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Research Article

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von Willebrand's Disease Antigen II

A NEW PLASMA AND PLATELET ANTIGEN DEFICIENT IN SEVERE VON WILLEBRAND'S DISEASE

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ABSTRACT Factor VIII-related antigen (VIIIag) is deficient in plasma and platelets of patients with severe von Willebrand's disease. This study reports a second von Willebrand's disease antigen (vWagII), distinct from VIIIag, that is also deficient in the platelets and plasma of patients with severe von Willebrand's disease. VIIIag and vWagII are separable by molecular exclusion chromatography, sucrose density gradient ultracentrifugation, and crossed immunoelectrophoresis. They show reactions of immunologic nonidentity with each other, and thus, do not share a precursor-product relationship. vWagII is released from normal platelets during blood clotting, accounting for a fourfold higher concentration of vWagII in serum over plasma.

INTRODUCTION

Classical von Willebrand's disease (vWD)¹ is characterized by a deficiency of Factor VIII procoagulant activity, ristocetin cofactor activity and Factor VIII-related antigen (VIIIag) (1). These entities may reside on one molecule or on more than one molecule which

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¹Abbreviations used in this paper: β -TG, β -thromboglobulin; CPD, citrate-phosphate dextrose; PPP, platelet-poor plasma; PRP, platelet-rich plasma; Vo, void volume; vWagII, von Willebrand's disease antigen II; vWD, von Willebrand's disease; VIIIag, Factor VIII-related antigen; VIII_c, factor VIII procoagulant activity; VIII_r, ristocetin cofactor activity.

co-purify under a variety of conditions (1-3). VIIIag is that antigen which reacts with heterologous antibody produced in rabbits to purified Factor VIII. VIIIag, also known as von Willebrand's disease antigen (vWagI), is present in normal or increased amounts in patients with hemophilia but absent or reduced in most patients with classical vWD. This report describes a second antigen, von Willebrand's disease antigen II (vWagII), which is deficient in the plasma and platelets of patients with severe vWD.

METHODS

Antisera to vWagII. The antisera used to identify vWagII were produced by two different methods in New Zealand white rabbits. In the initial method, lyophilized Factor VIII concentrate (lot B-17, American Red Cross Blood Research Laboratory, Bethesda, Md.) was subjected to molecular exclusion chromatography through Bio-Gel A-15M (Bio-Rad Laboratories, Richmond, Calif.). Concentration immunization of rabbits, and antiserum absorption were performed as previously described (4). After absorption with cryosupernatant plasma and IgM paraproteins, this antiserum contained an antibody to VIIIag, vWagII, and one additional platelet protein. The studies reported in this paper utilized this antiserum (antiserum I) because of its higher titer to vWagII.

Another antiserum (antiserum II) previously prepared by one of the authors (R.R.M.) at the University of Colorado Medical Center, Denver, Colo., was also studied and contained antibody to vWagII. This antiserum was produced as follows. Commercial Factor VIII concentrate (Profilate, Abbott Laboratories, Chemical Div., North Chicago, Ill.) was reconstituted with column buffer to a final concentration of 40 U VIIIag/ml. A 6-ml sample was subjected to gel filtration through Sepharose 4B (Pharmacia Fine Chemicals, Div. of Pharmacia Inc. Piscataway, N. J.) equilibrated with imidazole-saline buffer (0.02 M imidazole, 0.14 M NaCl, pH 6.5). The bed dimensions were 2.5 x 40 cm. The void volume (Vo) fractions were pooled, concentrated by Amicon filtration (PM 30 Amicon Corp., Lexington, Mass.), and injected into three New Zealand white rabbits. After boosting at 3-wk intervals, the rabbits were bled and the antiserum was obtained. This antiserum contained antibodies to VIIIag, vWagII, and six other nonidentified plasma proteins. This antiserum was then absorbed with equal volumes

of plasma from a patient with severe vWD (VIIIag <0.01 U/ml, Factor VIII procoagulant activity VIII_c <0.05 U/ml, VIII_r, Ristocetin cofactor activity <0.05 U/ml). After this absorption, only antibodies to VIIIag and vWagII were detected.

Antisera to marker proteins. Various plasma protein markers were identified by quantitative immunoelectrophoresis and crossed immunoelectrophoresis utilizing monospecific antisera. Antisera to C4, IgG, IgM, and fibrinogen were obtained through the Scripps Immunology Reference Laboratory, La Jolla, Calif. Other antisera were obtained commercially and include prothrombin, α_1 -antitrypsin, Factor XIII, and α_2 -macroglobulin (Behring Diagnostics, Somerville, N. J.).

Patient population. Plasma was obtained from 19 normal individuals and 24 patients with vWD. 8 of the 24 patients were classified as severe on the basis of no detectable VIIIag (<0.01 U/ml), VIII_c (<0.05 U/ml), and VIII_r (<0.05 U/ml). 16 of those with vWD had moderate vWD (VIIIag 0.080–0.34 U/ml). 13 of these 16 patients had VIIIag characterized by the presence of only those forms with more anodic migration on crossed immunoelectrophoresis. Two of the severe vWD patients were also studied after being transfused at a time when their VIII_c was 40% and their VIIIag was 20%.

Plasma preparation for VIIIag and vWagII analysis. Blood was drawn into citrate-phosphate dextrose (CPD) by adding 1.3 ml CPD to 8.7 ml of blood. Blood was centrifuged at 2,000 g for 30 min at 4°C. "Platelet-poor" plasma (PPP) was obtained by centrifuging CPD plasma at 5,000 g for 30 min at 4°C, and platelet-rich plasma (PRP) was obtained by centrifuging CPD blood at 200 g for 10 min at 4°C. CPD plasma plus inhibitors was obtained by immediately mixing fresh CPD blood with 10 U/ml heparin, 10 μ g/ml soybean trypsin inhibitor, 10 U/ml Trasylol (FBA Pharmaceuticals, New York), and 5 mM benzamidine.

