Studies of the Human Factor VIII/von Willebrand Factor Protein

III. QUALITATIVE DEFECTS IN VON WILLEBRAND'S DISEASE

HARVEY R. GRALNICK, BARRY S. COLLER and YVETTE SULTAN

From the Hematology Service, Clinical Pathology Department, Clinical Center, National Institutes of Health, Bethesda, Maryland 20014

ABSTRACT The Factor VIII/von Willebrand factor protein was characterized in two unrelated patients with von Willebrand's disease in whom procoagulant and Factor VIII/von Willebrand factor antigen levels were normal. In both patients evidence of an abnormal protein was observed on crossed antigen-antibody electrophoresis. In one patient the Factor VIII/von Willebrand factor protein eluted from Sepharose 4B in a position and distribution identical to normal with normal levels of procoagulant activity and antigen. However, the partially purified Factor VIII/von Willebrand factor protein had markedly reduced von Willebrand factor activity in a ristocetin assay. In the second patient the peak of Factor VIII/von Willebrand factor protein, antigen, and procoagulant activity eluted from a Sepharose 4B column with an estimated molecular weight of approximately half that of normal. This protein had no von Willebrand factor activity. In both patients the reduced Factor VIII/von Willebrand factor protein subunit was indistinguishable from normal on polyacrylamide gel electrophoresis. These studies indicate that in some patients with von Willebrand's disease there is a qualitative defect of the Factor VIII/von Willebrand factor protein; the total amount of protein, antigen, and procoagulant activity are normal while the von Willebrand factor activity is deficient.

INTRODUCTION

The human Factor VIII/von Willebrand (F.VIII/vWF)[1] protein in normals and hemophilia A has been characterized as a high molecular weight glycoprotein with a major subunit of 195,000–240,000 mol wt (1–6). In severe von Willebrand's disease (vWD), the deficiency of procoagulant and vWF activity (as judged by the ristocetin assay) is associated with a marked deficiency or absence of the F.VIII/vWF protein (7–8).

Immunologic studies of hemophilia A plasma or plasma concentrates have revealed a normal or elevated level of antigen despite the severe deficiency of procoagulant activity. In vWD there is usually a good correlation between the procoagulant F. VIII, antigen levels, and the vWF activity as judged by ristocetin-induced platelet aggregation (7). To account for these observations it has been suggested that there is a quantitative reduction of a normal protein in von Willebrand's disease while there is a normal amount of an abnormal protein in hemophilia A.

Recently four different vWD families have been described in whom some individuals were found to have normal procoagulant F. VIII and antigen levels but defective vWF activity (9–12). These findings were interpreted as being suggestive of a qualitative rather than a quantitative defect in the F.VIII/vWF protein. To assess this we investigated the F.VIII/vWF protein in two vWD patients from two different families who demonstrated normal levels of F.VIII/vWF antigen and procoagulant activity but deficient vWF activity.

Dr. Sultan's address is: Laboratoire d'Hemostase, Institut de Recherche sur les Maladies du Sang (Pr. Jean Bernard), Hôpital Saint-Louis, University of Paris, Paris, France.

Received for publication 28 January 1975 and in revised form 11 June 1975.

[1] *Abbreviations used in this paper:* F. VIII/vWF, Factor VIII/von Willebrand factor, the partially purified material from normal cryoprecipitate having the biologic properties of (*a*) correcting the coagulation defect in hemophilic plasma and (*b*) correcting the defect in platelet retention in a glass bead column and interacting with ristocetin to induce aggregation of von Willebrand's disease platelets or normal platelets made unresponsive to ristocetin; K_{av}, distribution coefficient, (elution volume of sample $- V_o)/(V_t - V_o)$; vWD, von Willebrand's disease; vWF, von Willebrand's factor.

TABLE I

Laboratory Data on Two vWF Patients, Their Families, and 50 Normal Subjects

Pedigree SEX	Bleeding time	Platelet retention	F.VIII procoagulant activity	F.VIII related antigen	vWF	Crossed antigen-antibody electrophoresis
	min	%	%	%	%	
vW I						
Parents						
I_1 M‡	6	53(S)	108	ND	ND	ND
I_2 F prop	16–20	10–15(B)	90	72–80	10–15	Abnormal
Children						
I_3 F	9	ND	115–136	120	100	Normal
I_4 M	7	31(S)	150	140	126	Normal
I_5 M	9–15	17(B)	130	100	42	Abnormal
I_6	>30	8(B)	9	0	0	No arc
I_7 M	20	16(B)	40	10	0	No arc
I_8 M	11	94(B)	110	85	41	Abnormal
I_9 F	>30	3(B)	10	0	0	No arc
I_{10} F	8	95(B)	115	115	57	Abnormal
I_{11} F	>30	12(B)	16	0	0	No arc
vW II						
Parents						
II_1 M	4	94(B)	125	112	50	Abnormal
II_2 F	7	86(B)	140	195	175	Normal
Child						
II_3 F prop	>30	10(B)	74–120	70–90	<5	Abnormal
Normal	<10	S, >25 B, >65	52–180	45–150*	56–120	

B, Bowie technique; ND, not done; prop, proposita; S, Salzman technique.

* Electroimmunoassay technique.

‡ Decreased.

METHODS

Patients. The normal individuals were volunteer blood donors. The patients with severe vWD had abnormal platelet retention upon passage of blood through a glass bead column, prolonged bleeding times, and low procoagulant F. VIII levels during several years of observation (7). Patient vW I_2 (a woman) has had occasional episodes of spontaneous or traumatic hemorrhage, while patient vW II_3 (a woman) has had a severe bleeding diathesis (see Table I). Procoagulant factor VIII level, antigen level, platelet retention, ristocetin-induced platelet aggregation, and bleeding time were tested on each vWD patient and on 50 normals. None of the patients in this study had received plasma or plasma concentrates for 3 mo before the studies performed on their cryoprecipitate or plasma. Patient vW II_3 received a preoperative infusion of cryoprecipitate (20 U procoagulant F. VIII/kg). This was a therapeutic infusion, and the patient was informed about the risk of hepatitis. The plasmas from which the infusion material was prepared were tested for hepatitis-associated antigen and were found to be negative. Neither patient was receiving any medications, especially oral contraceptives or drugs that might affect platelet function. Likewise, they were not under any stress such as pregnancy or central nervous system stimulation, or in any anxiety or hyperactive state.

Cryoprecipitate and plasma. Cryoprecipitate was prepared from the plasma obtained from a 1-U plasmapheresis performed on 15 normal individuals, three patients with severe vWD, and the two vWD patients (vW I and vW II) under study. The blood was collected in plastic bags containing 1/9 vol 4% sodium citrate (Fenwal Incorporated, Ashland, Mass.), and after centrifugation at 5,000 *g* at 4°C for 15 min, the plasma was removed and respun. The cell-free plasma (240±20 ml/plasmapheresis U) was immediately froze at −30°C. The cryoprecipitate was prepared in the smallest volume possible by thawing the plasma at 4°C overnight and centrifuging at 5,000 *g* for 15 min at 4°C. The supernatant material was decanted, and then the cryoprecipitate was warmed to 37°C. The volume was measured and brought to a total of 10 ml by the addition of supernatant plasma. The cryoprecipitate was prepared and used fresh for the following experiments. Both the cryoprecipitate and supernatant plasma were tested for antigen content. Hyland Method IV (a F. VIII concentrate prepared for human cryoprecipitate by polyethylene glycol precipitation of fibrinogen followed by glycine precipitation, Hyland Div., Travenol Laboratories, Inc., Costa Mesa, Calif.) was prepared and reconstituted as previously described (5).

Whole blood (30–40 ml) was collected in polypropylene tubes in 1/100 vol 40% sodium citrate. Cell-free plasma

was obtained and stored as above. In experiments with plasma, 10-ml portions were thawed at 37°C and used immediately for gel filtration experiments.

