

## von Willebrand factor binds to platelets and induces aggregation in platelet-type but not type IIB von Willebrand disease.

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### Research Article

Platelet-type von Willebrand disease (vWD) and pseudo-vWD are two recently described intrinsic platelet defects characterized by enhanced ristocetin-induced agglutination in platelet-rich plasma. A similar finding is also typical of type IIB vWD, where it has been related to a von Willebrand factor (vWF) rather than a platelet abnormality. Platelet aggregation induced by unmodified human vWF in the absence of other stimuli has been reported in pseudo-vWD. In this study we demonstrate that vWF induces aggregation in platelet-type but not type IIB vWD. Aggregation is observed when normal plasma cryoprecipitate or purified vWF are added to platelet-rich plasma. Cryoprecipitate also aggregates washed platelets, although at higher concentrations than required for platelet-rich plasma. Purified vWF, however, induces significant aggregation of washed platelets only when plasma is added. EDTA inhibits vWF-induced aggregation. Its effect can be overcome by calcium but much less effectively by magnesium ions. Unstimulated platelets in platelet-rich plasma from patients with platelet-type but not type IIB vWD bind 125I-vWF in a specific and saturable manner. All different sized multimers of vWF become associated with platelets. Both aggregation and binding exhibit a similar vWF concentration dependence, suggesting that a correlation exists between these two events. Removal of ADP by appropriate consuming systems is without effect upon such binding or upon vWF-induced aggregation. Thrombin-induced 125I-vWF binding to washed platelets is normal in [...]

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# von Willebrand Factor Binds to Platelets and Induces Aggregation in Platelet-type but Not Type IIB von Willebrand Disease

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**ABSTRACT** Platelet-type von Willebrand disease (vWD) and pseudo-vWD are two recently described intrinsic platelet defects characterized by enhanced ristocetin-induced agglutination in platelet-rich plasma. A similar finding is also typical of type IIB vWD, where it has been related to a von Willebrand factor (vWF) rather than a platelet abnormality. Platelet aggregation induced by unmodified human vWF in the absence of other stimuli has been reported in pseudo-vWD. In this study we demonstrate that vWF induces aggregation in platelet-type but not type IIB vWD. Aggregation is observed when normal plasma cryoprecipitate or purified vWF are added to platelet-rich plasma. Cryoprecipitate also aggregates washed platelets, although at higher concentrations than required for platelet-rich plasma. Purified vWF, however, induces significant aggregation of washed platelets only when plasma is added. EDTA inhibits vWF-induced aggregation. Its effect can be overcome by calcium but much less effectively by magnesium ions. Unstimulated platelets in platelet-rich plasma from patients with platelet-type but not type IIB vWD bind  $^{125}\text{I}$ -vWF in a specific and saturable manner. All different

sized multimers of vWF become associated with platelets. Both aggregation and binding exhibit a similar vWF concentration dependence, suggesting that a correlation exists between these two events. Removal of ADP by appropriate consuming systems is without effect upon such binding or upon vWF-induced aggregation. Thrombin-induced  $^{125}\text{I}$ -vWF binding to washed platelets is normal in platelet-type as well as type IIB vWD. These results demonstrate that a specific binding site for unmodified human vWF is exposed on unstimulated platelets in platelet-type vWD. The relatively high vWF concentrations required for aggregation and binding may explain the lack of significant *in vivo* aggregation and thrombocytopenia in these patients. Moreover, these studies provide additional evidence that platelet-type and type IIB vWD are different diseases with distinct pathogeneses.

## INTRODUCTION

von Willebrand factor (vWF)<sup>1</sup> is a large multimeric glycoprotein that circulates in plasma complexed with the Factor VIII procoagulant activity protein (1). Interaction of vWF with platelets is essential for normal primary hemostasis, as shown by the prolonged bleed-

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<sup>1</sup> *Abbreviations used in this paper:* VIII:C, Factor VIII procoagulant activity; CP, creatine phosphate; CPK, creatine phosphate kinase; EACA,  $\epsilon$ -aminocaproic acid;  $K_d$ , apparent dissociation constant; PMSF, phenylmethylsulfonyl fluoride; U, arbitrary unit of Factor VIII/vWF-related activities;  $V_0$ , void volume; vWD, von Willebrand disease; vWF, von Willebrand factor.

ing time in patients with von Willebrand disease (vWD) (1) and the Bernard-Soulier platelet defect (2).

vWF promotes platelet adhesion to exposed subendothelium (2) but its role in platelet-to-platelet interaction, i.e., platelet aggregation, is not defined. Binding of vWF to specific platelet membrane sites can be induced by the antibiotic ristocetin (3) and, as more recently described, by thrombin (4, 5) and ADP (6). Although platelet aggregation is observed under these circumstances (6, 7), the functional significance and the direct role of vWF have not been determined. Ristocetin is a nonphysiologic agent, and its action is not dependent on active platelet metabolism as it occurs with fixed platelets and in the absence of divalent cations (3). In the case of ADP and thrombin, both agents can activate platelets by vWF-independent pathways, as shown by normal aggregation in patients with severe vWD (8). Therefore, a direct correlation between vWF binding and aggregation has not been established using these two agonists.

Removal of terminal sialic acid residues from human vWF results in direct binding to platelets and aggregation (9). Similar results are observed with unmodified bovine and porcine vWF (10), suggesting that a specific receptor for vWF may be available on the platelet membrane in the absence of other stimuli. However, possible structural differences between the native human molecule and asialo-human, or unmodified bovine and porcine vWF might be responsible for this interaction, and no evidence exists that normal human vWF can interact directly with normal platelets.

Recently, two families with a new bleeding disorder of similar characteristics have been reported independently and designated as "pseudo-vWD" (11) or "platelet-type vWD" (12). In both instances, ristocetin-induced platelet agglutination was enhanced as in type IIB vWD (13). However, there was evidence for a platelet abnormality, as shown by increased binding of normal vWF to patient platelets in the presence of ristocetin (11, 12). Moreover, in pseudo-vWD platelet-rich plasma was aggregated directly by human vWF (11).