Washed platelets for vWagII analysis. PRP from 25 ml of CPD blood was obtained as above. The PRP was centrifuged at 2,000 g for 15 min at 20°C and the supernate was discarded. The platelets were resuspended in 25 ml of Krebs-Ringer buffer (5 mM KCl, 0.107 M NaCl, 0.02 M NaHCO₃, 9 mM Na₂ EDTA, 2 mM Na₂SO₄, pH 7.4) and centrifuged at 2,000 g for 14 min at 20°C. This procedure was repeated three additional times. After the final centrifugation, all the buffer was decanted and the platelets were resuspended in 0.1 ml barbital saline buffer (0.01 M barbital sodium, 0.015 M barbitoric acid, 0.125 M NaCl, 0.1% NaN₃, pH 7.4). This platelet concentrate was subjected to lysis by freezing (liquid nitrogen) and thawing (four times). Platelet fragments were removed by centrifugation at 11,000 g for 15 min at 20°C. An alternative method used modified Tyrode's buffer (0.0026 M KCl, 0.137 M NaCl, 0.001 M MgCl₂, 0.012 M NaHCO₃, 0.03 M adenosine; Sigma Chemical Co., St. Louis, Mo.), 0.1% dextrose, and 2% bovine serum albumin (Fraction V, Sigma Chemical Co.), pH 7.4, to wash the platelets. Lysis was performed as described above.

Crossed immunoelectrophoresis. Crossed immunoelectrophoresis was performed as previously described (5) except that the sample well was made larger to accept a 150- μ l sample. Since the antiserum concentration was very high (up to 26%), the slides needed to be washed in normal saline for 48 h before drying and staining.

A calibration curve for quantitating vWagII was constructed by plotting the area under the crossed immunoelectrophoresis precipitin peak on the arithmetic scale against serial dilutions of a plasma pool on the logarithmic scale. The plasma pool was obtained from 20 normal individuals and stored at -70°C. This served as the standard for vWagII, and was assigned the value of 1 U/ml. The area under the precipitin peak was obtained by multiplying the height of the peak times

the width at a point which bisects the height as previously described (6).

Quantitative immunoelectrophoresis of vWagII and VIIIag. Since the amount of protein applied to a slide was very large due to the high concentration of antibody and the large sample utilized, the standard quantitative immunoelectrophoresis of vWagII in plasma proved impossible. However, quantitative immunoelectrophoresis could be performed on vWagII that was partially purified by column chromatography or sucrose density gradient ultracentrifugation. This method could therefore be used to localize vWagII in these experiments. The antiserum concentration was the same as that used for quantitative crossed immunoelectrophoresis. The procedure for quantitative immunoelectrophoresis of VIIIag has been previously described (4).

Purification of VIIIag and separation from vWagII. Blood was drawn into CPD (13 ml/87 ml blood), 10 U/ml heparin, 10 U/ml Trasylol and 10 μ g/ml soybean trypsin inhibitor. The blood was then centrifuged at 3,000 g for 15 min at 20°C and the plasma was separated. Diisopropyl fluorophosphate was added to this plasma at a 0.002-M concentration. Aluminum hydroxide absorption was performed four times. In the first absorption, 5 ml Al(OH)₃ gel (Rehsorptar, Reheis Co., Inc., Phoenix, Ariz.) was added to 100 ml plasma and stirred gently at ambient temperature for 15 min. The Al(OH)₃ was removed by centrifugation at 25,000 g for 10 min at 20°C. The second, third, and fourth absorptions were performed in the same manner except the concentration of Al(OH)₃ was reduced to 1.5 ml/100 ml plasma. After the final absorption, the plasma was centrifuged at 90,000 g for 60 min. The supernatant plasma was flash frozen in liquid nitrogen and stored at -70°C until used. The cryoprecipitate was produced by thawing the plasma for 4–6 h in a stirred melting ice bath and the cryoprecipitate sedimented by centrifugation at 90,000 g for 20 min at 5°C. This precipitate was dissolved at 37°C, diluted to 5 ml with barbital saline buffer (pH 7.4), and centrifuged at 200,000 g for 30 min at 20°C. The sample below the lipid layer was carefully removed, taking care not to disturb any of the precipitate on the bottom of the tube, and subjected to agarose gel chromatography. Bio-Gel A-15M was packed into two columns connected in series with total dimensions of 2.5 \times 94 cm. The sample was eluted with Tris-saline buffer (0.05 M Tris, 0.15 M NaCl, and 0.02% NaN₃, pH 7.4). Fractions were collected in 2.6-ml aliquots and the optical density at 280 nm was recorded. Highly purified VIIIag eluted in the V₀ with semipurified vWagII eluting 2.5 \times V₀ along with other plasma proteins. The fractions containing the vWagII were concentrated using Aquacide II (Calbiochem, San Diego, Calif.) to 1/10th the original volume and are referred to as semipurified plasma vWagII.

Semipurification of platelet vWagII. Semipurified platelet vWagII was prepared by separating PRP from fresh CPD plasma at 200 g for 10 min at room temperature. The platelets were then sedimented at 2,000 g for 15 min and the supernatant plasma removed. These platelets were then washed six times using Krebs-Ringer buffer (4 mM KCl, 0.107 M NaCl, 0.02 M NaHCO₃, 2 mM Na₂SO₄, pH 7.4). The platelets were resuspended in a 5-ml barbital saline buffer (pH 7.4) and subjected to freeze-thaw lysis (four times). The particulate matter was removed by centrifugation at 90,000 g and the supernate was subjected to agarose gel chromatography as described above. The fractions containing vWagII were concentrated in the same fashion and are referred to as semipurified vWagII.

Electrophoretic mobility of vWagII. The relative electrophoretic mobility of vWagII was determined by crossed immunoelectrophoresis as described above except, the first dimension was run at a pH of 8.6 (rather than pH of 9.5) using

the same buffer. Other plasma proteins were run as external controls (i.e., parallel runs) and internal controls (using several antibodies in the second dimension and running normal plasma in the first dimension). Antiserum against fibrinogen, C4, prothrombin, and α_1 -antitrypsin were utilized and all runs were standardized to an albumin marker that was migrated 6.0 cm.