Alpha-chymotrypsin digestion of cryoprecipitate. Alpha-chymotrypsin (Worthington Biochemical Corp., Freehold, N. J.) from bovine pancreas was dissolved in 0.001 M hydrochloric acid at concentrations of 10 mg/ml just before use. The cryoprecipitate was digested at 24°C. Digestion of 9.6 ml of cryoprecipitate was initiated with 0.250 ml of chymotrypsin (final concentration 253 μg/ml of cryoprecipitate), and then further 0.03-ml additions were made (final concentration 30 μg/ml of cryoprecipitate) at 15–30-min intervals until digestion was completed as determined by prolongation of the thrombin time to more than 300 s. The digest was then immediately placed at 4°C and centrifuged twice at 27,000 *g*, and the supernate was applied to the column. Digestion of cryoprecipitate was always completed within 180–210 min. Samples of the cryoprecipitate digestion mixture were taken to monitor thrombin times, predigestion F. VIII, antigen, and vWF activity. At the end of the digestion samples were again taken for antigen, vWF activity, and F VIII levels. This reduced the volume by approximately 1.1–1.5 ml. When digestion was complete, the mixture was spun at 27,000 *g* at 4°C. Removal of the supernate from the small precipitate present resulted in the loss of approximately 0.4–0.7 ml. Thus, 8.0–8.5 ml of cryoprecipitate were actually applied to the column.

Procoagulant F. VIII and thrombin time assays. All coagulation tests were performed on a fibrometer (Bio-Quest Div., Becton, Dickinson & Co., Cockeysville, Md.). All column fractions were assayed undiluted for procoagulant F. VIII. All F. VIII determinations were measured by a one-stage assay (partial thromboplastin time) with hemophilic plasma (< 1% F. VIII activity) as substrate and celite as an activator (13). Units of procoagulant F. VIII activity were calculated by comparison with the amount contained in 1 ml of lyophilized plasma (AHG-PTC-PTA Reference Plasma, Hyland Div., Travenol Laboratories). Thrombin clotting times to monitor the alpha-chymotrypsin digestion were measured by adding 0.1 ml of the digestion mixture of undigested cryoprecipitate to 0.2 ml (2.5 U.S. U/ml) bovine thrombin solution (Parke, Davis & Company, Detroit, Mich.) at 37°C and measuring the time for clotting to occur. The thrombin time on undigested cryoprecipitate varied between 24 and 27 s.

Gel filtration. Gel filtration of the digested cryoprecipitate or intact plasma was performed on a 2.5 × 40-cm Pharmacia column packed with Sepharose 4B (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden). The void volume was determined with dextran blue 2000 (Pharmacia Fine Chemicals), fetal calf thymus DNA (Sigma Chemical Co., St. Louis, Mo.) and lipolysaccharide from *Salmonella typhosa* 0901 (Difco Laboratories, Detroit, Mich.). Columns were eluted at 4°C at a pressure of approximately 20 cm H_2O with buffer containing 0.05 M Tris (Schwarz/Mann ultra-pure) and 0.1 M sodium chloride adjusted with hydrochloric acid to pH 7.35. The flow rate of the column averaged 14 ml/h, and 1.6–1.8 ml fractions were collected. Protein was monitored by absorbance at 280 nm. Protein concentration was measured by the method of Lowry et al. (14). The Sepharose 4B column was calibrated with the following marker proteins: lipopolysaccharide of *S. typhosa,* (Difco Laboratories) human fibrinogen, (> 94% clottable protein) porcine thyroglobulin, (Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.) horse apoferritin, (Schwarz/Mann) human gamma globulin, (Schwarz/Mann) and human transferrin (Schwarz/Mann).

The void volume (V_0) of the column was 48 ml. Identical fractions were collected and concentrated from all column eluates: fraction 1, 48–61 ml; fraction 2, 62–75 ml; fraction 3, 76–89 ml; fraction 4, 90–103 ml; and fraction 5, 104–117 ml.

The pooled column fractions or in some instances individual column fractions were tested by polyacrylamide gel electrophoresis, ristocetin-induced platelet aggregation assay, and counterimmunoelectrophoresis. When necessary, normal column fractions were concentrated to 25–50% of the original volume by dialysis against powdered Ficoll (Pharmacia Fine Chemicals, Inc.) at 4°C. The column fractions from the severe vWD patients were concentrated to 10–25% of the original volume. All column fractions were tested for vWF activity the same day or frozen at −30°C and tested within 7 days.

Preparation of antibody to F. VIII. Antibody was developed in New Zealand rabbits and Nubian goats to purified F. VIII/vWF prepared from Hyland Method IV. (5) Crystallized rabbit albumin (at a final concentration of 5 mg/ml; Miles Laboratories, Kankakee, Ill.) was added to the V_0 fractions before concentration to stabilize the procoagulant activity. The rabbits were immunized at weekly intervals by three subcutaneous injections of purified protein (0.86–1.0 mg/injection), suspended in incomplete Freund's adjuvant (Difco Laboratories) and then they received three booster immunizations 1 mo apart. The goats were immunized with three weekly intramuscular injections (average of 2 mg/dose) of purified F. VIII/vWF without adjuvant. They then received booster immunizations once a month for 4 mo. The rabbits were bled by cardiac puncture, and the goats were bled by jugular venipuncture.

Blood from the animals was allowed to clot at 37°C for 4 h. The serum was separated by centrifugation at 5,000 *g* for 20 min and then heated to 56°C for 30 min. The heated serum was respun at 5,000 *g* for 30 min, and the supernatant serum was adsorbed with either (*a*) an 8% ethanol precipitate from 50 ml of plasma (per 10 ml of antiserum), from which a 3% ethanol precipitate had been removed and the crystallized rabbit albumin at 5 mg/ml antiserum; or (*b*) just the supernatant plasma from the cryoprecipitate of a patient with severe vWD (less than 3% F VIII activity) at a ratio of 9 parts antiserum to 1 part vWD supernatant plasma. The adsorbed antiserum was monospecific when tested in immunodiffusion, immunoelectrophoresis, or counterimmunoelectrophoresis against normal plasma, hemophilia A plasma, or their cryoprecipitates. A single precipitin arc was seen with the above-mentioned materials, while similar von Willebrand materials were unreactive. The only coagulation activities reduced by the antiserum were procoagulant F. VIII and the vWF.

Immunologic techniques. The electroimmunoassay of Laurell (15) was performed on plasma according to the technique of Zimmerman et al. (16). Pooled normal plasma (stored at −30°C) was used as the standard control.

Crossed antigen-antibody electrophoresis was performed on plasma according to the method of Laurell (17). The glass slides (3¼ by 4¼ in) were covered with 15 ml of 1% agarose in the Laurell buffer (sodium barbital 65 mM, barbital 11 mM, and calcium lactate 2 mM, pH 8.6). 10 μl of antigen were placed in a 4-mm-diameter well, and electrophoresis was carried out at 4°C for 5 h at 350 V (10–20 mA). After electrophoresis in the first dimension

and removal of the excess gel, 12 ml of 1% agarose containing either rabbit or goat anti-human F. VIII antibody (5–10 μl) were added to the remainder of the plate. After the gel had settled, electrophoresis was performed for 17–20 h in the second dimension under the same conditions. During each electrophoresis of patient plasma, a normal plasma was also tested to serve as a control. In some experiments one part of patient plasma was diluted with one part of pooled normal plasma. This mixture was then subjected to electrophoresis as above. After electrophoresis, the slides were washed, dried, and stained with Coomassie blue.

