

Demonstration and Characterization of Specific Binding Sites for Factor VIII/von Willebrand Factor on Human Platelets

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ABSTRACT The presence of specific Factor VIII/von Willebrand factor (FVIII/vWF) binding sites on human platelets has been demonstrated by using ^{125}I -FVIII/vWF and washed human platelets. Binding is ristocetin-dependent and increases in proportion to the concentration of ristocetin from 0.2 to 1 mg/ml. Binding of ^{125}I -FVIII/vWF to platelets can be competitively inhibited by unlabeled human or bovine FVIII/vWF, but not by human thrombin, fibrinogen, α_2 -macroglobulin, equine collagen, or a lectin of *Ricinus communis*. Scatchard analysis of binding data indicated that the dissociation constant of FVIII/vWF receptors is 0.45–0.5 nM. There are 31,000 binding sites per platelet at 1 mg/ml of ristocetin concentration. The optimal pH range for binding is from 7.0 to 7.5. At a concentration of 2 mM, EGTA inhibits 86% of the binding; however, 20 mM of Ca^{++} , Mg^{++} , or EDTA have little effect. Binding sites for FVIII/vWF were found only on platelets, and no significant binding was detected with human erythrocytes or polymorphonuclear leukocytes.

INTRODUCTION

Human Factor VIII/von Willebrand factor (FVIII/vWF)¹ is a plasma glycoprotein which has a mol wt $\approx 1.1 \times 10^6$ and is composed of an undetermined number of 200,000-dalton subunits linked covalently by disulfide bonds (1, 2). This glycoprotein has two

distinct biological activities: one being procoagulant activity (Factor VIII) which can specifically correct the blood coagulation defect of classical hemophilia patients; the other being platelet-aggregating activity, also known as von Willebrand factor activity, which is important for platelet plug formation at the site of vascular injury (3, 4). In von Willebrand's disease, a marked decrease in platelet-aggregating activity results in easy bruising, hemorrhage from mucous membranes, and a prolonged bleeding time (5); these defects can usually be corrected by administration of human FVIII/vWF concentrates. In 1973, Howard et al. (6) reported that the in vitro addition of an antibiotic, ristocetin, to normal platelet-rich plasma caused platelets to clump. In contrast, the addition of ristocetin promoted little or no clumping activity in the majority of platelet-rich plasma samples from patients with von Willebrand's disease (6). These observations were used to develop methods for quantitating plasma von Willebrand factor activity by measuring the rate or degree of aggregation of washed, normal human platelets to which aliquots of test plasma and ristocetin were added (7). At present, the mechanism by which FVIII/vWF protein interacts with platelets to cause aggregate formation in vivo or in vitro is still unknown. Nevertheless, immunofluorescent techniques (8) and the recovery of FVIII/vWF activity from platelet membrane (9) or platelet aggregates (10, 11) suggest that FVIII/vWF binds to human platelets. Such results prompted the hypothesis that binding of FVIII/vWF to specific sites on human platelets may be required for normal platelet aggregation to occur.

We now report evidence for the presence of specific binding sites for FVIII/vWF on human platelets, using a competitive, radiolabeled ligand binding assay. Some of the characteristics of these binding sites are also described.

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¹Abbreviations used in this paper: BSA, bovine serum albumin; FVIII/vWF, Factor VIII/von Willebrand Factor; PEG, polyethylene glycol; PMN, polymorphonuclear leukocyte(s); RBC, erythrocytes.

METHODS

Materials. Intermediate purity human FVIII/vWF concentrates (900 U/vial) and purified human thrombin (180 U/vial) were obtained from American National Red Cross, Bethesda, Md., and the Bureau of Biologics, Bethesda, Md., respectively. Bovine lactoperoxidase was obtained from Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif. A lectin of *Ricinus communis* (RC-60) was from Boehringer Mannheim Biochemicals, Indianapolis, Ind. Human fibrinogen (grade L) was purchased from A. B. Kabi, Stockholm, Sweden. Equine collagen was purchased from Hormon-Chemie, Munich, West Germany. Bovine serum albumin (BSA), EGTA, EDTA, and diisopropylfluorophosphate were purchased from Sigma Chemical Co., St. Louis, Mo. Ristocetin was purchased from Bio/Data Corp., Horsham, Pa. Carrier-free Na^{125}I (20 mCi/ml) was from New England Nuclear, Boston, Mass. α -Chymotrypsin (40 U/mg) was from Worthington Biochemical Corp., Freehold, N. J. Bio-Gel A-15m was from Bio-Rad Laboratories, Richmond, Calif. Sepharose 4B and Sephadex G-25 were from Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J. Lymphocyte separation medium and Hank's balanced salt solution were purchased from Bionetics Laboratory Products, Litton Bionetics Inc., Kensington, Md. and Gibco Diagnostics, Gibco Invenex Div., Chagrin Falls, Ohio, respectively. Plasma gel was from Roger Bellon Laboratoire, France. Polyethylene glycol (Carbowax 4000) was obtained from Union Carbide Corp., Carbon Products Div., New York. Highly purified bovine FVIII/vWF was a gift from Dr. E. W. Davie, Department of Biochemistry, University of Washington, Seattle, Wash. All other reagents were of analytical grade or the best grade available.