Sucrose density gradient ultracentrifugation. Sucrose density gradient ultracentrifugation was performed using a linear gradient (10–40% wt/vol sucrose). A 0.5-mol sample was applied to the top of a 10-ml gradient in a cellulose nitrate centrifuge tube (model 331370, Beckman Instruments, Inc., Palo Alto, Calif.). The gradients were centrifuged at 35,000 rpm using a SW-41 rotor in a Beckman L3-50 centrifuge (Beckman Instruments Inc.) for 18 h at 4°C. The gradients were eluted from the bottom in 0.33-ml aliquots. The sedimentation velocity of vWagII was obtained by running normal plasma that had been enriched in vWagII by the addition of either semipurified plasma vWagII or semipurified platelet vWagII. Sedimentation velocity of vWagII was determined by comparing it to other plasma proteins present in the sample (IgM, IgG, fibrinogen, prothrombin, and α_1 -antitrypsin). The location of the internal markers and vWagII was determined by quantitative electrophoresis.

Apparent molecular weight estimation by gel filtration. Sephadex G-200 (Pharmacia Div. of Diagnostics, Pharmacia Inc.) was equilibrated with Tris-saline buffer in a column with bed dimensions of 1.5×65 cm. The column was standardized using a calibration kit with molecular weight markers ranging from 13,700 to 158,000 daltons (Pharmacia Diagnostics, Div. of Pharmacia Inc.). Normal plasma was mixed with semipurified plasma vWagII or semipurified platelet vWagII and a 1-ml sample applied to the column. Fractions were collected in 2.5-ml increments. The elution volumes of vWagII, VIIIag, IgM, IgG, C4, prothrombin, α_1 -antitrypsin, Factor XIII, and α_2 -macroglobulin were determined by quantitative immunoelectrophoresis. The partition coefficient (K_{av}) was determined according to the formula $K_{av} = (\text{elution volume} - V_0) / \text{bed volume} - V_0$. A standard curve was constructed relating K_{av} to molecular weight.

Antibody against highly purified VIIIag. Highly purified VIIIag was prepared as described above. The fractions containing the middle portion of the VIIIag peak (295 U VIIIag/OD U) were used to immunize rabbits. Freund's complete adjuvant (Grand Island Biological Co., Grand Island, N. Y.) was emulsified with an equal volume of 80–150 μg of highly purified VIIIag and injected in multiple subcutaneous sites along the back of New Zealand white albino rabbits. Rabbits were boosted twice, using the same technique, at 3-wk intervals and the antiserum obtained by bleeding the rabbit from the ear artery.

Proteolytic degradation of highly purified VIIIag. Highly purified VIIIag was subjected to digestion with various proteolytic enzymes. These included streptokinase (500 U/mg plasminogen, Calbiochem), activated human plasminogen (150 $\mu\text{g}/\text{ml}$ courtesy of Dr. E. F. Plow), trypsin (bovine pancreas trypsin, 220 U/mg, Worthington Biochemical Corp., Freehold, N. J., lot TRTPCK-35K940), and porcine elastase (60 U/mg, Worthington Biochemical Corp., lot ESFF-56P343). VIIIag (80 $\mu\text{g}/\text{ml}$) was incubated at 37°C for 2½ h and for 18 h with plasmin (0.009 $\mu\text{g}/\mu\text{g}$ VIIIag), porcine elastase (0.1 elastase U/ μg VIIIag), and trypsin (0.016 U/ μg VIIIag). These conditions gave VIIIag digests containing either three immunologically distinct VIIIag fragments (elastase), two immunologically distinct fragments (trypsin digest), or one component with accelerated anodic mobility (plasmin digest)

when these digests were subjected to crossed immunoelectrophoresis. These digests were used to immunize rabbits.

Cellular lysates of nonplatelet blood cells. Erythrocytes, granulocytes, and lymphocytes were obtained in the following manner. Heparinized blood was obtained and diluted 1:2 in RPMI-1640 (Grand Island Biological Co.) and layered onto a barrier of Ficoll-Hypaque (P 1.074 g/ml) (7). The tubes were centrifuged at 2,260 g for 5 min at 20°C. The top plasma segment contained predominantly platelets. The cells at the interface were lymphocytes together with a few adherent cells and platelets. These cells were placed in culture (7). After 4 days, the nonadherent cells were 95% lymphocytes with no platelets present. This was the source of lymphocytes. Granulocytes were obtained from the buffy coat on top of the erythrocytes in the original Ficoll-Hypaque centrifugation step. There were 90% granulocytes with a few mononuclear cells. The erythrocytes were obtained from the bottom of the tube and were free of platelets and leukocytes. These erythrocytes, lymphocytes, and granulocytes were washed four times with Krebs-Ringer buffer (pH 7.4), subjected to freeze-thaw lysis, and centrifuged at 11,000 g for 15 min at room temperature to remove the particulate matter.

RESULTS

Detection of vWagII. The antisera used to detect vWagII also detected VIIIag. When antiserum I was used at a concentration of 0.6% in the second dimension of a crossed immunoelectrophoresis, only the VIIIag peak was produced (Fig. 1). No other precipitin peaks were detected. If, however, the sample applied to the well in the first dimension was increased sevenfold in the same sample volume and the antibody concentration in the second dimension increased 26-fold, the VIIIag precipitin peak was drastically reduced in height and another, more anodic, precipitin peak was detected (Fig. 1). This peak was called vWagII for reasons to be described. Antiserum II (produced at a different institution) behaved in a similar manner.

Both of these antisera clearly identified vWagII and its deficiency in patients with severe vWD.