In these studies we demonstrate that normal human vWF induces platelet aggregation in platelet-type but not type IIB vWD, thus establishing the distinct nature of these two abnormalities. Accordingly, specific binding of native human vWF to platelets in the absence of any additional stimulus occurs only in platelet-type vWD. Aggregation and binding require similar vWF concentrations, suggesting that they are related.

## METHODS

*Patients.* The patients with platelet-type vWD were one woman and three of her adult sons, all previously reported

in detail (12). The two unrelated patients with type IIB vWD were one man and one woman, both previously described (13). Healthy adult volunteers from the hospital staff served as normal controls. Informed consent was obtained from all donors, and experiments were performed in accordance with the Declaration of Helsinki.

*Sample collection and aggregation studies.* Blood was collected by a two-syringe technique using polypropylene syringes and 19-gauge needles, and immediately transferred into polypropylene tubes containing 1/10th final volume of 3.8% trisodium citrate. For some experiments, blood was drawn using the same citrate concentration but with the addition of apyrase (grade III, Sigma Chemical Co., St. Louis, MO) to achieve a final concentration of 5 U/ml (based on 5'-ATPase activity). For preparation of washed platelets, blood was collected into 1/6th vol of standard acid-citrate-dextrose anticoagulant. Platelets were always handled in polypropylene containers. Platelet-rich plasma was prepared by centrifugation at 900 *g* for 75 s at room temperature (22°–25°C), and platelet-poor plasma by centrifugation at 4,000 *g* for 20 min at 4°C. Samples not immediately tested were stored at –70°C until used. Platelet counts were performed on a Coulter S Plus (Coulter Electronics, Hialeah, FL) or by phase-contrast microscopy. Measurements of platelet aggregation and ATP release were made in a Chrono-log Lumiaggregometer (Chrono-Log Corp., Havertown, PA), using 0.45 ml of patient platelet-rich plasma and 0.05 ml of 40 mg/ml myokinase-free luciferin-luciferase (Sigma Chemical Co.). In some experiments, rubber spacers (Chrono-Log Corp.) were placed under the aggregometer cuvet permitting the use of final volumes of 0.25 ml. The cuvet was maintained at 37°C and the suspension stirred at 1,200 rpm with a magnetic stirrer. Release of ATP was quantitated by the addition of an internal ATP standard at the end of each experimental run. Rate of aggregation was determined from the maximal slope of the initial phase of aggregation, and calculated as change in percent light transmittance over time ( $\Delta\% T \text{ min}^{-1}$ ). The transmittance of platelet-poor plasma was assigned a value of 100% and that of platelet-rich plasma 0%. In the case of washed platelets, appropriate substitutions of buffer for plasma were used for the platelet-poor plasma channel.

*Preparations of washed platelet suspensions.* Washed platelet suspensions for these studies were prepared by the albumin density gradient technique of Walsh et al. (14), or by gel filtration of platelet-rich plasma through Sepharose 2B (Pharmacia Fine Chemicals, Piscataway, NJ). Washed platelets were finally maintained in modified calcium-free Tyrode buffer containing 137 mM NaCl, 2.7 mM KCl, 0.42 mM  $\text{NaH}_2\text{PO}_4$ , 11.9 mM  $\text{NaHCO}_3$ , 2 mM  $\text{MgCl}_2$ , 5.5 mM glucose, and buffered to pH 7.3 with 25 mM Tris HCl or 10 mM Hepes.

*Measurement of Factor VIII/vWF-related activities.* The Factor VIII procoagulant activity (VIII:C) was measured by a modification of the partial thromboplastin time using activated Factor XI (8). The ristocetin cofactor activity of vWF was measured using maximal rates of aggregation of formalin-fixed platelets (15). vWF antigen was quantitated by the Laurell rocket electroimmunodiffusion technique using rabbit antibody (15). All these measurements were expressed as arbitrary units (U) per milliliter. 1 U/ml corresponds to the amount of each of the Factor VIII/vWF-related activities present in 1 ml of a normal plasma pool.

*Purification of vWF.* Cryoprecipitate was prepared by the method of Pool et al. (16) using either 20–30 bags (~200 ml each) of human plasma from different donors or ~10 liters of plasma obtained by plasmapheresis of a single donor.

Sodium heparin (10 U/ml) was added to the plasma immediately after collection. The cryoprecipitate was resuspended at 37°C in 200 ml of a buffer consisting of 20 mM Tris, 20 mM sodium citrate (tribasic), 20 mM  $\epsilon$ -aminocaproic acid (EACA), pH 7, containing 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 10 U/ml Trasylol, and 10 U/ml sodium heparin. The redissolved cryoprecipitate was then adsorbed with  $\text{Al}(\text{OH})_3$  (17). The further purification steps were performed according to Newman et al. (18), as modified by Switzer and McKee (19). Gel filtration was performed on a 5 × 80-cm column of Sepharose CL-4B (Pharmacia Fine Chemicals) equilibrated with a buffer consisting of 20 mM imidazole, 100 mM NaCl, 10 mM sodium citrate (tribasic), 20 mM EACA, pH 6.5, and containing PMSF and Trasylol as indicated. vWF was present in the void volume ( $V_0$ ) protein peak along with VIII:C. The fractions corresponding to the ascending part of the  $V_0$  peak were pooled and concentrated by dialysis against polyethylene glycol (average  $M_r = 40,000$ ). The vWF preparations used in these experiments had a protein concentration of between 0.42 and 1.1 mg/ml and a specific activity of 116–128 U of ristocetin cofactor, 117–135 U of vWF antigen and 47–52 U of VIII:C/mg of protein. The purity of the final product was assessed by electrophoresis in sodium dodecyl sulfate (SDS)-5% acrylamide (5% cross-linking) gels after reduction by 2-mercaptoethanol (20).

