Variant von Willebrand’s Disease Type B—Revisited

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Results of investigations of the factor VIII (FVIII) of a patient with an unusual variant form of von Willebrand’s disease (vWD) are presented. A two-peak crossed-immuno-electrophoresis (CIE) pattern was seen when fresh plasma was electrophoresed, but the CIE pattern became normal by incubating the plasma at 37°C for more than 72 hr. The two peaks on CIE were separated by cryoprecipitation: the slow-moving peak precipitating and the fast-moving forms of FVIII remaining in the cryosupernate. An additional protein band was seen on multimeric analysis of the platelet-rich plasma (PRP) from this patient did not respond to ristocetin, but agglutinated normally in response to botrocetin. Multimeric and CIE analysis of the FVIII post agglutination and 125I-FVIII binding studies to normal formalin-fixed platelets indicated that this patient’s FVIII interacted normally with botrocetin but failed to interact with ristocetin. These data strongly suggest that the sites on the FVIII molecule or the multimeric forms involved for ristocetin and botrocetin are different and that the ristocetin reaction is more closely aligned to the physiologic function of FVIII.

Factor VIII (FVIII) is a complex plasma glycoprotein with at least two distinct physiologic functions, procoagulant activity (VIII:C) and von Willebrand factor activity (VIII:vWF); corresponding antigenic activities are coagulant antigen (VIII:CAg) and FVIII-related antigen (VIII:Ag). Our present understanding of the functions of FVIII has evolved from both basic biochemical research using purified materials1 and from a study of the nonfunctional or abnormal FVIII molecules that are synthesized by patients with hemophilia A or variant forms of von Willebrand’s disease (vWD).

Patients with classical or type I vWD have either undetectable FVIII-related protein or similarly reduced levels of all functions associated with the FVIII molecule. A number of patients have been reported with the clinical features of severe vWD but with normal levels of VIII:Ag and VIII:C and reduced or absent VIII:vWF;2-10 these patients have been classified by some as variant or type II vWD. Patients with variant vWD generally have an alteration in their crossed-immuno-electrophoretic (CIE) patterns, with a shift to the faster migrating anodal forms of FVIII.5,7,12 A carbohydrate deficiency or abnormality has been demonstrated in the purified FVIII from some of these patients using periodic acid Schiff (PAS) staining of sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE),13,15 by measurement of the sialic acid content,14 precipitation by the lectin concanavalin-A (Con-A),12,16,17 and binding to 125I-Con-A.18

Recently, several groups have demonstrated by electrophoretic means that the normal FVIII molecule is composed of a large number of multimeric subunits.19-21 On the basis of FVIII multimeric patterns and the response of the patient’s platelet-rich plasma (PRP) to ristocetin, variant von Willebrand’s disease has been subdivided by Ruggeri and Zimmerman19 into type IIA and IIB. In type IIA, only the smaller multimeric forms of FVIII are present in both plasma and platelets, and minimal VIII:vWF activity is detected.19,21 In type IIB, on the other hand, the minimum concentration of ristocetin required to induce agglutination of PRP is reduced compared to normal, and both small and intermediate size FVIII multimers are present in the plasma, but the large polymeric forms are absent. The platelet FVIII multimeric pattern in type IIB is normal.19,23

This article presents further experimental information on the FVIII of a patient with a variant form of vWD with normal levels of both VIII:C and VIII:Ag and undetectable levels of VIII:vWF measured using the ristocetin cofactor assay (VIII:RCof). This patient was first described in 1973 as vWD type B9 and was again reported in 1979.18 Further investigations show that the abnormality of the FVIII of this patient remains unique, and thus warrants a different classification to other variants of vWD so far described.

Materials and Methods

Patient

Clinical and routine laboratory data for this patient (A.A.) have been reported previously.19 The patient’s plasma levels, VIII:C, and VIII:Ag were within the normal range, while VIII:RCof was undetectable, platelet adhesion to glass beads reduced, and the bleeding time prolonged.
Materials
Ristocetin was kindly provided by Lundbeck & Co., Copenhagen, Denmark. The method of Read et al. was used to purify venom coagglutinin (botrocetin) from Bothrops jararaca venom (Sigma Chemical Co., St. Louis, Mo.). Botrocetin was used for platelet aggregation at a concentration that caused visible macroscopic aggregation in 20 sec. Proteolytic inhibitors used were 1 mM phenyl-methyl sulphonyl fluoride (PMSF) (Sigma), 10 U/ml heparin (Glaxo, Melbourne, Australia); 0.1 mg/ml soya bean trypsin inhibitor (Sigma); and 10 U/ml trasyol (Bayer, Germany). Radiolabeled rabbit anti-human FVIII-IgG was prepared by immunofinity chromatography as previously described.