Counterimmunoelectrophoresis was performed on lantern slides 3¼ by 4 in precoated with 1 ml of melted 1% agarose in barbital buffer 0.025 M, pH 8.6. 16 ml of 1% agarose dissolved in the same buffer were then applied to the plate. Antigen wells were cut with a number 3 cork bore to contain 0.04 ml of material. Antibody wells were cut to contain 0.006 ml. The antigen and antibody wells were separated by 3 mm. After the application of antigen and antibody (goat or rabbit anti-human F. VIII/vWF), constant current (30 mA) was supplied for 90 min at 4°C with a 50 mM barbital buffer in the troughs. After electrophoresis, the plate was read immediately, washed in 0.85% sodium chloride for 48 h and in distilled water for 24 h, dried, and stained with 0.5% Amido-schwartz and then read again. Relative quantitation of antigen was obtained by recording the reactivity of dilutions of the test material (made in 50 mM barbital buffer). Samples of plasma pools from 30 normal blood donors stored at −30°C were sporadically tested in the assay; however, a pool of lyophilized plasma (AHF-PTC-PTA Reference Plasma, Hyland Div., Travenol Laboratories) and plasma from a patient with severe vWD (procoagulant F. VIII—3%, antigen undetectable) were used as controls in all of the studies. The lyophilized plasma used during this study was lot No. 3403V004A1, procoagulant F. VIII (1.06 U/ml). All hemophilic and vWD column fractions were analyzed with comparable normal material. Specific antigenic activity (antigen per unit protein) was defined as the highest reaction dilution of a column fraction divided by that fraction's absorbance at 280 nm.

The Laurell technique was used for measuring the F. VIII/vWF antigen in all of the studies of the families. In addition, in the propositae of both families and in the father of vW II_A, the counterimmunoelectrophoresis technique was used. Antigen measurements of all of the cryoprecipitate and cryoprecipitate column fractions were measured by the counterimmunoelectrophoresis technique.

Acrylamide gel electrophoresis. Acrylamide gel electrophoresis was performed with the reagents and methods described by Canalco, Inc., Rockville, Md. Electrophoresis was performed in 5% (5.13 *T*, the concentration of acrylamide and bisacrylamide, 2.5% *C*, the percentage cross-linking) acrylamide gels containing 0.1% sodium dodecyl sulfate (Pierce Chemical Co., Rockford, Ill.) according to Shapiro et al. (18), as previously described (5). Portions of the column pools were incubated for 3 h at 37°C in 1% SDS–0.1 M sodium phosphate buffer, pH 7.1, containing 0.025% sodium azide with or without the addition of the reducing agent dithioerythritol at a final concentration of 5 mM (Mann Research Labs, Inc., New York). 10–35 μg of protein from fraction 1 and 2 of vW I and the normal cryoprecipitate and fraction 2 of vW II were applied to the gel. 50–100 μg (in 100 μl) of protein from fraction 1 of vW II were applied to the gel. The maximum amount of protein applied to the gel from any fraction was 100 μg in 100 μl. Proteins used as standards were those previously described (5) and in addition the subunit of rabbit muscle myosin (mol wt, 194,000). The rabbit muscle myosin was a gift of Drs. Judith Andersen and Patrick McKee, Durham, N. C.

Platelet retention. Platelet retention studies were performed by the method of Bowie et al. (19) and by the method of Salzman (20). Results were expressed as the percentage of platelet retained in the column.

Platelet aggregation with ristocetin. Platelet aggregation studies on citrated (1/100 vol 40% citrate) platelet-rich plasma (PRP) adjusted to 300,000 platelets/mm^3 were performed in a Chrono-log aggregometer (Chrono-Log Corp., Broomall, Pa.) as originally described by Born and Cross (21). Ristocetin was supplied by Abbott Laboratories, North Chicago, Ill. In the assay of the patients' PRP response to ristocetin, 0.4 ml of PRP was allowed to warm to 37°C for 30 s, and then 0.05 ml of ristocetin was added (final concentration 1.5 mg/ml). Plasma and column fractions were tested for their vWF activity (i.e., the ability to support ristocetin-induced platelet aggregation) in one of two ways: (*a*) A 50-μl portion of the test material was added to 0.3 ml of PRP from a patient with severe vWD (who had no response to ristocetin at a final concentration of 1.8 mg/ml), and then 15 μl of ristocetin (50 mg/ml dissolved in 0.15 M NaCl, 0.01 M barbital, pH 7.4) were added. (*b*) Normal platelets were treated in one of two ways (see below) so that they no longer aggregated in response to ristocetin at the dose tested. This assay consisted of the addition of a 50-μl portion of the test material to 0.4 ml of the washed platelets and, after a 1-min incubation, the addition of 10–20 μl of ristocetin (50 mg/ml).

Normal platelets were made unresponsive to ristocetin by washing or by gel filtration followed by washing. In the first instance, washing was performed by the technique of Zimmerman.[2] 50 ml of fresh whole blood were collected in 1/10 volume of acid-citrate dextrose and centrifuged at 1,800 *g* at 25°C for 20 min. The platelet-poor plasma was removed and measured and replaced with an equal volume of Tyrode's buffer modified to contain 2% bovine serum albumin (Pentex, Sigma Chemical Co.) and 0.03 M adenosine (Sigma Chemical Co.). The cells and platelets were gently resuspended and centrifuged again. This procedure was repeated four times (1,800 *g* at 25°C for 20 min) with the last centrifugation performed at 250 *g* at 25°C for 15 min, and the platelet-rich buffer supernate was removed. A final centrifugation at 1,100 *g* for 1 min reduced the red blood cell contamination. Platelet-rich buffer adjusted to 300,000/mm^3 was kept at room temperature and used immediately. The assay procedure consisted of adding 50 μl of test material to 0.4 ml of the platelet-rich buffer and then adding 20 μl (50 mg/ml) of ristocetin (final concentration 2.1 mg/ml).

The other method for prepared ristocetin-unresponsive platelets was modified from the technique of Olson et al. (22). 1- and 2-day-old platelet concentrates from the NIH Clinical Center Blood Bank (prepared in acid citrate dextrose) were centrifuged at 1,200 *g* for 3 min to decrease red blood cell contamination. The platelets were then gel-filtered on a 2.5 × 30-cm siliconized glass column packed to a height of 20 cm with Sepharose 2B, as described by Tangen and Berman (23). Treatment of the gel, packing the column, gel filtration, eluant flow (1 ml/min), and buffer (without albumin) were as described (23). The

[2] Zimmerman, T. Personal communication.

gel-filtered platelet fractions with platelet counts greater than 300,000/mm³ were pooled, and the volume was brought to 50 ml with buffer, adjusted to 3 mM Na_2 EDTA, and then centrifuged at 2,500 *g* for 10 min. The platelet button was then resuspended in the original Tangen buffer (without albumin) to a final concentration of 300,000 platelets/mm³. The assay procedure consisted of incubating 0.4 ml of washed GFP with 50 μl of test material for 1 min and then adding 10 μl of a 50 mg/ml solution of ristocetin (final concentration 1.1 mg/ml).

Mixing experiments were performed to determine if fractions 1 and 2 of vW I and vW II inhibited ristocetin: induced platelet aggregation. Controls (lyophilized normal human plasma and fraction 1 from normal cryoprecipitate) were tested undiluted, diluted 1:1 with buffer, or diluted 1:1 with fractions 1 and 2 of vW I and vW II. One part of normal plasma (lyophilized) and one part of either vW I_3 or vW II_3 plasmas were mixed and incubated at 37°C for 15 min and then tested for vWF activity. Expected values were calculated as the average of the components and compared with the observed value. If the difference between expected and observed values was greater than 15%, this was interpreted as evidence of inhibition or augmentation.

To quantitate the vWF, the initial slope of aggregation for lyophilized reference plasma (Hyland AHF-PTC-control) and various dilutions of this material (1/1–1/28) were determined. A standard curve was prepared by plotting the initial slope versus the percentage of plasma in the sample on semilogarithmic graph paper. The standard curve was determined at the beginning and at the end of each group of assays, and results were averaged. It was observed that the washed platelets spontaneously regained their aggregation response to ristocetin after standing at room temperature for several hours. Periodic controls (washed platelets and ristocetin) were run throughout the assay procedure, and the test series was terminated if aggregation was observed. The initial slopes produced by the unknown test materials were converted into percentages of those obtained for the lyophilized normal plasma by reference to the standard curve. All test materials were diluted so that they fell near the middle of the standard curve. The sensitivity of the assay varied between less than 3% and less than 6% of the normal lyophilized plasma added to the mixture. Plasma from 50 voluntary blood donors had a mean of 88±18% vWF activity compared to the lyophilized plasma. Two frozen pools of normal plasma had 84% and 87% vWF compared to the lyophilized plasma. No sample was assigned a value below the lower limits of the assay sensitivity. The vWF activity was expressed as percent of normal plasma and specific vWF activity as percent of normal plasma per 0.1 OD_{280}.