**Radioiodination of purified vWF.** This was accomplished with  $^{125}\text{I}$  by the method of Fraker and Speck (21) to a specific activity of between 0.46 and 6.8 mCi/mg. The preparations of  $^{125}\text{I}$ -vWF were characterized by electrophoretic analysis in unreduced SDS-agarose gels (22) as well as in reduced SDS-acrylamide gels (see above).

**Binding experiments.** In a typical binding assay, platelets, at the indicated concentration, were incubated at room temperature with varying concentrations of  $^{125}\text{I}$ -vWF. In experiments performed with platelet-rich plasma, no stimulus was added to the platelets. Fibrinogen levels (23) in the patient plasmas were all within the normal range. In experiments performed with washed platelets, 0.25 NIH U/ml of purified  $\alpha$ -thrombin (the generous gift of Dr. J. W. Fenton II) was used as a stimulus. In parallel mixtures, nonspecific binding was determined in the presence of a 25- to 50-fold excess of unlabeled vWF. After 30 min, 50  $\mu\text{l}$  of the platelet suspension (in duplicate for each experimental point) was layered onto 400  $\mu\text{l}$  of 20% sucrose in modified Tyrode buffer containing 2% bovine serum albumin (Sigma Chemical Co.), pH 7.3. 1-ml conical polypropylene tubes were used. The samples were centrifuged for 4 min at 13,000  $g$  at room temperature in an Eppendorf microcentrifuge (Brinkmann Instruments, Westbury, NJ), the tips of the tubes containing the sedimented platelets were cut with a scalpel, and the platelet-associated radioactivity was measured. Characterization of this binding assay has been published in detail (5). The line best fitted to the experimental points of each binding curve was obtained by means of a nonlinear fit program, and least-squares regression lines for double-reciprocal plots were obtained using proportionate standard error weighting (24), using a desk-top computer model 85, (Hewlett-Packard Co., Palo Alto, CA). The  $^{125}\text{I}$ -vWF that became bound to platelets was analyzed by means of SDS-agarose electrophoresis after lysing the platelet pellets with 5% SDS for 15 min at 60°C, as previously described (5).

## RESULTS

**Characterization of purified vWF.** The purified and radiolabeled  $^{125}\text{I}$ -vWF used in these experiments

appeared homogeneous on SDS-polyacrylamide gel electrophoresis under reducing conditions (Fig. 1). Unreduced  $^{125}\text{I}$ -vWF was composed of a series of multimers with a pattern similar to that observed in normal plasma (Fig. 8, lane 1).

**vWF-induced platelet aggregation.** Platelets in platelet-rich plasma from all four patients with platelet-type vWD aggregated in response to the addition of highly purified vWF and single-donor cryoprecipitate (Fig. 2). However, only one patient showed aggregation in response to normal plasma at the highest possible concentration used (0.225 ml into 0.5 ml final volume). There was no evidence of spontaneous aggregation of patient platelets upon prolonged (10 min) observation in the aggregometer under stirring con-

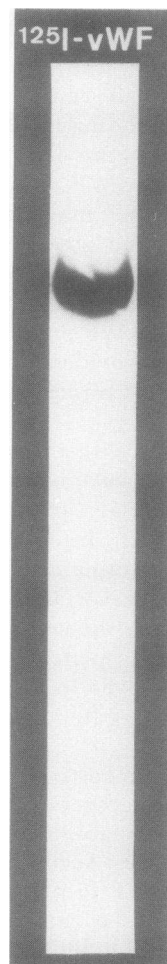


FIGURE 1 Electrophoretic analysis of purified vWF. Direct autoradiography of purified vWF radiolabeled with  $^{125}\text{I}$  ( $^{125}\text{I}$ -vWF) after reduction with 2-mercaptoethanol and electrophoresis in a 5% acrylamide slab gel using a discontinuous buffer system. Cathode at the top.

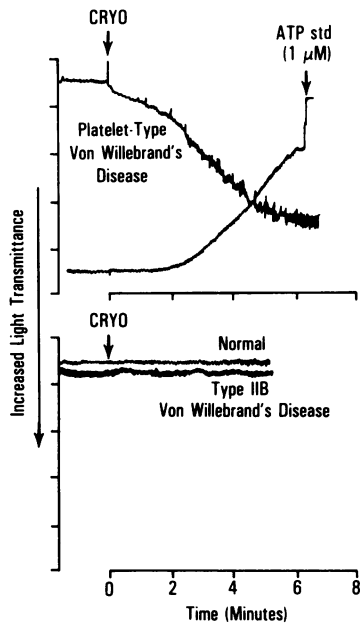


FIGURE 2 vWF-induced platelet aggregation and release reaction in platelet-type vWD. 0.45 ml of patient platelet-rich plasma (186,000/ $\mu$ l) and 0.05 ml of luciferin-luciferase (40 mg/ml) were added to a cuvet maintained at 37°C with constant stirring (1,200 rpm) in a Chronolog Lumi-aggregometer. At arrow, 0.03 ml (1/18th final volume) of single-donor cryoprecipitate was added at a final concentration of 1.2 U/ml ristocetin cofactor activity. *Upper trace*: aggregation. *Lower trace*: continuously monitored ATP release using luciferin-luciferase reaction. Addition of 1  $\mu$ M ATP internal standard at end of run indicates a 2.4  $\mu$ M increase of ATP during platelet release reaction. (*Upper panel*). Lack of aggregation in platelet-rich plasma from a normal individual and a patient with type IIB vWD upon addition of 1/5th final volume of the same cryoprecipitate (4.3 U/ml ristocetin cofactor activity). (*Lower panel*).

ditions and in the absence of added vWF. Aggregation induced by vWF was characteristically immediate, mono- or biphasic, and accompanied by the release of ATP in amounts comparable to those seen with epinephrine, collagen, or ADP stimulation (Fig. 2). No aggregation was observed in five normal volunteers or in the two patients with type IIB vWD under identical conditions (Fig. 2). All these experiments were performed within 3 h from blood sampling and with alternation of normal and patient samples in the aggregometer.