Deficiency of vWagII in severe vWD. When plasma from patients with severe vWD was subjected to identical crossed immunoelectrophoresis, neither the VIIIag nor the vWagII peaks were detected (Fig. 2). When plasma from patients with moderate vWD (i.e., 8–34%) were tested, the vWagII peak was present. Patients, with a form of vWD characterized by a more anodic migration of the VIIIag peak, were tested and had easily detectable vWagII (Fig. 2). Quantitation of vWagII was accomplished by measuring the area under the peak as described in Methods. Levels of 6% vWagII or greater produced identifiable precipitin peaks. Table I gives the VIIIag and vWagII results in 19 normal individuals and the patients with vWD. None of the severe vWD patients had detectable vWagII (i.e., <0.06 U/ml plasma vWagII). All but one of the moderate vWD patients had vWagII detected. The moderate vWD patient who had <0.06 U/ml vWagII

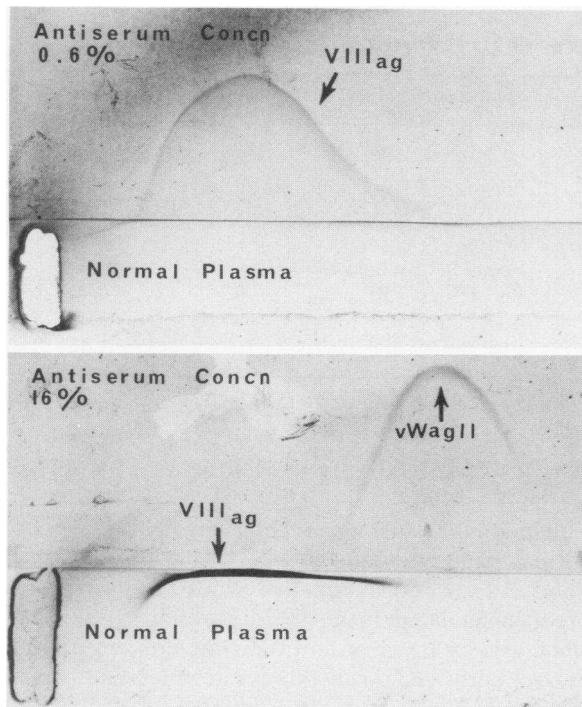


FIGURE 1 Crossed immunoelectrophoretic analysis of normal plasma using two concentrations of antibody. At the lower antiserum concentration only the VIIIag precipitin peak is seen. When the antiserum concentration is increased, the VIIIag peak is drastically decreased and the vWagII peak is clearly identified.

had two different plasma samples tested and neither had detectable vWagII levels. This patient was otherwise not distinguishable from other patients with moderate vWD. Two patients with severe vWD were studied at 60 min, 12, 24, and 48 h after they received transfusions with cryoprecipitate (1 bag/5 kg). No vWagII could be detected in their plasma even when their VIIIag was >30% and their VIII_c was >40%. Fig. 3 shows the relationship between vWagII and VIIIag in normal individuals and patients. In the individuals studied, a weak but significant linear relationship exists for the normal individuals with a correlation coefficient of 0.71 ($P < 0.01$). There was no linear relationship for the vWagII levels of the patients with moderate vWD (correlation coefficient 0.034; not significant).

Effect of clotting on vWagII. When normal serum was compared to normal plasma from the same individual, vWagII levels were fourfold greater. To evaluate the source of this rise, experiments were designed to evaluate the effect of clotting. PRP, PPP, CPD plasma, and CPD whole blood were recalcified in glass tubes with 30 μ l of 1 M CaCl₂/ml plasma and permitted to clot at 37°C for 30 min before serum

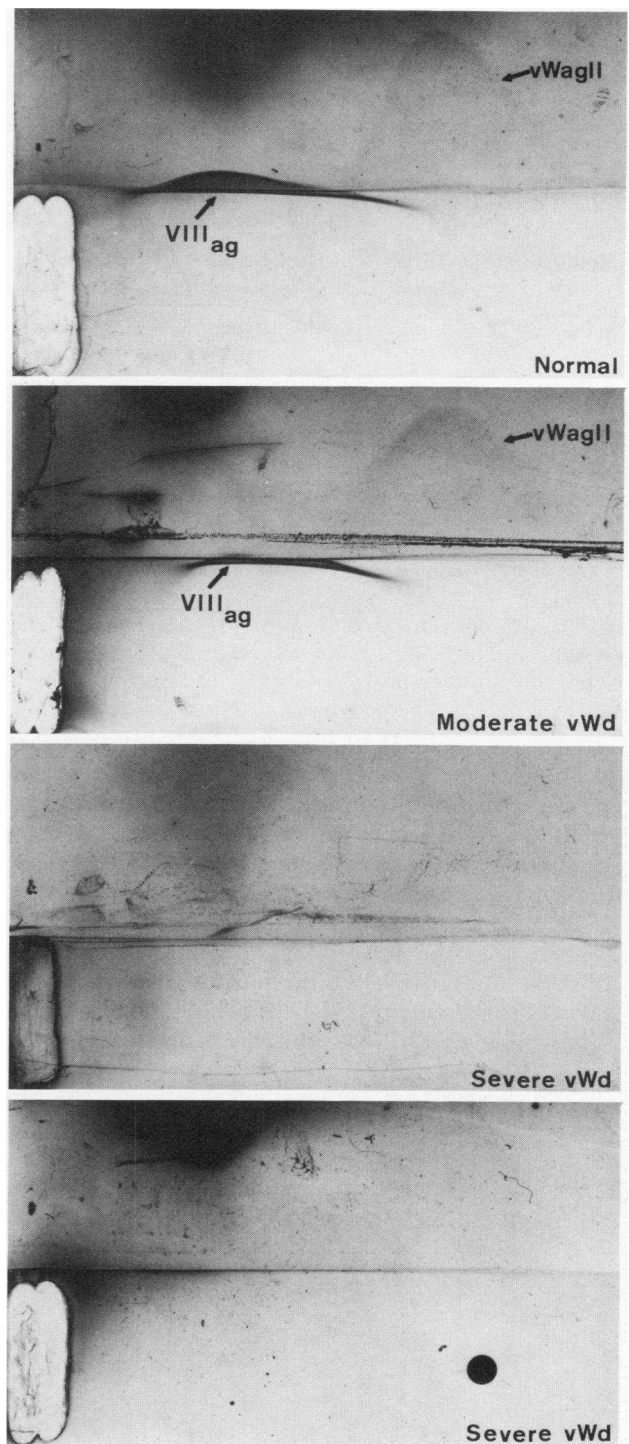


FIGURE 2 Crossed immunoelectrophoretic analysis of plasma from a normal individual, an individual with moderate vWD, and two individuals with severe vWD. The vWagII peak is undetectable in the severe vWD plasmas. The less anodic forms of VIIIag are absent from the moderate vWD plasma. No VIIIag is detectable in the severe vWD plasmas.