RESULTS

Patients. The laboratory data on the two patients (vW I_2 and vW II_3) and their family members, along with the results on 50 normals, are presented in Table I. Patient vW I_2, an orphan, is a woman with a mild bleeding disorder studied at Hôpital Saint-Louis, Paris, France. She has a prolonged bleeding time (20 min), abnormal platelet retention, and deficient vWF (10% and 12% respectively); however, her F. VIII level varies between 90–100%, and her F. VIII-related antigen varies between 72–100%. She has borne 11 children (2 are dead). Of the 9 living children, 2 are normal (I_3 and I_4). 4 children (I_6, I_7, I_9, I_{11}) have severe bleeding disorders with marked procoagulant and F.VIII/vWF antigen deficiencies, long bleeding times, and reduced platelet retention and ristocetin-induced platelet aggregation. 3 children (I_5, I_8, I_{10}) have mild reductions of their vWF activity and abnormal crossed antigen-antibody electrophoresis while their procoagulant and antigen levels are normal. The husband (I_1) of the proposita, now deceased, had normal bleeding time, platelet retention, and procoagulant F. VIII activity. He was asymptomatic.

Patient vW II_3 has a history of excess menstrual and post-traumatic bleeding. Her mother and four siblings are asymptomatic, but her father has a history of excessive bleeding after trauma. As he had grown older, this bleeding diathesis has become more benign. This (vW II_3) patient is childless. Coagulation studies (including ristocetin aggregation, F.VIII/vWF antigen and crossed antigen-antibody electrophoresis) of the mother and two sisters are normal, while the father has a borderline low vWF and an abnormal crossed antigen-antibody electrophoresis pattern (similar to vW II_3). She has been cared for at the Hôpital Saint-Louis, Paris, France. Her laboratory data includes 74–120% F. VIII procoagulant activity, 70–90% antigen, 5% vWF, and 10% platelet retention. Platelet aggregation to ADP, epinephrine, and collagen were normal, as was platelet factor-3 availability. Her response to a therapeutic infusion of cryoprecipitate (20 U of F. VIII procoagulant activity/kg) given preoperatively is depicted in

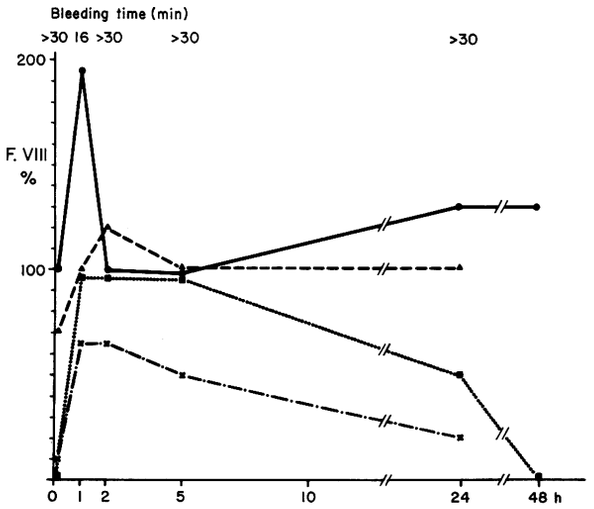

FIGURE 1 Cryoprecipitate infusion in vW II_3. Cryoprecipitate (20 U/kg) was infused into patient vW II_3. F. VIII procoagulant activity (●——●), F. VIII-related antigen (F. VIII/vWF) (▲---▲), platelet retention to glass bead column (×-·-×), ristocetin-induced aggregation (■·····■), and bleeding times were measured serially over 48 h.

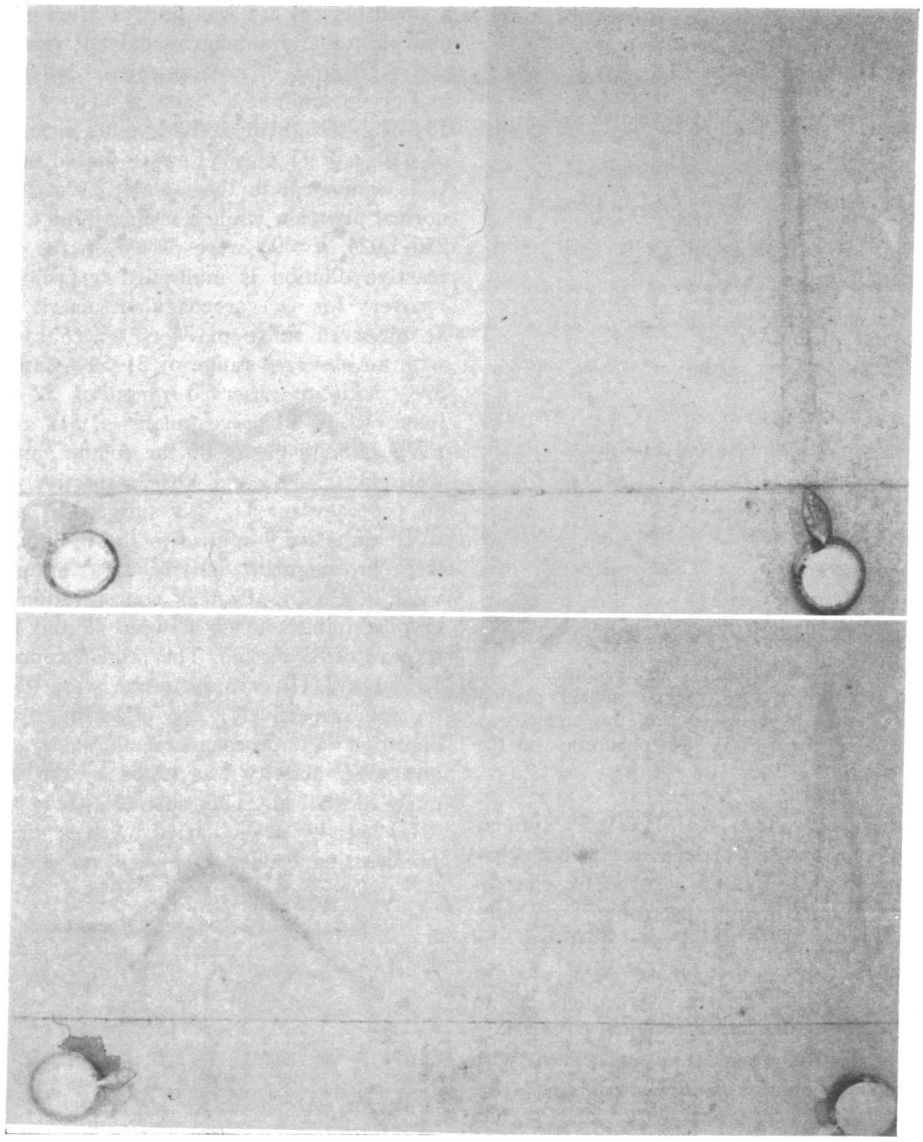

FIGURE 2 Crossed antigen-antibody electrophoresis of plasma from vW I_2 (top) and normal pooled plasma (bottom). During the first electrophoretic run, the anode was to the right; and during the second electrophoretic run, the anode was at the top. At the far right are the electroimmunoassays of normal and vW I_2 plasma. Details of the electrophoresis are described in Methods.