Platelet aggregation induced by vWF in patient platelet-rich plasma was dependent upon the presence of free divalent cations. EDTA, 2.5 mM, added before the addition of cryoprecipitate, blocked aggregation. Aggregation did, however, ensue upon the subsequent addition of excess calcium (5 mM), and was largely complete before clotting of plasma was observed. Mag-

nesium at a 10 mM concentration had a minimal effect in overcoming the effect of 2.5 mM EDTA.

Washed platelets from normal donors were not aggregated by the addition of normal plasma cryoprecipitate. Washed patient platelets, on the contrary, exhibited aggregation in response to cryoprecipitate, but only at higher agonist concentrations than necessary in platelet-rich plasma and with a slower time course. Washed patient platelets suspended in modified Tyrode buffer containing CaCl<sub>2</sub> (0, 0.1, 1.0, or 2.5 mM) showed little or no aggregation upon the addition of purified vWF (Fig. 3 A). In contrast, when washed patient platelets were mixed with platelet-poor plasma (60% of final volume), strong and rapid aggregation was observed in response to purified vWF. The response was identical whether normal (Fig. 3 B) or patient (Fig. 3 C) platelet-poor plasma was used. Such a response was again not seen in washed platelets from normal volunteers. The lack of response of patient platelets in the absence of plasma persisted even when the pH was raised to the values observed in the mixtures with platelet-poor plasma (pH 7.8–8.1). Washed platelets resuspended in platelet-poor plasma showed strong aggregation patterns in response to ADP or epinephrine throughout the course of these experiments.

The possible involvement of ADP in vWF-induced

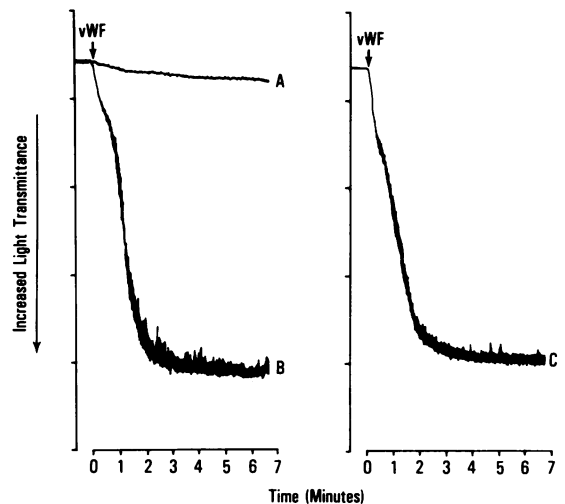


FIGURE 3 vWF-induced aggregation of washed platelets in platelet-type vWD. Gel-filtered patient platelets in modified Tyrode buffer (0.100 ml of 780,000/ $\mu$ l suspension) added to aggregometer cuvet containing (A) 0.125 ml modified Tyrode buffer plus 0.025 ml of 25 mM CaCl<sub>2</sub> in modified Tyrode buffer (final CaCl<sub>2</sub> concentration following addition of platelets, 2.5 mM); (B) 0.150 ml of citrated platelet-poor plasma from a pool of normal donors; or (C) 0.150 ml of the patient's own citrated platelet-poor plasma. Purified vWF (2.9 U/ml ristocetin cofactor activity, final concentration) added at arrow.

aggregation of patient platelets was evaluated by collecting blood in the presence or absence of the ADP-splitting enzyme, apyrase. When blood was collected in citrate containing apyrase (5 U/ml final concentration), the resulting platelet-rich plasma showed rapid and complete reversal of aggregation induced by 8  $\mu$ M ADP (Fig. 4, A and B). In contrast, vWF-induced aggregation of the same platelet-rich plasma was completely unaffected by apyrase (Fig. 4, C and D).

The multimeric structure of the vWF added to the patient platelets was relevant in determining the aggregation response (Fig. 5). When single-donor cryoprecipitate with intact multimeric structure (*left panel, left lane*) was added to patient platelets (1.2 U/ml ristocetin cofactor activity final concentration), strong aggregation resulted (*right panel, cryoprecipitate*). In contrast, commercial concentrate devoid of the larger multimers (*left panel, right lane*) added to these platelets (2.0 U/ml ristocetin cofactor activity final concentration) lacked comparable aggregating activity (*right panel, commercial concentrate*). The commercial concentrate failed to produce aggregation even at a final concentration of 4 U/ml ristocetin cofactor activity, and produced only very mild aggregation at 6 U/ml. Prior addition of the commercial concentrate, moreover, did not inhibit aggregation resulting from the subsequent addition of vWF possessing normal multimeric structure.

Platelet aggregation induced in platelet-rich plasma by both cryoprecipitate and purified vWF was concentration dependent. Both the rate and extent of platelet aggregation increased with increasing concentrations of vWF, until a plateau was reached. When the maximal rate of aggregation was plotted against the final concentration of vWF in the mixture (measured as ristocetin cofactor activity), a dose-response curve was obtained (Fig. 6). The apparent dissociation constant ( $K_d$ ) of the vWF-platelet interaction was estimated from double-reciprocal plots of the aggregation data (Fig. 6). These values appeared of the same order of magnitude when either cryoprecipitate vWF were used (Table I).

*Specific binding of  $^{125}$ I-vWF to unstimulated platelets.* When  $^{125}$ I-vWF was added in increasing concentrations to the platelet-rich plasma of two patients with platelet-type vWD, saturable binding was observed in the absence of any other stimulus (Fig. 7 A). In contrast, platelet-rich plasma from the normal volunteers and the two patients with type IIB vWD showed only nonsaturable binding comparable to the nonspecific binding observed with platelet-type vWD platelets in the presence of excess unlabeled vWF. Nonspecific binding corresponded to <20% of total binding observed in platelet-type vWD (Fig. 7 A). The difference in  $^{125}$ I-vWF binding between normal and patient platelets