Purification of FVIII/vWF protein. The purification procedures for human FVIII/vWF protein from intermediate purity concentrates were the same as previously described from this laboratory (12) with some slight modifications. Polyethylene containers were used throughout, except where it is specifically stated that siliconized glassware was used. Lyophilized, intermediate purity FVIII/vWF concentrate, containing 1,800 U of Factor VIII procoagulant activity, was dissolved in 150 ml of distilled water. The pH of the solution was adjusted to 6.1 with 0.02 M citric acid and then diluted with 2 vol 0.02 M sodium citrate (pH 6.1). Polyethylene glycol (PEG), 40% (wt/vol) in H_2O , was added dropwise to this solution to give a final PEG concentration of 5%. After 15 min, the solution was centrifuged at 7,000 *g* for 10 min. The supernate was removed, and 40% PEG was added to bring the final PEG concentration to 12%. The solution was left at room temperature for 30 min and then centrifuged at 7,000 *g* for 10 min. The precipitate was placed on ice; gently washed once with ice-cold 0.02 M Tris-HCl buffer (pH 7.4); made 8% in ethanol, to remove residual PEG; and then dissolved in 25 ml of a 0.05-M Tris-HCl, 0.15-M NaCl, 1 mM-diisopropylfluorophosphate, and 0.02% NaN_3 solution (pH 7.4). This solution was applied to 4% agarose (Bio-Gel A-15m) packed in a 5 \times 50-cm siliconized glass column. The column was eluted with a solution of 0.05 M Tris-HCl, 0.15 M NaCl, and 0.02% of NaN_3 (pH 7.4) at a flow rate of 25 ml/h. The void-volume fractions, which contained the protein peak having FVIII/vWF activity, were pooled and precipitated at 4°C by adding solid ammonium sulfate until 35% saturation was attained. After 8 h at 4°C the precipitate was collected by centrifugation at 12,000 *g* for 10 min and dissolved in 0.025 M cacodylate, 0.15 M NaCl buffer (pH 6.8). Residual ammonium sulfate in the precipitate was removed by dialysis at 4°C against the same

buffer. The purified FVIII/vWF was stored at 4°C in a final concentration of 1.5–2.5 mg/ml. The purity of the final product was analyzed by electrophoresis on sodium dodecyl sulfate-6M urea-5% polyacrylamide gels before and after reduction by β -mercaptoethanol (12, 13).

Radioiodination of FVIII/vWF. Highly purified FVIII/vWF was labeled with ^{125}I by the Sepharose 4B-lactoperoxidase method of David and Reisfeld (14). The iodination was performed by mixing the solutions in the following sequence: 50 μl of Na^{125}I (1 mCi/0.1 ml) in 0.1 N NaOH; 50 μl of 0.1 N HCl; 10 μl of 1.1 mM KI; 100 μl of 50% (vol/vol) Sepharose 4B-lactoperoxidase suspended in 0.01 M sodium phosphate, 0.1 M NaCl buffer (pH 7.0); and 0.5 ml of FVIII/vWF (2 mg/ml) dissolved in 0.01 M sodium phosphate, 0.1 M NaCl buffer (pH 7.0). The reaction was initiated by adding 5 μl of 6.7 mM H_2O_2 ; the solution was then left at room temperature and stirred frequently for the next 30 min. The reaction was quenched by adding 15 μl of 2.5 M NaN_3 . The solution was centrifuged, and the supernate was applied to a 1 \times 15-cm Sephadex G-25 column which had been equilibrated with 0.025 M cacodylate, 0.15 M NaCl buffer (pH 6.8), and pretreated with 2 ml of 1% BSA dissolved in the same buffer. The column was eluted with 0.025 M cacodylate, 0.15 M NaCl buffer, and 0.5-ml aliquots were collected and counted in a gamma counter (model 4000, Beckman Instruments, Inc., Fullerton, Calif.). Usually, two peaks were obtained: the first peak was iodinated FVIII/vWF, and the second was free ^{125}I . The fractions of the first peak which contained the iodinated FVIII/vWF were pooled, and the specific activity of the preparation was determined by measuring the radioactivity and the protein concentration (15). This iodination procedure routinely yielded ^{125}I -FVIII/vWF with a specific radioactivity of 0.3–0.4 $\mu\text{Ci}/\mu\text{g}$. The platelet-aggregating activity of the iodinated FVIII/vWF was assayed by the ristocetin-induced platelet aggregation method as described previously (16).

