abnormality of vWF limited to its ability to bind FVIII), we tentatively suggest it be named not simply by a new letter but by the term “Normandy” after the patient's birth province.

Because no complete family study could be done before now, we have no precise information on the transmission of this abnormality. Since none of the parents or the two children had hemorrhagic problems, if the abnormality is due to a defect of the vWF gene and not to a posttranscriptional abnormal event, the following hypotheses can be presented: (1) mutation occurred of a dominant character of the vWF gene of one of the two chromosomes 12 of the patient, and none of the children received this chromosome from their mother; (2) there is, as is presumed in type IIC vWD patients, a double heterozygosity for two mutant alleles inherited in an recessive manner: one could be a “null” vWF gene and the other a gene inducing the synthesis of abnormal vWF; (3) there is homozygosity of a recessive abnormality of the vWF gene. The last two hypotheses fit in with the patient's son's preliminary data (normal level of vWF but decreased level of FVIII), given that he could be homozygous for the vWF gene abnormality explaining the defect of the FVIII/vWF interaction. However, the homozygosity of our patient seems to be the most likely hypothesis because her parents were probably blood-related.

The study of this patient's DNA with specific probes of the vWF gene will soon be undertaken to search for a possible mutation or deletion of the nucleotide sequence. The characterization of such a defect could enable a possible abnormality to be inferred at the level of the vWF molecule itself. Another more direct approach involves locating the vWF structural abnormality of this patient. The vWF domain binding to FVIII was located on the N-terminal part of the plasma vWF subunit20-22 and persists in the tryptic or plasmic 34-Kd fragment made up of 272 AA containing several intrachain disulfide bonds and one N-glycanic chain.23-26 Our research, currently in progress, is therefore directed toward a comparison of the tryptic fragments obtained from normal and patients' vWF.

Although the molecular abnormality of vWF, and possibly of its gene, still have to be defined, we can assume that we demonstrated a new autosomal defect explaining FVIII deficiency. Therefore, we believe that certain patients diag-
nosed as mild hemophilia A, for whom there is no data clearly indicating an FVIII half-life of around 10 hours after transfusion or a family history of obvious sex-linked recessive transmission, may in fact have the same vWF abnormality as the patient described here. A current study is being done to reinvestigate such patients to determine whether their plasma vWF is able to bind FVIII.

REFERENCES


11. Frazer PJ, Speck JC: Protein and cell membrane iodination with a sparingly soluble chloroamide, 1,3,4,6-tetrachlor-3a,6a-diphenyl glycoluril. Biochem Biophys Acta 80:840, 1978


31. Furlong RA, Chesham J, Peake IR: The combined use of monoclonal antibody-based enzyme-linked immunosorbent assays (ELISA) for factor VIII antigen (VIIIAg) and von Willebrand factor antigen (vWF:Ag) for the detection of carriers of haemophilia A. Clin Lab Haematol 10:295, 1988

32. Hoyer LW: Factor VIII, in Colman RW, Hirsch J, Marder