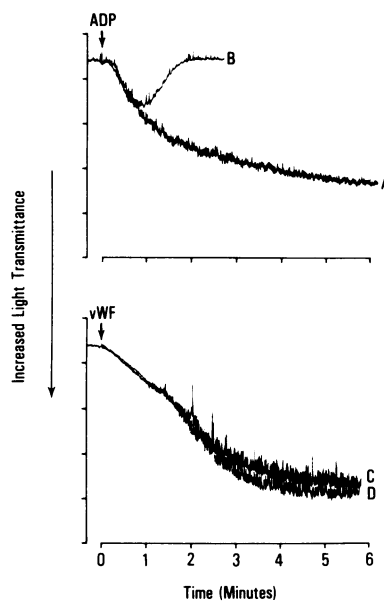


FIGURE 4 Lack of effect of apyrase on vWF-induced aggregation in platelet-type vWD. 0.25 ml of platelet-rich plasma (156,000/ $\mu$ l) from a patient with platelet-type vWD was prepared either from blood collected in citrate alone (0.38% final concentration, curves A and C) or in citrate plus apyrase (5 U/ml final concentration, curves B and D). ADP (8  $\mu$ M final concentration) or purified vWF (2 U/ml ristocetin cofactor activity, final concentration) added at arrow in *upper* or *lower* panels, respectively.

was maintained even when blood was collected in citrate containing apyrase (5 U/ml final concentration). For better comparison with aggregation dose-response curves (Fig. 6), specific binding was also plotted against the concentration of added  $^{125}$ I-vWF expressed as ristocetin cofactor activity (Fig. 7 B). The apparent  $K_d$  derived from double-reciprocal plots of the experimental binding data (Fig. 7 B) was of the same order of magnitude as the apparent  $K_d$  derived from aggregation dose-response curves (Table I).

All multimers present in the  $^{125}$ I-vWF added to the platelet-rich plasma (Fig. 8, lane 1) became associated with platelet-type vWD platelets, as shown by electrophoretic analysis of bound material following solubilization of platelet pellets (Fig. 8, lane 3). No multimers were seen in the lysates of control platelets under the same conditions (Fig. 8, lane 2).

*Binding of  $^{125}$ I-vWF to thrombin-stimulated washed platelets.* Thrombin-stimulated washed platelets from two patients with platelet-type vWD bound  $^{125}$ I-vWF in a manner similar to normal platelets (Fig. 9). The amount of vWF bound was more than three times that bound in platelet-rich plasma without added thrombin (compare Figs. 7 and 9). Removal of ADP either by

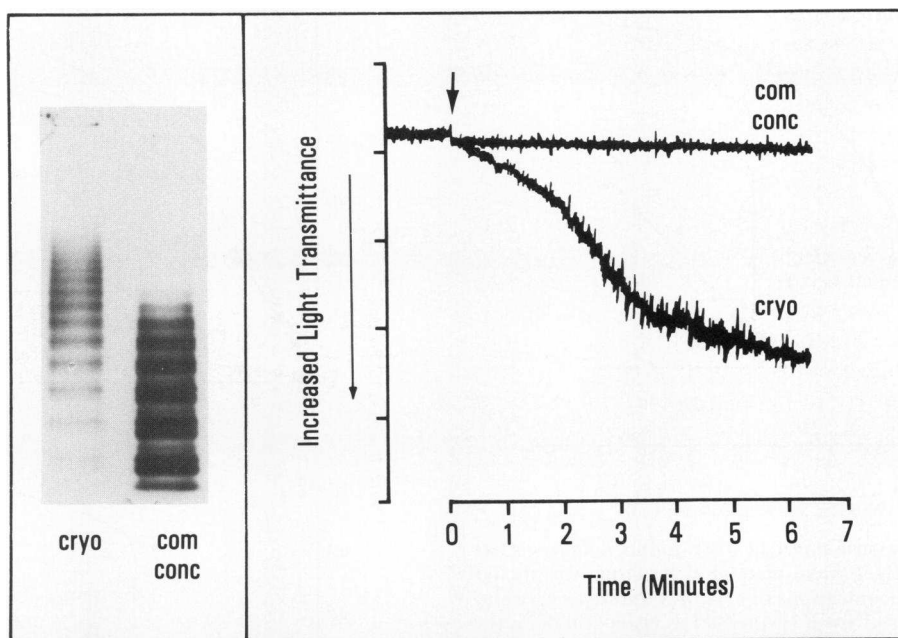


FIGURE 5 Dependence of aggregation response on multimeric structure of vWF in single-donor cryoprecipitate and commercial concentrate. Cryoprecipitate (*left* lane), prepared by the method of Pool et al. (16), was diluted to  $\sim 1$  U/ml of vWF protein (measured by Laurell electroimmunodiffusion). Commercial concentrate (*right* lane), obtained from Alpha Therapeutics, South Pasadena, CA, was diluted to  $\sim 3$  U/ml. The samples were then analyzed in 1.6% agarose-0.1% SDS, as described, and detection of vWF multimers was achieved using specific radiolabeled antibodies (see reference 22). Note the absence of larger vWF multimers in the commercial concentrate, in spite of the higher vWF concentration. Cathode at the top. (*Left* panel.) Washed patient platelets ( $\sim 200,000/\mu\text{l}$ , final concentration) resuspended in citrated platelet-poor plasma showed strong aggregation in response to the cryoprecipitate (1.2 U/ml ristocetin cofactor activity) but not to the commercial concentrate (2.0 U/ml ristocetin cofactor activity). The arrow indicates addition of cryoprecipitate (cryo) or commercial concentrate (com conc). (*Right* panel.)

apyrase (5 U/ml) or by the combination of 7.5 mM creatine phosphate and 12 U/ml creatine phosphate kinase (CP/CPK) resulted in 57–71% reduction of  $^{125}\text{I}$ -vWF binding to thrombin-stimulated patient platelets. These results were similar to those observed with normal platelets. The apparent  $K_d$  for binding to thrombin-stimulated washed platelets, as derived from double-reciprocal plots of experimental data (Fig. 9), was about an order of magnitude lower than the apparent  $K_d$  for binding to platelets in patient platelet-rich plasma without added thrombin (Table II). In the two patients with type IIB vWD, the thrombin-induced binding of vWF to platelets was also similar to normal (Table II).