TABLE I
Plasma vWagII and VIIIag

	vWagII	VIIIag
	U/ml	U/ml
Normal individuals (19)	0.87±0.33* (0.46–1.44)‡	0.98±0.27 (0.58–1.58)
Moderate vWD (16)	0.49±0.24§ (<0.06–1.19)	0.19±0.09 (0.08–0.34)
Severe vWD 1	<0.06	<0.01
2	<0.06	<0.01
3	<0.06	<0.01
4	<0.06	<0.01
5	<0.06	<0.01
6	<0.06	<0.01
7	<0.06	<0.01
8	<0.06	<0.01

* Mean±SD.

‡ Range.

§ If the patient with <0.06 U/ml vWagII was eliminated, the mean would be 0.52±0.21 for the remaining 15 patients.

^{||} Only 1 of 16 was <0.29 U/ml.

separation. Table II shows the results of these experiments. The vWagII level of normal CPD plasma was unaffected by immediately adding proteolytic inhibitors (soybean trypsin inhibitor, Trasylol, benzamide, and heparin). Recalcification of CPD blood caused a fourfold increase in vWagII. Recalcification of PPP, however, did not change the level of vWagII compared to the starting plasma. Recalcification of PRP, on the other hand, produced a fourfold rise in vWagII suggesting this to be a platelet effect. To test this hypothesis, PRP was subjected to four times freeze-

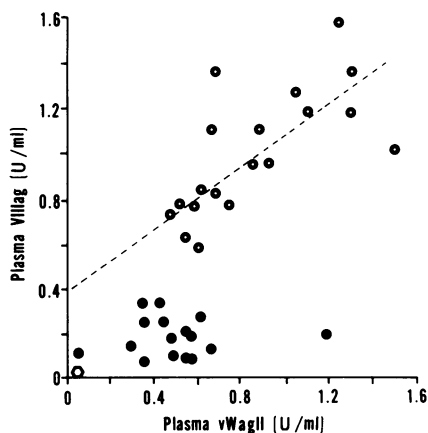


FIGURE 3 The comparison of vWagII concentrations with those of VIIIag in normal plasmas (○), individuals with moderate vWD (●), and severe vWD (◻). The hexagon represents eight individuals who had undetectable levels of both antigens.

TABLE II
Effect of Clotting on vWagII

	vWagII*
	U/ml
Plasma, CPD without inhibitors	1.12
Plasma, CPD with inhibitors	1.16
Serum	4.83
Recalcified whole blood	4.53
Recalcified PRP	5.02
Recalcified PPP	1.02
PRP, freeze-thaw lysis	4.83

* The average of duplicate determinations.

thaw lysis and a similar fourfold rise in vWagII was detected.

vWagII in washed normal and severe vWD platelets. Normal platelets were obtained from 50 ml of CPD blood and washed six times in Krebs-Ringer buffer before resuspension in a 100- μ l barbitol saline buffer. After lysis by freezing and thawing, crossed immunoelectrophoresis showed this lysate to contain vWagII at a concentration of 20 U/ml (see Fig. 4). When

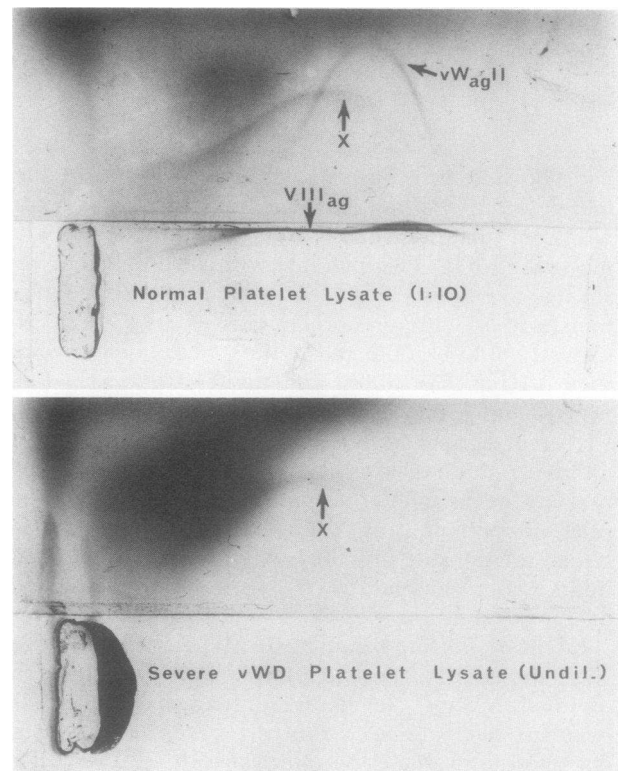


FIGURE 4 Crossed immunoelectrophoretic analysis of platelet lysate from a normal individual and a patient with severe vWD. A third antigen "X" is identified in both the normals and severe vWD (see text).

platelets were obtained from four patients with severe vWD in the same fashion (Fig. 4), there was no vWagII detected (i.e., <0.06 U/ml). Single samples of platelet lysate in three of these four patients were studied with three or more separate determinations of platelet vWagII. The fourth individual had platelets sampled and vWagII studied on four separate occasions. None of these studies revealed any detectable vWagII in the platelet lysate of individuals with severe vWD. In addition, a concentrate of vWD platelet membranes (after freeze-thaw lysis) also did not contain vWagII when tested on crossed immunoelectrophoresis. Patients with severe vWD therefore have <0.3% of normal platelet vWagII. In the process of evaluating platelets, it was found that antibody to a third antigen (present in both normal and severe vWD platelets and labeled "X" in Fig. 4) was present in antiserum I. This antigen showed no identity with either VIIIag or vWagII. Further characterization of this unknown antigen (X) has not been undertaken since it is present in both normal and severe vWD platelets.