Routine Methods
Blood was collected into 3.8% trisodium citrate (1/10 volume) and processed at room temperature (RT). PRP was prepared by centrifuging citrated blood at 200 g for 20 min and platelet-poor plasma (PPP) was prepared by centrifuging at 2000 g for 10 min. Serum was obtained by allowing whole blood to clot in glass tubes at 37°C for 2 hr, then storing overnight at 4°C before centrifuging at 2000 g for 10 min. Cryoprecipitate was prepared by the method of Pool and Shannon. Platelet aggregation of PRP was carried out at a platelet concentration of 250–300 x 10^3/μl using a Paytons dual channel aggregometer with stirring at 37°C. Fresh washed platelets were prepared by a modification of the albumin cushion technique of Walsh. VIII:C activity was assayed using a one-stage clotting technique, with the standard curves constructed using a standard supplied by Commonwealth Serum Laboratories (CSL). VIII:Ag was measured immunoelectrophoretically by the technique described by Zimmerman et al. VIII:RCof was assayed using ristocetin by the formalinized fixed platelet (FFP) method and VIII-botrocetin cofactor (VIII:BOcof) by botrocetin using paraformaldehyde-fixed platelets. VIII:CAg was assayed immunoradiometrically using a solid-phase technique and a combination of human anti-VIII:C IgG and rabbit anti-human FVIII-IgG. Radio-cross-immunoelectrophoresis (radio-CIE) for VIII:Ag was carried out using a mixture of 125I-anti-FVIII and unlabeled anti-FVIII as previously described. A pool of plasma from 20 normal volunteers was snap frozen in 0.5 ml aliquots and stored at −70°C. This pool was used for the standard curve construction for VIII:Ag, VIII:RCof, and VIII:CAg.

Anti-FVIII Serum
Rabbit anti-FVIII was raised against highly purified normal FVIII or purified patient FVIII as previously described.

Purification and Radiolabeling of FVIII
FVIII was purified from either normal or patient citrated plasma by the method described by Montgomery and Zimmerman using a Bio-Gel A-15 column (1.5 x 100 cm) as the final purification step. Control purified FVIII was radiolabeled with 125I by the lactoperoxidase method using an Enzymplex kit (E-Y Laboratories, San Mateo, Ca.). This 125I-FVIII retained its VIII:RCof and was used for platelet binding studies.

Binding of 125I-FVIII to Platelets
This test was performed in Eppendorf tubes coated with PPP from a patient with severe vWD. FFP (50,000/μl) were incubated with either 0.154 M sodium chloride (N. NaCl) or patient or control purified FVIII and either ristocetin (0.5 mg/ml) or botrocetin for 10 min at RT. 125I-FVIII (5,000 cpm/50 μl) was then added and the incubation continued for a further 10 min. The platelets were pelleted and counted in a γ counter (LKB) (total cpm). Nonspecific binding was measured by carrying out the above incubations in the absence of botrocetin or ristocetin (nonspecific cpm), and the sample prepared for counting. Specific binding of 125I-FVIII to platelets was calculated as total cpm minus nonspecific cpm.

Multimeric Analysis of FVIII
The procedure outlined below is based on the method described by Meyer et al. FVIII was purified from 1 ml of plasma by counter current immunoelectrophoresis against a monospecific rabbit anti-human FVIII in 1% agarose dissolved in 0.075 M barbitone buffer, 0.01 M EDTA, pH 8.6. Electrophoresis was carried out overnight at 15°C at 10 mA/gel (8.5 x 10 cm gels). The precipitant line was cut out and washed for 24 hr in 0.15 M NaCl, 0.02% sodium azide at RT. The sample was then dialyzed against 8 M urea, 0.5% SDS, for 24 hr at 37°C, then against distilled water for 15 min.