## DISCUSSION

The addition of normal vWF to platelet-rich plasma from four patients with platelet-type vWD resulted in platelet aggregation that was calcium dependent, irreversible, and accompanied by the platelet release

reaction. Therefore, unmodified human vWF per se is, in these patients, a sufficient stimulus to initiate platelet activation. The larger molecular forms of vWF appear to be necessary to induce aggregation of patient platelets, as shown by the lesser efficacy in this regard of a commercial concentrate deficient in the larger multimers normally found in plasma and cryoprecipitate. Such a finding is in agreement with the hypothesis that these (12) and similar patients (11) lack the larger, but not intermediate and small multimers of vWF in plasma because of preferential absorption of the former to their abnormal platelets.

The aggregation response of platelet-type vWD platelets to the addition of vWF was concentration dependent. These results can be interpreted in accordance with the general occupancy theory by which, in this case, aggregation of platelets is directly proportional to the binding of vWF. A similar approach has been used by others to analyze the fibrinogen-dependent platelet aggregation induced by ADP (25). In

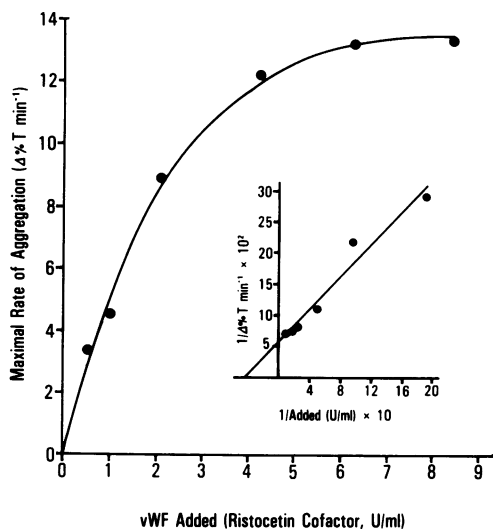


FIGURE 6 Dose-response curve of vWF-induced aggregation in platelet-type vWD. Patient platelet-rich plasma was mixed with increasing concentrations of cryoprecipitate to give the final concentrations of vWF (expressed as ristocetin cofactor activity) indicated. Experimental conditions were as described in Fig. 2. Rate of aggregation was determined from the maximal slope of the initial phase of aggregation, and calculated as change in percent transmittance over time ( $\Delta\% T \text{ min}^{-1}$ ). Transmittance of platelet-poor plasma was taken as 100% and that of platelet-rich plasma as 0%. The *inset* represents the double reciprocal plot of the experimental data.

agreement with this hypothesis, we found that addition of  $^{125}\text{I}$ -vWF to platelet-rich plasma from patients with platelet-type vWD resulted in specific and saturable binding independent of any additional stimulus. Specificity was proven by using a homogeneous vWF preparation and by performing the experiments directly in platelet-rich plasma. This assured that other plasma proteins known to interact with platelets, and notably

TABLE I  
 $K_d$  of vWF-induced Aggregation and  $^{125}\text{I}$ -vWF Binding to Platelets in Platelet-type vWD

	$K_d$ Ristocetin cofactor activity
	U/ml
Aggregation with cryoprecipitate	2.50
Aggregation with purified vWF	1.67
Binding of $^{125}\text{I}$ -vWF	1.95

Experiments were all performed with platelet-rich plasma from patient I. No other stimulus was added to platelets in addition to the vWF. Apparent  $K_d$  values were generated by computer analysis of double-reciprocal plots, and expressed as vWF concentration measured as ristocetin cofactor activity in arbitrary units (U) per ml.

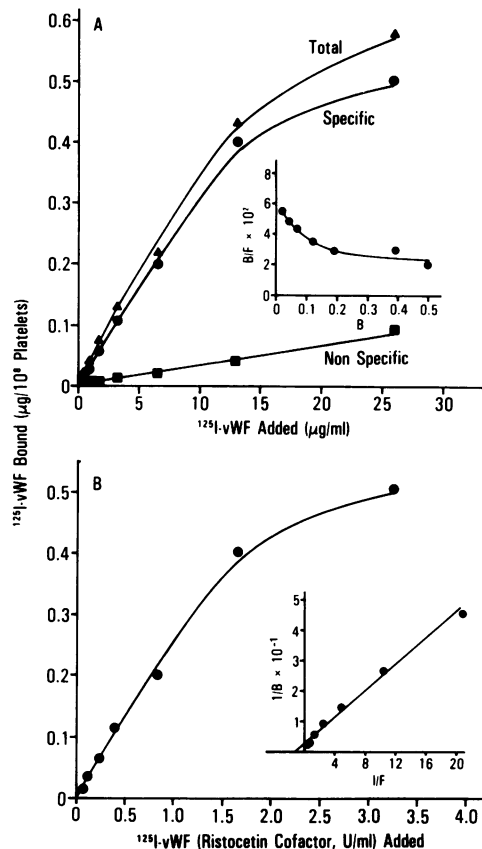


FIGURE 7 Binding of  $^{125}\text{I}$ -vWF to platelets in platelet-type vWD. Patient platelet-rich plasma was mixed with increasing concentrations of  $^{125}\text{I}$ -vWF diluted in modified Tyrode buffer, pH 7.3, containing 2% bovine serum albumin. The final platelet count was  $10^8/\text{ml}$ , and the ratio of plasma to buffer was kept constant. No additional stimulus was added, and the mixtures were incubated at room temperature ( $22^\circ\text{--}25^\circ\text{C}$ ) for 30 min without stirring. After sedimentation of the platelets by centrifugation through a 20% sucrose layer, the platelet-associated radioactivity was measured. (A) The amount of  $^{125}\text{I}$ -vWF bound was calculated from the specific activity of the ligand. Nonspecific binding was determined in the presence of a 50-fold excess of unlabeled vWF and was subtracted from total to calculate specific binding. The *inset* represents the Scatchard plot analysis of specific binding. B is the fraction of  $^{125}\text{I}$ -vWF bound to platelets, and F is free  $^{125}\text{I}$ -vWF in the mixture. (B) Specific binding as shown above, but added vWF is expressed as ristocetin cofactor activity for better comparison with Fig. 6. The *inset* represents the double-reciprocal plot of specific binding. 1/F is the reciprocal of free  $^{125}\text{I}$ -vWF in each mixture, and 1/B is the reciprocal of bound  $^{125}\text{I}$ -vWF.

fibrinogen (25), were in significant molar excess over added vWF.