Preparation of highly purified VIIIag and its physical separation from vWagII. Highly purified VIIIag was obtained by the method described. The elution pattern of the cryoprecipitate is shown in Fig. 5. Since the starting plasma contains 1 U VIIIag/30 OD U and the V_0 peak is found at 8 U VIIIag/0.027 OD U, this represents >8,500-fold purification. An 80- μ g sample of this material was reduced and examined by 5% sodiumdodecyl sulfate-polyacrylamide gel electrophoresis. A representative gel is shown in Fig. 5. Five rabbits were immunized three times with 150–200 μ g of highly purified VIIIag given at 3-wk intervals. The antiserum produced in all five individual rabbits was monospecific for VIIIag without absorption as determined by Ouchterlony analysis and crossed immunoelectrophoresis using high antiserum concentration (18%) in the second dimension. The titer of anti-VIIIag was fourfold greater than the anti-VIIIag titer of the antiserum used to

identify vWagII yet no vWagII or other antigens were detected.

Semipurification of plasma and platelet vWagII. Fig. 5. shows the clear physical separation of plasma vWagII from VIIIag by 4% agarose gel chromatography. The vWagII was included in 4% agarose and was eluted at 2.5 times the V_0 and at a concentration of 4.25 U vWagII/0.13 OD U (i.e. 1,200-fold purification). Semipurified platelet vWagII was obtained as described in Methods from a washed platelet lysate. The vWagII eluted in the same position of 4% agarose and was \approx 600-fold purified. To evaluate the immunologic relationship of platelet and plasma vWagII, a tandem crossed immunoelectrophoresis was performed using the method described by Kroll (8). Fig. 6 shows the results when semipurified plasma vWagII was run in tandem with a platelet vWagII concentrate. A reaction of immunologic identity, as described by Axelsen et al. (9) was seen between platelet and plasma vWagII.

Electrophoretic mobility of vWagII. Platelet and plasma vWagII were found to have identical electrophoretic mobility. To compare their mobility to the mobility of other known plasma proteins, crossed immunoelectrophoresis was performed with the pH of the first dimension changed to the more commonly used pH of 8.6. Table III shows the relative mobility at pH 8.6 in 0.9% agarose at ambient temperature. The mobility is between that of prothrombin (an α_2 -globulin) and α_1 -antitrypsin (an α_1 -globulin) under these conditions the R_f (migration distance of antigen/migration distance of Evans blue bovine serum albumin marker) of vWagII was 0.68 and the R_f of VIIIag 0.285.

Sedimentation velocity of vWagII. Sucrose density gradient ultracentrifugation was performed on semipurified platelet vWagII and semipurified plasma vWagII and compared to the sedimentation velocity of various known plasma proteins. The sedimentation velocity of semipurified vWagII run in parallel with markers was identical to

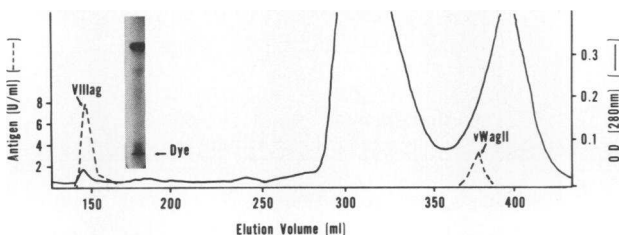


FIGURE 5 Elution profile of agarose gel filtration step of VIIIag purification. The inset is a 5% sodium dodecyl sulfate-polyacrylamide gel of eluting in the V_0 . This material was reduced in sodium dodecyl sulfate-urea before gel electrophoresis. VIIIag elutes in the V_0 and vWagII at $2.5 \times V_0$.

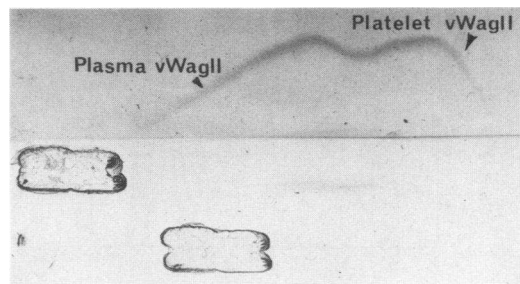


FIGURE 6 Tandem crossed immunoelectrophoretic analysis of plasma and platelet vWagII with a reaction of immunologic identity between platelet vWagII and plasma vWagII.

TABLE III
Electrophoretic Mobility at pH 8.6 in 0.9% Agarose

	Distance migrated	R_f^*
	cm	
Albumin	6.0	1.0
Fibrinogen	0.51	0.09
C4	1.54	0.26
Prothrombin	3.87	0.65
α_1 -Antitrypsin	4.79	0.80
vWagII	4.10	0.68
VIIIag	1.7	0.285

* R_f = (migration distance of unknown)/(migration distance of albumin marker).

its sedimentation velocity when mixed with normal plasma to provide internal markers. Fig. 7 shows the results for platelet and plasma vWagII with the internal markers of IgM, fibrinogen, IgG, prothrombin, and α_1 -trypsin. The distribution of vWagII was identical for platelet and plasma vWagII and corresponded to an average sedimentation velocity of 4.8S. The average S rate for VIIIag in plasma was 21S.

Behavior of vWagII in Sephadex G-200. Although the determination of the true molecular weight of vWagII awaits its further purification, gel filtration in Sephadex G-200 was carried out to compare its behavior to the partition coefficients (K_{av}) and molecular weights of known globular plasma proteins. Fig. 8 illustrates the elution profile of vWagII. The elution of platelets and plasma vWagII was almost identical. When compared to the internal plasma protein markers, the behavior was similar to that expected for a globular protein of 135,000 daltons. The K_{av} for vWagII was

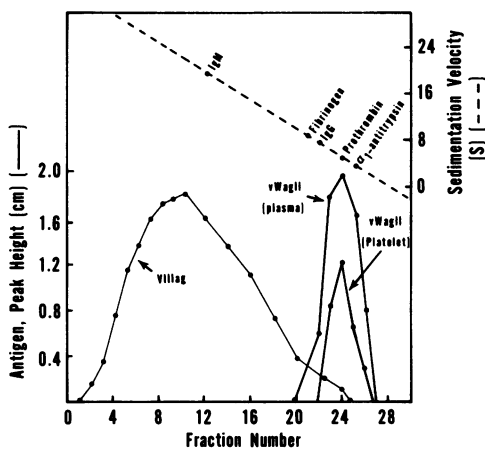


FIGURE 7 Sucrose density gradient analysis of vWagII and VIIIag and comparison with the sedimentation velocity of various plasma proteins.