The samples (200 μl) were mixed with 5 μl of equal volumes of 10% SDS, 8 M urea, 0.05% bromphenol blue, glycerol, and 60 μl applied to wells cut in slabs of 1% agarose (10 x 10 cm) in 0.075 M sodium barbitone, 0.01 M EDTA, 6 M urea, 0.2% SDS, pH 8.6, poured onto Gel Bond (Marine Colloids, U.S.A.). The gels were electrophoresed at 15°C at 12.5 mA/gel with a running buffer of 0.075 M sodium barbitone, 0.01 M EDTA, 0.2% SDS, pH 8.6. The gels were fixed in 25% isopropanol, 10% acetic acid, and then stained with 0.25% Coomassie blue and destained with 7% acetic acid. Alternatively, the gels were fixed for 1 hr as above, then washed in barbitone-buffered saline for 4 hr, incubated with 125I-anti-FVIII (rabbit) (100,000 cpm/ml) overnight at RT. They were then washed in N NaCl, dried under vacuum, and autoradiographed.

RESULTS
Routine FVIII Assays
The patient’s plasma levels of VIII:C, VIII:Ag, and VIII:CAg were 1.0, 0.73, and 1.5 U/ml, respectively, while VIII:RCof (using ristocetin) was undetectable, and platelet adhesion to glass beads was greatly reduced, as reported previously. Binding of VIII:Ag from this patient to Con-A was also reduced as previously reported.

Two-Dimensional Immunoelectrophoresis
Crossed-Immunoelectrophoresis Patterns
Crossed-immunoelectrophoresis (CIE) of the patient’s plasma showed an unusual precipitant pattern compared to normal pooled plasma (Fig. 1, A and B). The immunoprecipitant arc was composed of two peaks, a normal, slow-migrating peak and a second smaller, fast-migrating peak. A similar pattern was observed when the CIE was carried out using unlabeled rabbit anti-human FVIII and staining with Coomassie blue. Similarly, abnormal CIE patterns were obtained using three other rabbit anti-human FVIII sera prepared against purified normal FVIII in this laboratory, a commercial anti-FVIII serum (Behringwerke), and a rabbit anti-FVIII raised against the patient’s purified FVIII.
The CIE pattern for the patient’s serum was not significantly different from the plasma pattern.

Effects of Incubation on the Crossed-Immunoelectrophoretic Pattern

Normal pooled plasma and patient plasma were incubated at 37°C and aliquoted at intervals for CIE analysis. A fresh sample of patient plasma showed a two-peak pattern (Fig. 2A), which on incubation at 37°C in the absence of proteolytic inhibitors (detailed under Materials and Methods) for up to 144 hr, progressively lost the fast-moving peak and changed to a more normal CIE pattern (Fig. 2, B–D). Similar changes occurred in the presence of proteolytic inhibitors.

There was no change in the CIE pattern of normal

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**Fig. 1.** Radio-crossed-immunoelectrophoresis of (A) normal pooled plasma and (B) plasma from the patient with variant vWD.

**Fig. 2.** Effect of incubation at 37°C on the radio-CIE pattern. (A–D) Plasma from the patient with variant vWD. (A) A fresh sample. (B) incubated at 37°C for 48 hr. (C) incubated at 37°C for 96 hr. (D) incubated at 37°C for 144 hr. (E) fresh sample of normal pooled plasma. (F) normal pooled plasma incubated at 37°C for 144 hr.
pooled plasma during incubation at 37°C for 144 hr, either in the presence or absence of proteolytic inhibitors (Fig. 2, E and F).

**Separation of Anodal Peak**

Figure 3 demonstrates that preparation of cryoprecipitate and cryosupernate from citrated patient PPP effectively separates the two precipitant peaks seen on the patient's CIE pattern. The fast migrating anodal peak remains in the cryosupernate (Fig. 3B), while the slower migrating forms precipitate (Fig. 3C).

Mixing experiments with various combinations of normal and patient cryoprecipitate and cryosupernate were carried out. Mixing patient cryoprecipitate with normal cryosupernate produced a normal CIE pattern, while a mixture of either patient or normal cryoprecipitate and patient cryosupernate produced a CIE pattern indistinguishable from that of the patient plasma. These experiments suggest that the anodal peak of the patient is composed of polymeric forms of FVIII absent from normal plasma, or alternatively, of increased concentrations of low molecular weight (mol wt) polymers that are present in small concentrations in normal plasma. The latter is more likely, since the distance from the starting well to the precipitant arc of the CIE pattern for the VIII:Ag of the cryosupernant for patient and normal is 33 and 30 mm, respectively. This suggests that the two peaks were composed of polymers of similar molecular weight.