Evaluation of aggregation dose-response curves and binding isotherms, directly representing experimental points, revealed that the concentrations of vWF required for both phenomena to occur and approach



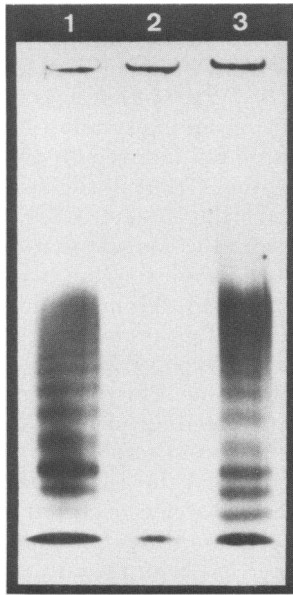


FIGURE 8 Electrophoretic analysis of  $^{125}\text{I}$ -vWF bound to platelet-type vWD platelets. Lane 1,  $^{125}\text{I}$ -vWF used as ligand in the experimental mixtures; lane 2,  $^{125}\text{I}$ -vWF bound to normal platelets; lane 3,  $^{125}\text{I}$ -vWF bound to platelet-type vWD platelets. Bound  $^{125}\text{I}$ -vWF was analyzed after lysis of the platelet pellets with 5% SDS for 15 min at  $60^\circ\text{C}$ . The same amount of  $^{125}\text{I}$ -vWF was added to normal and patient platelet-rich plasma. Analysis was performed by means of electrophoresis in 1.6% agarose slab gels containing 0.1% SDS. After electrophoresis, gels were fixed, washed, dried, and exposed.

saturation were similar. Analysis of these data to derive apparent  $K_d$  values was attempted. Analysis according to Scatchard (26) resulted in curvilinear plots. However, in the absence of direct experimental evidence for the existence of two (or more) binding sites involved in this interaction, we considered that extrapolation of  $K_d$  and total binding values for putative high and low affinity binding sites was an arbitrary procedure (27). This is particularly true for a heterogeneous protein like vWF, since curved Scatchard plots are to be expected with multimeric ligands. Such curved plots may reflect different affinities of individual oligomers and not necessarily heterogeneity of binding sites (28).

Analysis by double-reciprocal plots resulted in significant linear correlations. Two major theoretical problems are inherent in this type of graphic representation: (a) excessive statistical weight of the points corresponding to the lowest ligand concentrations (less accurate measurements); (b) underestimation of the tendency to curvature, if actually present (29). The use of proportionate standard error weighting in the linear regression analysis (24) partly obviates the first

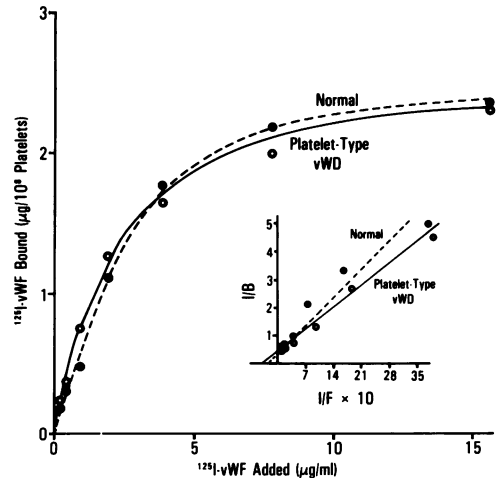


FIGURE 9 Specific binding of  $^{125}\text{I}$ -vWF to washed platelets after thrombin stimulation. Washed platelets resuspended in modified Tyrode buffer, pH 7.3, containing 2% bovine serum albumin were mixed with increasing concentrations of  $^{125}\text{I}$ -vWF in a constant volume. The final platelet count was  $0.5 \times 10^8/\text{ml}$ . Thrombin was added at a final concentration of 0.25 U/ml and mixtures were incubated at room temperature ( $22^\circ\text{--}25^\circ\text{C}$ ) for 30 min without stirring. After sedimentation of the platelets by centrifugation through a 20% sucrose layer, the platelet-associated radioactivity was measured. Specific binding was calculated as indicated in Fig. 7. The inset represents the double-reciprocal plot of specific binding. Note the different order of magnitude on the coordinates as compared with the inset in Fig. 7B.  $1/F$  is the reciprocal of free  $^{125}\text{I}$ -vWF in each mixture, and  $1/B$  is the reciprocal of bound  $^{125}\text{I}$ -vWF.

problem. As to the second point, in the absence of direct experimental evidence, the observation of linear double-reciprocal plots is not sufficient to conclude that different vWF multimers bind with the same affinity to a single class of binding sites on platelets. Nevertheless, the same vWF preparation was used in all these studies, thus eliminating the possible influence on the results of varying the multimeric structure of the ligand. Therefore, the similarity of the apparent  $K_d$  derived from double-reciprocal analysis suggests that binding and aggregation are related. Moreover, the finding of relatively high apparent  $K_d$  values is in agreement with the observation that normal platelet-poor plasma, as opposed to materials enriched in vWF (cryoprecipitate or purified vWF preparations), fails to induce platelet aggregation in some of these patients. More definitive studies must await the isolation of individual vWF multimers of definite size. This is, at present, technically unfeasible.