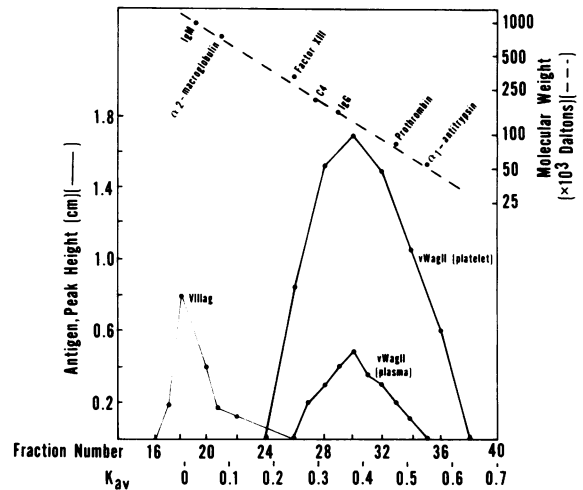


FIGURE 8 Elution profile of VIIIag, plasma vWagII, and platelet vWagII on Sephadex G-200 and comparison with the molecular weight of various plasma proteins.

0.0375. VIIIag was present in the V_0 as would be expected.

Immunologic relationship between vWagII and VIIIag. As noted above, antibody produced to highly purified VIIIag does not react with vWagII, indicating the immunologic distinctness of these two entities. To further investigate this, the antiserum containing both anti-VIIIag in high titer and anti-vWagII was absorbed with purified VIIIag to reduce the anti-VIIIag titer. Fig. 9 shows the crossed immunoelectrophoresis of normal plasma. VIIIag and vWagII precipitin peaks cross giving a reaction of immunologic nonidentity. Evaluation of immunologic relationships on crossed immunoelectrophoresis has been reviewed (9, 10). In

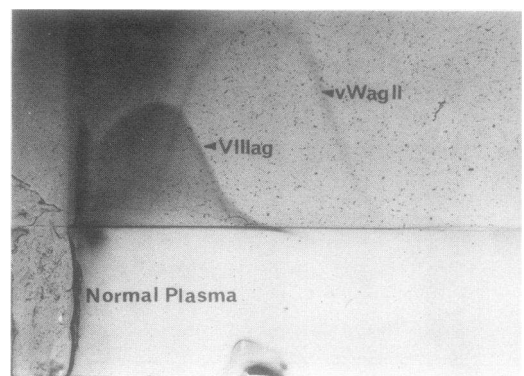


FIGURE 9 Crossed immunoelectrophoretic analysis of normal plasma after partial absorption of the antiserum with purified VIIIag. Electrophoresis time for the first dimension was reduced so vWagII would not migrate as far as usual and thus allow easier comparison with VIIIag. The crossing of the VIIIag and vWagII precipitin lines denotes a reaction of immunologic nonidentity.

fact, further absorption with purified VIIIag showed complete elimination of the VIIIag peak with no change in the anti-vWagII titer (see Fig. 10).

Highly purified Factor VIII was digested with plasmin, elastase, and trypsin. None of the VIIIag fragments produced by these proteolytic enzymes showed immunologic identity with vWagII. In addition, antisera to these digests were produced in rabbits. None of the antisera produced a vWagII precipitin peak when tested against normal plasma utilizing crossed immunoelectrophoresis.

Absence of vWagII in erythrocytes, granulocytes, and lymphocytes. Erythrocyte, granulocyte, and lymphocyte suspensions were washed and subjected to lysis by freezing and thawing. None of these preparations contained detectable amounts of vWagII.

Effect of temperature and anticoagulant. Since the temperature of blood after the collection and type of anticoagulant used have been shown to markedly affect the amount of platelet Factor IV (11, 12), or β -thromboglobulin (13, 14) released from platelets, various anticoagulants, and temperatures were used in the collection of specimens for vWagII analysis. With EDTA, CPD, citrate, and heparin as anticoagulants, identical amounts of vWagII were present in plasma and this was unaffected by incubation of the samples at 4°, 37°, or 22°C for 30 min before centrifugation of the whole blood.

DISCUSSION

This study reports the presence of a newly recognized plasma and platelet antigen, vWagII. This antigen is not detected in either the platelets or plasma from individuals with severe vWD (eight individuals). It is present in all but one of the patients with moderate or variant forms of vWD tested and all normal individuals tested (34 individuals).

The antisera used to recognize this antigen has been produced using two different methods and utilizing Factor VIII concentrates from two different sources as starting material. Neither of these two methods included a step to render the starting sample free of platelets or platelet fragments. Thus platelet fragments would elute in the V_0 of both procedures. Other investigators have found antisera, produced in a similar fashion, to contain antibodies to platelet proteins (1, 15, 16). We feel the presence of the vWagII antibody in our antiserum is due to the presence of platelet fragments in the immunizing materials. In fact, when VIIIag was purified as described in Methods ("purification of VIIIag and separation from vWagII") no antibody to vWagII was produced in any of the five rabbits immunized with V_0 material. The probable reason for this was the inclusion of a high-speed ultracentrifugation step before applying the sample to the column. Thus,