Fig. 1. There was an immediate rise (1 h) in F. VIII procoagulant activity, followed at 24–48 h by a more gradual rise in procoagulant activity. Within the first 2 h there was shortening of the bleeding time and complete correction of both the platelet retention (adhesiveness) and ristocetin-induced platelet aggregation. The platelet retention and ristocetin-induced platelet aggregation remained normal for 5 h and then returned to abnormal values. The procoagulant F. VIII returned to the base-line values at 1 h and then at 24 h rose 30%. The antigen levels peaked at 2 h and then returned to a level 40% above baseline at 5 h, where it remained until the end of the study.

Washed platelets from both patients demonstrated normal ristocetin aggregation when incubated with either normal plasma or column fraction 1 from normal cryoprecipitate. The two patients and selected family members demonstrated normal platelet aggregation with collagen, ADP, and epinephrine and PF-3 availability.

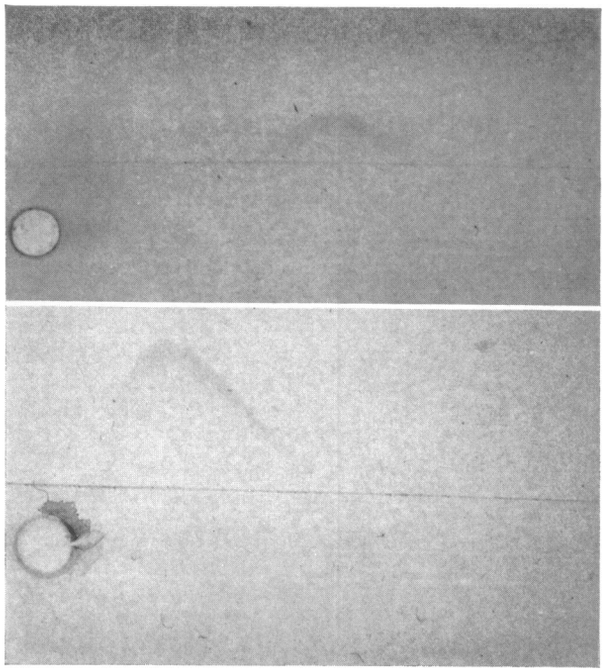

FIGURE 3 Crossed antigen-antibody electrophoresis of plasma from vW II_3 (top) and normal pooled plasma (bottom). In the first electrophoretic run, the anode was to the right; and during the second electrophoretic run the anode was at the top.

Immunologic studies. The F.VIII/vWF antigen level was normal on multiple occasions in both patients' plasma and cryoprecipitate, assayed by electroimmunoassay and counterimmunoelectrophoresis techniques. There was excellent agreement between the two assay techniques.

Although the precipitin patterns were identical to normal in the electroimmunoassay, unique patterns in crossed antibody electrophoresis were observed with both patients' plasma. In the crossed electrophoresis the precipitin arc in vW I_3 was composed of two distinct peaks, the more cathodal one appearing identical with the single peak seen with 10 individual normal plasmas and a pool of 30 normal plasmas (Fig. 2). Children I_5, I_8, and I_{10} had similar migration and precipitin arcs. A broad precipitin arc was observed with vW II_3's plasma, marked by much greater anodal migration than normal (Fig. 3). A similar pattern was observed with vW II_3's father (vW II_2), while the two sisters and mother of vW II (vW II_1) had normal crossed antibody electrophoresis. Two asymptomatic brothers were unavailable for testing.

A mixture of patient and normal plasma was examined with both patients. In patient vW I_2 this resulted in augmentation of the more cathodal portion of her precipitin arc while the more anodal portion of the precipitin arc remained unchanged. In patient vW II_3 a small normal arc was present after mixing with normal plasma and a long anodal tail remained as before.

Gel filtration of cryoprecipitate and plasma. In normal cryoprecipitate a mean of 43 U (range 20–75, $n = 15$) of procoagulant activity and a mean of 99 U (range 65–120, $n = 9$) of vWF were placed on the column (1 U is equivalent to the amount of material in 1 ml of normal plasma), while a mean of 768 antigen U (range 256–1,024, $n = 9$) were placed on the column (highest reactive dilution is multiplied by volume). The mean recovery for (*a*) procoagulant activity was 49% with an observed range of 34–63%; (*b*) for antigen 42% with an observed range of 31–50%; and (*c*) for vWF 39% with an observed range of 32–44%. With patient vW I_2, 50 procoagulant U, 512 antigen U, and 8 vWF U were placed on the column, and the recoveries were 45%, 44%, and 43%, respectively. With vW II_3, 36 procoagulant U, 282 antigen U, and less than 6 vWF units were applied to the column. The recoveries were procoagulant activity 41%, antigen 47%, and 0 vWF (<6%). Protein concentration of the normal cryoprecipitate varied between 75 and 102 mg/ml, with a mean of 85 mg/ml. The protein concentration of vW I_3 and vW II_3 cryoprecipitate were 95 mg/ml and 85 mg/ml, respectively. The effect of alpha-chymotrypsin digestion on procoagulant F.VIII activity, antigen, and vWF activity was tested by the techniques previously described. The post-digestion procoagulant F.VIII activity never varied by more than ±11% of the predigestion value. Likewise, no appreciable loss or

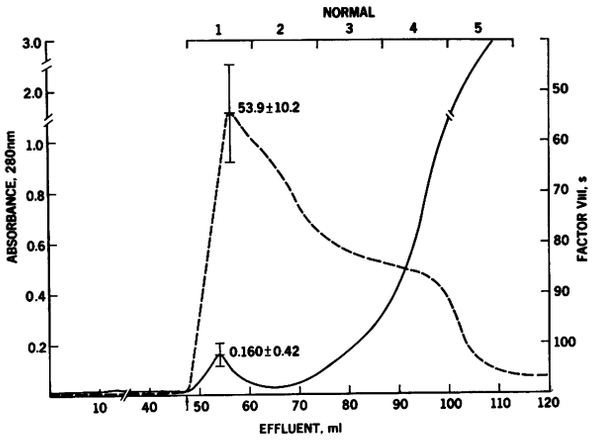

FIGURE 4 Chromatograph of normal cryoprecipitate on Sepharose 4B. Plasma from 240±20 ml were frozen and thawed to form cryoprecipitate. The cryoprecipitate was digested with alpha-chymotrypsin and placed on the column. The dashed line represents F. VIII activity and the solid line absorbance at 280 nm. The arrow represents the void volume. The bars over the protein and procoagulant activity reflect the mean±SD. The brackets at the top of the figure indicate the fractions 1–5, pooled for further testing (see Methods).

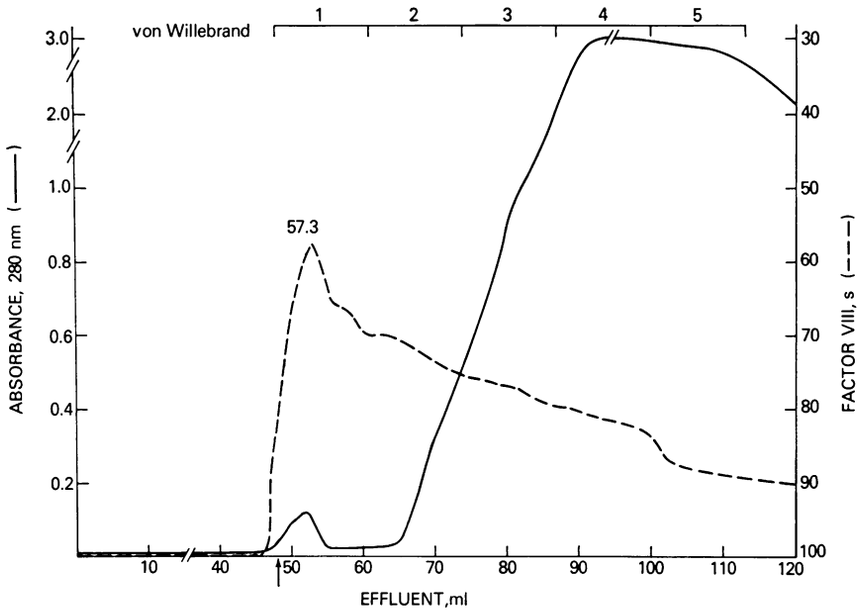

FIGURE 5 Chromatograph of cryoprecipitate from vW I_2. The preparation and methods are identical to those described in Fig. 4 and Methods.

gain of vWF or antigen activity was noted with alpha-chymotrypsin digestion of cryoprecipitate.