No evidence for direct interaction of normal or type IIB vWD platelets with normal vWF was observed. As already suggested by studies of ristocetin-induced vWF binding (11–13), these results establish that the vWF

TABLE II  
*K<sub>d</sub>* of <sup>125</sup>I-vWF Binding to Unstimulated and  
 Thrombin-stimulated Platelets

Patients	<i>K<sub>d</sub></i>		<i>r</i>
	vWF	Ristocetin cofactor activity	
	μg/ml	U/ml	
Platelet-rich plasma (Unstimulated)			
Platelet-type vWD I	15.2	1.95	0.975
Platelet-type vWD II	28.9	3.70	0.982
Washed platelets (Thrombin-stimulated)			
Platelet-type vWD I	1.86	0.24	0.980
Platelet-type vWD III	2.34	0.30	0.988
Type IIB vWD I	2.07	0.27	0.977
Type IIB vWD II	2.99	0.39	0.981
Normal I	3.81	0.49	0.922
Normal II	3.45	0.44	0.984

Apparent *K<sub>d</sub>* values were generated by computer analysis of double-reciprocal plots and expressed as vWF concentrations in micrograms per milliliter, or measured as ristocetin cofactor activity in arbitrary units (U) per ml for better comparison with aggregation studies. The correlation coefficient (*r*) of each linear regression plot is indicated. No specific binding to unstimulated platelets in platelet-rich plasma was observed in normal and type IIB vWD. Different Roman numerals identify different patients or normal subjects.

receptor abnormality presumably present on platelet-type vWD platelets is not shared by type IIB vWD, thus confirming the distinct pathogenesis of these two disorders.

Thrombin stimulation of platelets from patients with platelet-type vWD resulted in specific and saturable binding of vWF approximately threefold greater than that observed when vWF was added to platelet-rich plasma without any additional stimulus. The *K<sub>d</sub>* derived from double-reciprocal plots were approximately one order of magnitude smaller than those derived from binding curves generated with platelet-rich plasma in the absence of added thrombin. In this regard, experimental evidence has recently been obtained to show that the vWF-binding site induced by thrombin and ADP is different from that induced by ristocetin (30). Therefore, the possibility exists that the thrombin-induced binding site is also different from the abnormal one present on unstimulated platelets from patients with platelet-type vWD, thus explaining the different binding characteristics. Thrombin-induced binding was similar to normal in both platelet-type and type IIB vWD, thus suggesting that the vWF-binding site involved in this interaction is normal in both disorders.

In recent studies, Fujimoto and Hawiger (6) and Ruggeri et al. (30) have shown that ADP can induce the binding of vWF to human platelets. Thrombin-induced binding of vWF, moreover, is inhibited in the presence of ADP-consuming systems (4, 30). In platelet-type vWD, thrombin-induced binding of vWF was inhibited both by the ADP-splitting enzyme, apyrase, and the ADP-converting system, CP/CPK. This finding is consistent with the concept that the mechanism of thrombin-induced vWF binding is normal in these patients. In contrast to thrombin-induced binding, ADP was not required for the characteristic interaction of vWF with platelet-type vWD platelets in unstimulated platelet-rich plasma. This was demonstrated both with respect to the binding of <sup>125</sup>I-vWF and to the aggregation of platelets in response to vWF. Such results indeed suggest that the ADP-independent binding of vWF to these platelets occurs at a different site than the one involved in thrombin- and ADP-induced binding. The latter has been recently associated with the glycoprotein IIB/IIIa complex (30).

The presence of free calcium ions was necessary for vWF-induced aggregation in the platelet-rich plasma of these patients. Inhibition of aggregation by EDTA could be overcome by calcium, but much less effectively by magnesium. This calcium dependence for platelet aggregation (and secretion) induced by vWF in platelet-type vWD stands in contrast to the calcium-independent agglutination of normal platelets induced by vWF in the presence of ristocetin (31). Washed patient platelets suspended in buffer containing a wide range of calcium concentrations (0–2.5 mM) failed to aggregate in response to purified vWF when plasma was not added. Adjustment of pH in the buffer to that observed in mixtures of washed platelets and platelet-poor plasma did not alter this result. It seems, therefore, that a plasma cofactor other than calcium or pH is involved in vWF-induced aggregation of platelet-type vWD platelets. Normal plasma, as well as the patients' own plasmas, were equally effective in this regard.

Direct autoradiographic studies with <sup>125</sup>I-vWF showed that all multimeric forms of vWF bind to platelet-type vWD platelets in platelet-rich plasma. However, commercial concentrate deficient in the larger vWF multimers was relatively ineffective in inducing aggregation of these platelets. Therefore, it appears possible that although even the smaller vWF multimers bind, they are less effective in promoting aggregation of platelet-type vWD platelets. It is of interest that ristocetin also induces binding of all vWF multimers to normal platelets (30), although it is primarily the larger multimers that are thought to be involved in ristocetin-induced platelet agglutination (32, 33).

The existence of available binding sites for vWF on unstimulated normal human platelets can be inferred from the specific binding of human asialo-vWF (9) or unmodified bovine and porcine vWF (34). However, these binding sites are normally not occupied by unmodified human vWF, suggesting that the affinity for the intact human molecule is too low for any significant interaction to occur at physiological ligand concentrations. In platelet-type vWD, however, a congenital structural change in or near the binding site might be responsible for the observed interaction of platelets with unmodified human vWF in the absence of other stimuli.

Patients with platelet-type vWD and pseudo-vWD may show borderline thrombocytopenia (11, 12), and consistently lack the larger vWF multimers in plasma (11, 12). The apparent  $K_d$  derived from aggregation dose-response curves and binding isotherms show that a relatively high concentration of normal vWF (>1.5 U/ml of ristocetin cofactor activity) is necessary for inducing aggregation at half-maximal rate. Since the plasma levels of ristocetin cofactor activity in these patients are usually well below these values, presumably due to absorption of vWF onto the abnormal platelet membrane, platelet consumption in vivo may be a self-limiting phenomenon. Removal of the larger vWF multimers from plasma without platelet aggregation may explain the prevalence of a bleeding rather than thrombotic tendency in these patients. Similar considerations might be relevant in deciding the amount of plasma or plasma derivatives to be used for therapeutic purposes. Achieving plasma levels of vWF sufficient to stop or prevent bleeding, and yet below the critical concentrations that induce aggregation, might help prevent severe thrombocytopenia in these patients.

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