![Fig. 3. Radio-CIE of (A) variant vWD plasma; (B) cryosupernate from variant vWD; (C) cryoprecipitate from variant vWD; (D) normal pooled plasma; (E) normal cryosupernate; (F) normal cryoprecipitate.](image-url)
Multimeric Structure

Figure 4A shows the multimeric pattern of normal FVIII after incubation with ¹²⁵I-rabbit anti-FVIII, and Fig. 4B shows a comparable pattern for patient FVIII. The arrow indicates a protein band present in the patient’s plasma that was absent from all samples of normal plasma examined. A protein band in a similar position was present in increased concentrations on multimeric analysis of the supernate from the patient compared to a control sample, otherwise the protein patterns were identical.

Comparison of Botrocetin and Ristocetin

Platelet Aggregation

The patient’s PRP did not aggregate in response to ristocetin at final concentrations between 0.2 and 2.0 mg/ml. Similarly, fresh washed or FFP failed to aggregate in the presence of patient plasma or purified patient FVIII at ristocetin concentrations of 1.0 or 1.5 mg/ml. Mixtures of patient plasma or purified FVIII and normal PRP all aggregated normally in response to ristocetin (1.5 mg/ml) added after a 10-min incuba-
tion at 37°C. Therefore, the patient’s plasma does not contain an inhibitor to ristocetin or VIII:RCof.37

In contrast to ristocetin, botrocetin induced a normal aggregation response of the patient’s PRP (Fig. 5, curve B). Also, fresh washed platelets or normal FFP aggregated normally in the presence of patient plasma in response to botrocetin.

Qualitative Analysis of FVIII Post-Agglutination

Washed normal platelets were resuspended in either normal PPP or patient PPP and aggregated in the presence of ristocetin (1.5 mg/ml) or botrocetin. The platelets were removed by centrifugation and the FVIII remaining in the supernatant subjected to (1) CIE and (2) multimeric analysis.

Crossed-immunoelectrophoresis. The CIE pattern of patient FVIII was unchanged after incubation with ristocetin (no aggregation occurred) (Fig. 6B). By comparison, the slowly migrating forms of FVIII were absent from normal plasma following platelet agglutination by ristocetin (Fig. 6E). On the other hand, after botrocetin induced aggregation of normal washed platelets in the presence of patient PPP, only the fast-migrating anodal peak of the patient’s FVIII remained (Fig. 6C). The comparable experiment using normal PPP is shown in Fig. 6F, where a reduction of all forms of the FVIII molecule was observed.

Multimeric analysis. No change in the multimeric pattern of the patient’s FVIII was observed following incubation with 1.5 mg/ml of ristocetin compared to the saline control, using either Coomassie blue staining or incubation with 125I-anti-FVIII. By contrast, agglutination of normal PRP in response to ristocetin caused a loss of both the high and intermediate mol wt FVIII polymers using Coomassie blue staining; however, these multimeric forms of FVIII were visible using the 125I-anti-FVIII overlay technique. Botrocetin caused similar changes in both patient and control FVIII, with a loss of the intermediate mol wt polymers.

Blocking of 125I-FVIII-Binding to FFP

In the presence of botrocetin, purified FVIII from the patient or control blocked subsequent platelet binding of 125I-FVIII in a dose-dependent manner (Fig. 7A), indicating that patient FVIII interacts normally with botrocetin and platelets. By contrast, the patient’s purified FVIII caused only minimal blocking of 125I-FVIII binding to platelets in the presence of ristocetin at high antigen concentrations (Fig. 7B, A.A.). Normal FVIII blocked ristocetin-induced binding in a dose-dependent manner (Fig. 7B, control). These data strongly suggest that the site for the ristocetin interaction of the patient’s FVIII is abnormal or absent.

DISCUSSION

The patient described in this report fits the diagnosis of variant vWD, in that she has a moderate to severe bleeding disorder, a prolonged skin bleeding time, reduced glass bead adhesion, and undetectable levels of VIII:RCof activity, and normal plasma levels of VIII:Ag, VIII:C, and VIII:CAg.