The protein and procoagulant elution patterns of normal cryoprecipitate have been previously described (2–6). A low level of protein associated with the bulk of the procoagulant activity begins to elute at the void volume (Fig. 4). The peak procoagulant activity and protein appeared 6.0±1.0 ml and 5.0±1.0 ml, respectively, after the void volume (mean±SD, $n = 15$). The peak procoagulant activity resulted in a F. VIII clotting time of 53.9±10.2 s (mean±SD, observed range 33.9–73.3 s or 10–41% of pooled normal plasma, $n = 15$).

The procoagulant F.VIII and protein elution patterns of cryoprecipitate from vW I_2 were the same as normal (Fig. 5). However, vW II_3's elution pattern differed from normal in several respects. A reduced amount of both protein and procoagulant activity eluted at the void volume, with a broad, slowly rising plateau of protein (with no real peak) eluting over the first 25 ml (Fig. 6). In parallel with the slow rise of protein, the F. VIII level also rose slowly to a peak of 64.1 s. While this peak activity was in the normal range, it eluted at 18 ml after the void volume (rather than at 6.0±1.0 ml, seen with normals), giving it an estimated mol wt of 840,000 (K_{av} 0.23; K_{av} = distribution coefficient) rather than the estimated 1,600,000 (K_{av} 0.04) found in normals.

Normal, vW I_2, and vW II_3 plasmas (10 ml) were each chromatographed on the same Sepharose 4B column as the cryoprecipitate. The patterns of procoagulant and antigen elution of these plasmas were identical to those found with their respective cryoprecipitates. The amounts of procoagulant and antigen in the plasma column fractions were reduced when compared to the cryoprecipitates in proportion to the reduced amount placed on the column. Thus, unaltered plasma and alpha-chymotrypsin-digested cryoprecipitate had similar elution patterns for protein, procoagulant activity, and antigen for both patients vW I and vW II.

Polyacrylamide gel electrophoresis. Fractions 1 and 2 from normal cryoprecipitate had a protein that did not enter a 5% gel, but after reduction, a subunit of approximately 235,000 mol wt was observed (Fig. 7). The polyacrylamide gel patterns of fractions 1 and 2 in vW I_2 were the same as normal (Fig. 8). However, vW II_3 had a markedly different polyacrylamide gel pattern (Fig. 9). With fraction 1 no protein was present at the top of the gel, and no subunit of any size appeared after reduction. A large amount of protein was, however, observed at the top of the gel with fraction 2, which yielded an apparently normal subunit upon reduction. Fraction 3 appeared to have a very small amount of the subunit present.

Immunologic investigation of the column fractions by counterimmunoelectrophoresis. The reproducibility of counterimmunoelectrophoresis was tested by the use of lyophilized normal plasma pool and a severe von Willebrand's plasma. These were included in all counter-immunoelectrophoresis assays. When column fraction 1 of known protein content from Hyland IV was used as antigen, the sensitivity of the assay was 1.5–1.0 μg/

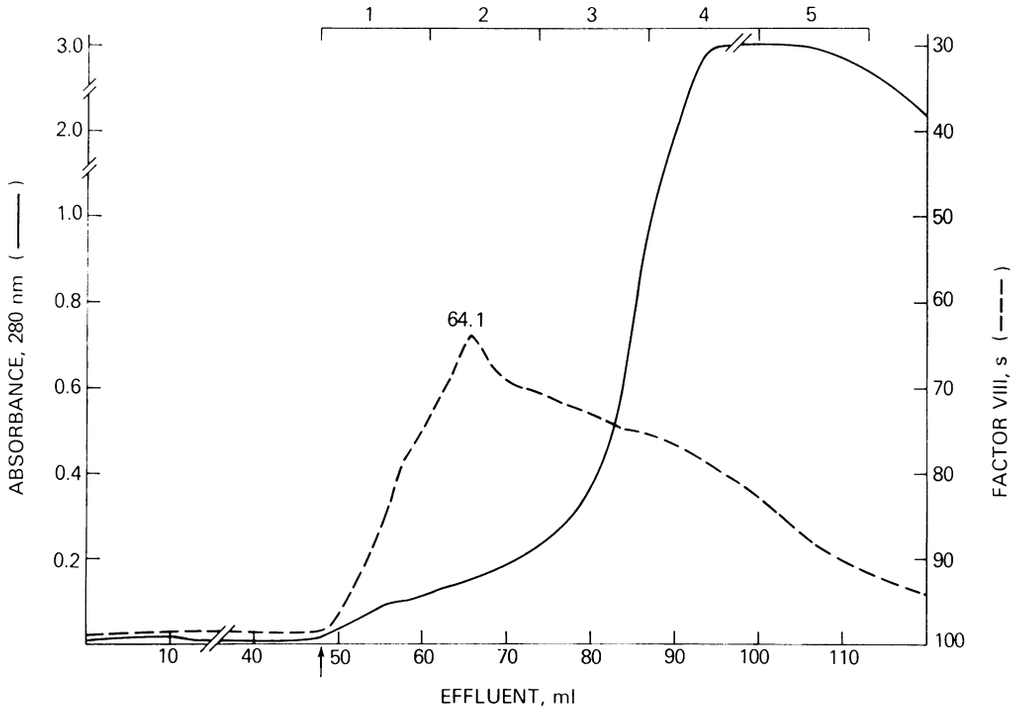

FIGURE 6 Chromatograph of cryoprecipitate from vW II_3. The preparation and methods are identical to those described in Fig. 4 and Methods.

ml. The primary standard for this assay was the pooled lyophilized normal plasma. The range for the pooled, frozen normal plasma was 1/32, 1/16, and 1/16, with three different normal pools, while the highest dilution reactivity with the lyophilized pool (observed throughout the study) was 1/32 in 19/24 assays, 1/16 in 5/24 assays. The vWD plasma was always unreactive. When vW I and vW II cryoprecipitate fractions were tested on two separate occasions, the highest reactive dilutions were identical on each electrophoresis. The endpoint

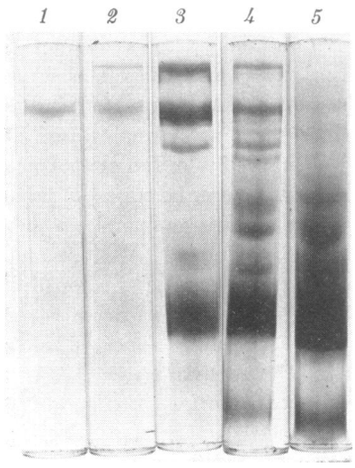

FIGURE 7 Polyacrylamide gel electrophoresis of normal column fractions 1–5. The column fractions were reduced with diethylerythritol and electrophoresis was performed in the presence of sodium dodecyl sulfate. Note the subunit in fraction 1 and to a lesser degree in fraction 2, and not clearly seen in any other fractions.

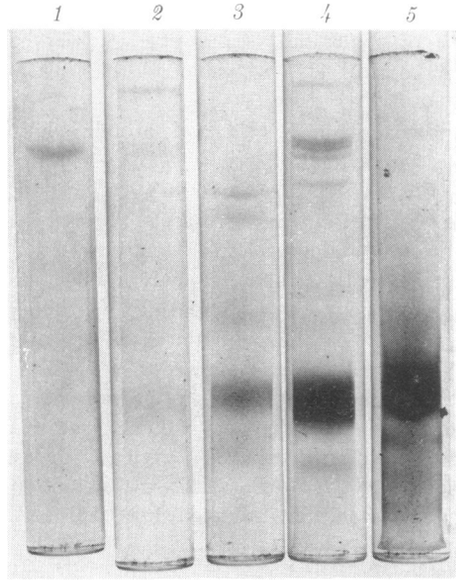

FIGURE 8 The polyacrylamide gel electrophoresis patterns of column fractions from vW I_2.