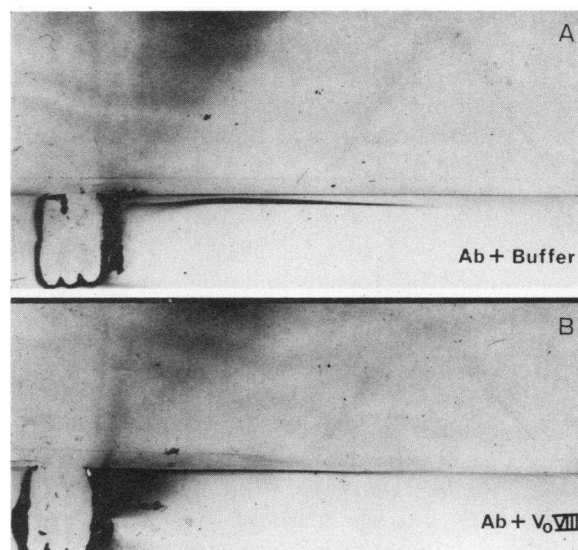


FIGURE 10 Crossed immunoelectrophoretic analysis of plasma before (A) and after (B) complete absorption of anti-VIIIag antibody with purified VIIIag. The vWagII precipitin arc (A and B) was unaffected by prior absorption with VIIIag, though the heavy VIIIag precipitin arc (A) is now completely absent (B).

platelet fragments were removed and there was no vWagII in the region of the V_0 , though it did elute in the region of $2.5 \times V_0$ as shown in Fig. 5.

There are a number of abnormalities that have been described in vWD. These abnormalities include low VIII_c (17–19), low VIIIag (20–22), low VIII_r (23–25), long bleeding time (14, 26–28), and decreased platelet adhesiveness (29, 30). In Erik von Willebrand's initial description of vWD and in follow-up studies on these patients, the bleeding diathesis in vWD was felt to be due to a platelet abnormality (26, 27). Since the advent of platelet aggregation studies, numerous authors have attempted to define a platelet aggregation defect in vWD. Aggregation to epinephrine, ADP, collagen, and thrombin have been normal (31, 32). The abnormal tests that were felt to reflect a platelet abnormality—such as the bleeding time, platelet adhesiveness, and aggregation induced by ristocetin—have subsequently been felt to be secondary to a plasma defect. Normal plasma, cryoprecipitate, and purified Factor VIII have been shown to correct or partially correct these abnormalities (33–25). It has been felt, therefore, that intrinsic platelet function is normal (31–33). A study of platelet membrane glycoproteins did not detect an abnormality in the platelets of patients with vWD (36). However, both VIIIag (15, 16, 37) and vWagII are deficient in the platelets of individuals with severe vWD, though no functional abnormalities can, at present, be ascribed to this lack.

VIIIag and vWagII are physically separable from each other by electrophoresis in agarose, molecular exclusion chromatography, and sucrose density gradient ultracentrifugation. They are also antigenically distinct. Antisera from rabbits immunized with highly purified VIIIag fail to recognize vWagII. In addition, highly purified VIIIag does not absorb any of the antibody to vWagII (Fig. 10). Of major significance is the observation that VIIIag and vWagII show a reaction of nonidentity on crossed immunoelectrophoresis of normal plasma (Fig. 9). These latter observations provide direct evidence that vWag II is not a proteolytic digestion product of VIIIag (or vice versa). If vWagII were a fragment of VIIIag it might lack some of the antigenic determinants of native VIIIag and (or) might express neoantigens. However, it would also retain some of the antigens expressed by native VIIIag. Therefore, the two would show a reaction of partial identity and antisera raised against one would recognize the other. Not only did native VIIIag fail to show any antigenic relationship to vWagII, neither did its proteolytic digestion products. In addition, antisera raised to Factor VIII proteolytic digestion products (see Methods) have likewise failed to identify vWagII. A variety of proteolytic inhibitors added to blood immediately after venipuncture failed to affect vWagII levels and clotting of PPP failed to increase vWagII content. Thus, it is unlikely that vWagII is generated in vitro or in vivo as the result of VIIIag proteolysis. However, the absence of antigenic determinants shared by vWagII and VIIIag does not exclude the possibility that both are products of a heretofore unidentified common precursor.

Like vWagII, VIIIag is present in plasma and platelets of normal individuals and absent from those with severe vWD (15, 16, 37). Recent studies of Nachman et al. (38) provide evidence that VIIIag is synthesized by the megakaryocyte. Whether this is true for vWagII remains to be determined.

Although vWagII is released from platelets during clotting, it is unlikely that the presence of vWagII in plasma is the result of in vitro platelet leakage during the drawing of the blood and subsequent plasma separation. Incubation of the blood at 4°, 20°, or 37°C for 30 min before separation of plasma did not cause an increase of vWagII content. One would expect such a leakage to be time- and temperature-dependent as it is for β -thromboglobulin (β -TG) (see below). It is possible that the presence of vWagII in normal plasma is secondary to platelet breakdown or release in vivo. vWagII is not present in erythrocytes, lymphocytes, or granulocytes. Study of other cells is in progress.

There appears to be a weak correlation between vWagII and VIIIag concentration in normal individuals and in patients with severe vWD (Fig. 4). Although patients with moderate forms of vWD as a group have

decreased levels of VIIIag and vWagIII, there is no demonstrable correlation between the two in the group studied.

Although vWagII is released from platelets during clotting as are β -TG (13, 14) and platelet Factor IV (11, 12), it appears to be distinct from them. Platelet Factor IV concentration in serum is 1,000-fold greater than that in plasma, whereas vWagII is only fourfold greater. β -TG levels are increased up to 80-fold in plasma from PRP prepared in EDTA at 20° compared to 4°C. Use of heparin as anticoagulant results in a sixfold rise in β -TG in plasma from PRP prepared at 4°C compared to PRP from EDTA-anticoagulated blood. Plasma vWagII concentration, however, is unaffected by the anticoagulant used (CPD, citrate, EDTA, or heparin) and is unaffected by incubation of whole blood at 4°, 20°, or 37°C for 30 min with any of these anticoagulants. The electrophoretic mobility of vWagII is more anodic (α -mobility) than either β -TG (β -mobility) or platelet Factor IV (γ -mobility).

The function of vWagII is unknown at present. Nevertheless, the description of its deficiency in severe vWD imposes a new level of complexity on the genetics and pathobiology of this disorder.

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