The patient’s CIE pattern is different from other variant patterns, where increased anodal migration is usually seen with a reduction in the larger multimeric forms of FVIII. We previously described this patient as showing a normal CIE pattern, however, the increased sensitivity of the radio-CIE technique has enabled visualization of the two-peak pattern in samples stored for several years. The samples used in the earlier study were subjected to repeated freeze–thawing and incubation at 37°C. This treatment has now been shown to produce a normal CIE pattern in this patient’s PPP (Fig. 2).

Armitage and Rizza reported a family where several members exhibited a two-peak CIE VIII:Ag pattern with normal FVIII-related functions. Gralnick et al. also described a patient with type II vWD with a
Fig. 6. CIE of FVIII following aggregation of normal washed platelets resuspended in either normal PPP (D–F) or patient PPP (A–C) and incubated with stirring at 37°C with (A and D) NaCl, (B and C) ristocetin, and (C and F) botrocetin.

Fig. 7. (A) Binding of purified $^{125}$I-FVIII to platelets in response to botrocetin is blocked by increased concentrations of VIII:Ag purified from control plasma and variant vWD plasma, A.A. (B) Blocking of purified $^{125}$I-FVIII to platelets in response to 0.5 mg/ml ristocetin by VIII:Ag purified from control plasma and variant vWD plasma, A.A.
two-peak CIE pattern and reduced VIII:vWF. However, these patterns were different in appearance from the one we are reporting, in that the fast-migrating peak was the dominant peak, while for our patient the reverse pattern is seen. It is clear that these patients and the patient described in this report do not fit into the current classification of vWD.

Investigation of the FVIII of this patient demonstrates that there are differences between botrocetin and ristocetin in their interaction with FVIII and platelets. This patient has normal VIII:vWF assayed by botrocetin and undetectable levels when assayed by ristocetin (Fig. 5). Earlier reports\textsuperscript{24,31} postulated that botrocetin mimics ristocetin and may be used as an alternate reagent for VIII:vWF assay; however, clearly these are two alternative probes to study VIII:vWF.

Three lines of evidence indicate that the patient’s FVIII interacts normally with botrocetin: (1) a normal aggregation response of PRP to botrocetin (Fig. 5); (2) the loss of large and intermediate mol wt forms of FVIII after interaction of normal washed platelets, botrocetin, and patient FVIII shown by both CIE (Fig. 6C) and multimeric analysis; and (3) the ability of purified patient FVIII to block subsequent binding of $^{125}$I-normal FVIII to normal platelets in the presence of botrocetin (Fig. 7). On the other hand, a similar argument indicates that the patient’s FVIII fails to interact with ristocetin. No platelet agglutination occurred in response to ristocetin in the presence of patient plasma or purified patient FVIII. Secondly, both the CIE pattern and the multimeric analysis of the patient’s FVIII after ristocetin interaction was indistinguishable from a saline-treated sample (Fig. 6, A and B). Third, purified patient FVIII caused only minimal blocking of subsequent $^{125}$I-FVIII binding to FFP (Fig. 7B). These studies and the multimeric analysis of the patient’s FVIII with only minimal differences from normal FVIII suggest that the abnormality of this FVIII molecule is highly specific for the site of ristocetin interaction. This site on the FVIII molecule and/or the multimeric composition of FVIII are of physiologic importance, as evidenced by the bleeding diathesis of this patient.

The precise abnormality of this patient’s FVIII remains to be elucidated. However, the relative normality of the multimeric profile, the two-peak CIE pattern, and the specific abnormality for the site of ristocetin interaction suggest either of the following hypotheses. The abnormality is a failure of the FVIII subunits to form normal polymers in the intermediate molecular weight region, thereby giving rise to an increased concentration of very low mol wt polymers (those present in the anodal peak). This hypothesis would imply that ristocetin interacts with the intermediate-sized polymers and that such polymers are as important or more important than the large forms of FVIII for ristocetin interaction. Alternatively, the abnormality may be a specific error of the carbohydrate side-chain of the FVIII molecule, since the ability of the patient’s VIII:Ag to bind Con-A is reduced.\textsuperscript{16,18} This could be postulated to result in an abnormality in the interaction of FVIII with the physiologic counterpart of ristocetin and an inability of the FVIII protein to form a normal sequence of polymers.

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