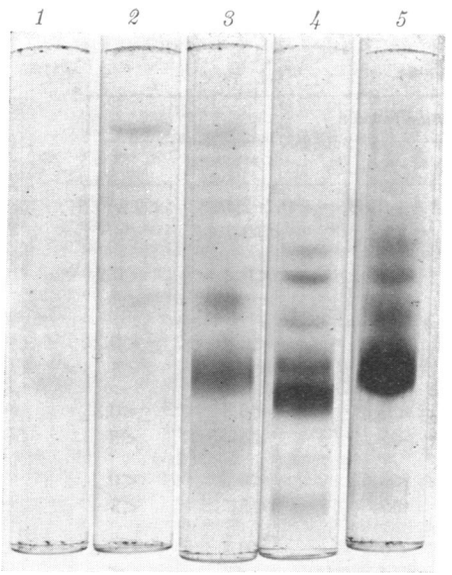

FIGURE 9 Polyacrylamide gel electrophoresis patterns of cryoprecipitate column fractions from vW II_3. Note that in fraction 1 there is no subunit; however, in fraction 2 a large amount of subunit is present, with a lesser amount in fraction 3. Details of electrophoresis are described in Methods and Ref. 5.

was a clear precipitin arc, and there was no difficulty in determining negative and positive dilutions.

Patient vW I_2's specific antigenic activity pattern and antigen content were indistinguishable from normal (Table II). The peak specific activity was in the first fraction, with fraction 2 containing approximately 30% of that present in fraction 1. A small amount of antigen was present in fraction 3, but none was detectable in the other column fractions. Patient vW II_3, however, had only a very small amount of antigen in fraction 1 while fraction 2 contained 18.4 antigen U (4.0 SD above the normal mean). Fraction 3 contained 1.2 antigen U while fraction 4 and 5 contained no detectable antigen.

Assay of vWF activity. In normals, the peak of vWF activity is in fraction 1, with fraction 2 having about 20% of the peak activity (Table III). The other fractions have negligible amounts of activity. In patient vW I_2 a small amount of vWF was found in fractions 1 and 2, while in vW II_3 no vWF activity was detected in any column fraction. In mixing experiments, column fractions 1 and 2 or plasma of vW I_2 and vW II_3 did not inhibit the ristocetin-induced platelet aggregation of normal column fraction 1 or normal plasma.

TABLE II
Antigen Units in Cryoprecipitate Column Fractions

		Fraction				
		1	2	3	4	5
Source						
Normal (7)	a	31.8±7.6 (22.8–41.6)	8.0±2.6 (5.5–11.4)	3.9±1.1 (1.0–6.1)	UR*–1.9	UR–0.7
	b	8–16	2–4	1–4	UR–2.0	UR–2.0
vWD, severe	a	UR	UR	UR	UR	UR
	b	UR	UR	UR	UR	UR
vWD, severe	a	UR	UR	UR	UR	UR
	b	UR	UR	UR	UR	UR
vWD, severe	a	2.1	UR	UR	UR	UR
	b	1	UR	UR	UR	UR
vWD I_2	a	26.5	9.5	2.8	UR	UR
	b	8	4	2	UR	UR
vW II_3	a	3.1	18.4	1.2	UR	UR
	b	1	8	2	UR	UR

a. All values are antigen units expressed as the highest reactive dilution per 1.0 OD of the test sample at 280 nm. Mean±SD for seven normals. b. Reciprocal of the highest reactive dilution. The three patients with severe vWD had no detectable antigen, bleeding times >30 min, procoagulant levels below 5%, and absent platelet retention and ristocetin aggregation. These three previously reported patients have been included for comparison (8).
* Unreactive.

TABLE III

Restoration of Ristocetin-Induced Aggregation of Normal Washed or Gel Filtered Platelets by Column Fractions

Source		Column fraction 1	2	3	4	5
Normal* (7)	a‡	53.2±15.6	9.2±4.2	1.6±0.68	0.2–2.1	<0.2–0.5
	b‡	140	28.8	8.2	<10	<5
vWD, severe	a	<3.0	<2.0	<0.3	<0.2	<0.2
	b	<5	<5	<5	<5	<5
vWD, severe	a	<3.0	<3.0	<0.8	<0.2	<0.2
	b	<5	<5	<5	<5	<5
vWD, severe	a	4.2	<2.0	<0.7	<0.3	<0.3
	b	7	<5	<5	<5	<5
vW I_2	a	5.3	2.0	<0.6	<0.3	<0.2
	b	16	12	<5	<5	<5
vW II_3	a	<3.0	<2.0	<0.3	<0.2	<0.2
	b	<5	<5	<5	<5	<5

The severe vWD patients are described in Table II.

* All values are expressed as the percentage of normal plasma. For all values greater than 100% of normal plasma, the sample was diluted until the slope could be read from the standard curve.

‡ a. These values are the percentage of normal plasma per 0.1 OD of the test sample at 280 nm. Mean±SD for seven normals. b. These values are the mean percentage of normal plasma for each fraction: fraction 1, 48–61 ml; fraction 2, 62–75 ml; fraction 3, 76–89 ml; fraction 4, 90–103 ml; fraction 5, 104–117. Mean of seven normals.

DISCUSSION

The classic diagnostic criteria for vWD include a long bleeding time, low factor VIII, reduced platelet retention, and an autosomal dominant inheritance pattern. Recently, Zimmerman et al. (16) and Stites et al. (24) demonstrated immunologically that in vWD plasma the F. VIII-related antigen is reduced or absent, while Howard and Firkin (25), and Weiss et al. (7) noted a deficiency in vWD of a plasma factor necessary for ristocetin-induced platelet aggregation. Thus an immunologic deficiency of the F.VIII-related antigen and absent or reduced ristocetin aggregation factor have been added to the diagnostic abnormalities in vWD.

We studied two patients in whom there were dramatic disparities in the diagnostic criteria for vWD. Both had normal F.VIII procoagulant activity and F.VIII/vWF antigen but markedly abnormal ristocetin-induced platelet aggregation, platelet retention, and bleeding times. The first patient (vW I_2), an orphan, has two apparently normal children, four children (two male, two female) who have severe vWD with all the "classical" findings, including low F. VIII, low antigen, and reduced ristocetin-induced platelet aggregation, and three asymptomatic children with an apparent qualitative defect. The father of this family (now deceased) was studied before antigen levels and vWF levels were available. However, his bleeding time, procoagulant level, and platelet retention were normal. This does not exclude the possibility that this parent still had a qualitative defect in his F.VIII/vWF protein. Although the inheritance in the vW I family is not clear without complete studies of the father, we postulate that neither parent is a homozygote (two normal children) and that the affected children inherit either a qualitative defect and/or quantitative defect of the F.VIII/vWF protein. The apparent quantitative defect may reflect a double heterozygous qualitative defect appearing phenotypically as severe classical vWD. An analogous situation occurs in individuals heterozygous for hemoglobin C and S. The individual heterozygous for just on abnormal hemoglobin (i.e., C or S) is asymptomatic or very mildly clinically affected with reduced levels of A hemoglobin, while double heterozygotes (C and S) present a clinical picture of anemia and hemolysis and the absence of normal A hemoglobin (26). In addition, if an individual is doubly heterozygous for hemoglobin S (a qualitative defect) and β-thalassemia (a quantitative defect), the clinical picture can be quite variable with a syndrome similar to homozy-

gous sickle cell disease to no clinical disability, depending on the type of inherited thalassemia defect (27).

The inheritance pattern in family vW I has significant implications for the genetics of vWD. We offer one hypothesis that explains the data but recognize that others are possible. We believe that both parents are heterozygotes, with the mother carrying one normal vW gene and one vW gene that codes for a qualitatively abnormal protein. The father carried one normal vW gene and one vW gene that coded for a qualitatively abnormal protein (that may be different from the mother's defective protein). We further speculate that the immunologic and vWF biologic activities are related to a random multimeric combination of the gene products (subunits) from both parents; that the multimeric form is necessary (but not necessarily sufficient) for VIII coagulant activity; that while the abnormal subunits are capable of interacting with normal subunits, they are incapable of interacting with each other; that the mother's normal-abnormal combination has full immunologic and VIII coagulant supporting activity but only partial vWF activity; and the father's combination retained normal VIII supporting and platelet retention activity (immunologic and ristocetin data are unavailable). Thus, the parents could produce offspring having each of the phenotypes observed. In fact, we recently have found another family similar to family vW I where the mother has a defect similar to the proposita (vW I_2) and her children (two sons) have severe classical vWD. The father's bleeding time, procoagulant factor VIII, and platelet retention are normal; however, his vWF and F. VIII-related antigen are 25% of normal. Thus, the inheritance in the vWD I family is not clear without complete studies of the father. The affected children (seven out of nine) inherit either a qualitative defect or a quantitative defect of the F.VIII/vWF protein. The latter defect probably reflects a double heterozygous defect appearing phenotypically as severe classical vWD. Precisely the same reasoning would apply if the father had a quantitative (thalassemia-like) defect and the mother's abnormal subunits could not interact with each other.

The infusion of cryoprecipitate in vW II resulted in a hemostatic response different in some aspects from those previously described in vWD (28, 29). The initial peak (1 h) and rapid decline in procoagulant factor VIII are unusual, as is the initial rise and subsequent plateau of the antigen. The total correction of the platelet retention (adhesiveness) and ristocetin-induced platelet aggregation persist for 5 h, while the bleeding time is only partially corrected at 1 h. The delayed rise of procoagulant activity (24 and 48 h) of 30% over preinfusion levels is a well-recognized occurrence. It is unclear why this patient has such an unusual hemostatic response to cryoprecipitate infusion (compared to other patients with vWD). One possibility may be that most reported infusion studies are performed in patients with very low or undetectable levels of procoagulant or antigenic F. VIII and that different responses may be seen in patients with moderate reductions or normal levels of procoagulant and antigen F. VIII levels (see ref. 29). Bennett et al. demonstrated an increased catabolism of the normal human F. VIII/vWF antigen in vWD (28). It is possible that the metabolic rate differs markedly in patients with vWD and that the observed response to transfusion in vW II_3 indicates a wide spectrum of increased utilization or metabolism of the normal F.VIII/vWF protein in the vWD syndrome. Whether metabolism is governed by plasma levels, alterations in the F.VIII/vWF protein, or end organ mechanisms is unclear.

We believe these patients have qualitative abnormalities of their F.VIII/vWF. This hypothesis is strongly supported by the dramatic abnormalities found in crossed antigen-antibody electrophoresis, suggesting a dual population of molecules in one case and an altered single population in the other. The F.VIII/vWF protein in vW I_2 appeared normal by gel filtration, procoagulant activity, polyacrylamide gel electrophoresis, subunit size, and antigen content; however, in vW II_3, her plasma and her purified F.VIII/vWF protein (from cryoprecipitate) eluted with an estimated mol wt varying between 1,200,000 and 810,000. This contrasts with the normal procoagulant, antigen, and F.VIII/vWF protein peaks, which all have estimated mol wts of approximately 1,600,000. Since the subunit appears to have the same mol wt as normal, it would appear the F.VIII/vWF protein in vW II is composed of a smaller number of subunits. Despite the presence of both the protein and antigen, however, none of the column fraction from vW II supported ristocetin-induced platelet aggregation. Qualitatively identical results were obtained when these patients' plasmas were chromatographed instead of cryoprecipitate.

Holmberg and Nilsson (30) described a group of vWD patients with normal levels of F. VIII-related antigen. These patients did not, however, have a delayed procoagulant response to a transfusion of fraction I_0, and the disease appeared to be transmitted on the X chromosome. Firkin et al. (9) described a vWD patient with normal levels of F. VIII and F. VIII-related antigen, while her bleeding time, platelet retention, and ristocetin-induced platelet aggregation were abnormal. Kernoff et al. (10) investigated a patient with a normal level of antigen and a reduced procoagulant level and found that the patient's cryoprecipitate had an altered precipitin peak in electroimmunoassay and a more anodal migration in crossed antigen-antibody electro-

phoresis. Another unique feature in this patient was the low yield of F. VIII-related antigen in a 3% ethanol precipitate. Family studies were not performed in these two cases (9, 10). Thompson et al. (11) described three patients in one vWD family who had normal F. VIII-related antigen and procoagulant activity and postulated a qualitative defect in the F.VIII/vWF molecule. Peake et al. (12) reported six vWD patients in whom the F. VIII and antigen level varied but in general were normal or only slightly reduced. Four of these patients' plasma were tested in two-dimensional crossed immunoelectrophoresis at pH 9.2. The plasma precipitin arcs were found to be more anodal than those of normal plasma. Mixtures of patient and normal plasma revealed a double peak with no evidence of spur formation (nonidentity).

Our two patients seem to be different from that reported by Kernoff et al. in that the majority of the F.VIII/vWF antigen and procoagulant activity is present in their cryoprecipitates. They differ from the patients described by Holmberg and Nilsson since vW I seemed to transmit the disease as a dominant trait and vW II responded to a cryoprecipitate infusion like a patient with classic vWD. The crossed antigen-antibody electrophoresis patterns of vW I_2 and vW II_3 plasma differ from those described by Peake et al., although we perform our electrophoresis at pH 8.6 rather than 9.2. Differentiation of our patients from those described by Firkin et al. and Thompson et al. is impossible without more information.

The investigation of the F.VIII/vWF protein in vWD reveals a spectrum of molecular defects. In some patients there is a complete absence of the protein by immunologic and biochemical parameters and its biologic activities, while in most vWD patients the level of F.VIII/vWF antigen, procoagulant activity, and vWF activity are all reduced in parallel (7, 16). In a small population of vWD patients there is a marked deficiency of vWF activity but normal levels of the F.VIII/vWF antigen and procoagulant activity. The variability in the level of and biologic function(s) of the F.VIII/vWF protein in vWD are similar to the abnormalities found in other congenital disorders of proteins such as fibrinogen (31, 32).

In the fibrinogenopathies, synthetic defects result in afibrinogenemia, hypofibrinogenemia, and dysfibrinogenemia. To date catabolism of fibrinogen has been normal in these conditions, except for one instance of dysfibrinogenemia where the abnormal fibrinogen had a short half-life (33). The catabolic rate of the vW F.VIII/vWF protein in vWD is unknown; however, Bennett et al. have reported a shortened half-life of the normal F.VIII/vWF antigen in vWD patients compared to hemophiliacs (28). Whether this increased metabolism or utilization of the normal F.VIII/vWF antigen in vWD is indicative of a hypercatabolic state of the F.VIII/vWF protein and/or is in any way responsible for the vWF and procoagulant deficiency is unknown at the present time.

The recent studies of the F.VIII/vWF protein suggest that a variety of molecular defects may be responsible for the disorder known as vWD. Our previous study of patients with severe vWD showed a parallel absence of protein, antigen, vWF, and biologic activities. The lack of correlation between the protein (or antigen) and sites for procoagulant activity, vWF, and antigenic reactivity indicate that these sites are not identical, so that a defect in the vWF site may not affect either the antigenic or the procoagulant activity. Thus the F.VIII/vWF protein and the antigenic material present in some vWD patient, although quantitatively normal, is qualitatively abnormal.

ACKNOWLEDGMENTS

Ms. Georgia Jackson, Mary Cregger, Jacqueline Simeon, Joyce Bagley, and Dawn Mullarkey provided excellent technical assistance. Mrs. Wanda Chappell and Elsie Yanchulis were generous with their time and efforts in the procurement of plasma and cryoprecipitate. Mrs. Lynda Ray and Marty Auman provided valuable secretarial assistance.

Hyland Laboratories kindly provided Hyland Method IV and Abbott Laboratories kindly provided ristocetin.

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