Preparation of washed platelets, erythrocytes (RBC), and polymorphonuclear leukocyte (PMN). With a two-syringe technique, 18 ml of venous blood were drawn into 2 ml of 3.8% sodium citrate. After immediate mixing, the blood sample was centrifuged at 250 *g* for 10 min at room temperature to obtain platelet-rich plasma and sedimented RBC. The RBC were saved while the plasma was further centrifuged at 6,000 *g* for 10 min at room temperature to sediment the platelets. The platelet pellet was washed five times by resuspending it in 16 ml of 0.025 M Tris-HCl, 0.15 M NaCl, 0.01 M EDTA (pH 7.4), and then centrifuging at 6,000 *g* for 10 min at room temperature. After the last wash, the platelets were resuspended in 0.025 M Tris-HCl, 0.15 M NaCl, 0.1% BSA (pH 7.4). The RBC were washed in a similar fashion except that the centrifugation was carried out at 300 *g*. To each milliliter of packed RBC, 8 ml of washing buffer was added.

PMN were prepared according to the method of Bøyum (17). 20 ml of venous blood was drawn into a heparinized syringe which contained 5 ml of plasma gel. After mixing the blood with the gel, the syringe was placed vertically in an incubator at 37°C. RBC sedimented in the syringe by 60 min. The PMN-rich plasma was collected from the top of the syringe and layered on top of a tube containing 12 ml of the lymphocyte separation medium. The PMN pellet was collected by centrifuging at 400 *g* for 30 min at room temperature, washed twice in Hank's balanced salt solution (pH 7.4), and resuspended in the same solution. The concentration of cells in the suspension was determined with a hemacytometer and phase-contrast microscopy and adjusted to the final desired concentration with an appropriate volume of the same buffer.

FVIII/vWF binding assay. The assay was done at room temperature in 12×75 -mm polystyrene culture tubes in a final vol of 0.5 ml. Each incubation tube contained the following: $0.1 \mu\text{g}$ ^{125}I -FVIII/vWF in $100 \mu\text{l}$ 0.025 M Tris-HCl, 0.15 M NaCl, 0.1% BSA (pH 7.4); selected amounts of unlabeled FVIII/vWF in $100 \mu\text{l}$ of the above buffer; 0.5 mg ristocetin in $100 \mu\text{l}$ of the same buffer as above, but without BSA; and $100 \mu\text{l}$ of the above buffer. Incubations were begun by adding $100 \mu\text{l}$ of platelet suspension (5×10^6 cells/ $100 \mu\text{l}$, unless otherwise stated). After reaching equilibrium, the incubations were stopped by adding 1 ml of ice-cold 0.025 M Tris, 0.15 M NaCl buffer (pH 7.4). Tubes were immediately centrifuged at $3,500 \text{ g}$, 4°C , for 20 min to separate the bound from the free FVIII/vWF. The supernate which contained free FVIII/vWF was aspirated, and the pellet which contained bound FVIII/vWF was counted. The assay for each point was performed in duplicate. The variation between duplicate assays was always $<4\%$. The specific binding of ^{125}I -FVIII/vWF to platelets was determined by performing incubations in the presence and absence of excess amounts of unlabeled FVIII/vWF ($30 \mu\text{g}$). The bound ^{125}I -FVIII/vWF which could not be displaced by the presence of excess amounts of unlabeled FVIII/vWF represented nonspecific binding. The difference in radioactivity between incubations with and without excess unlabeled FVIII/vWF was defined as specific binding. The free ^{125}I -FVIII/vWF is derived by subtracting the bound counts from the total counts in each incubation mixture. In this study, the nonspecific binding was only 2–3% of the total ^{125}I -FVIII/vWF contained in each incubation mixture. It was found that filtration methods cannot be used to separate the bound FVIII/vWF from the free FVIII/vWF because the FVIII/vWF protein sticks to the millipore or glass fiber filter. Dissociation of ^{125}I -FVIII/vWF binding from platelets was studied at room temperature by adding unlabeled FVIII/vWF ($50 \mu\text{g}/0.5 \text{ ml}$) into incubations which had reached equilibrium. Specific binding was then determined at the specified time intervals. Unlabeled FVIII/vWF was not added to the control tubes. The increase of nonspecific binding with time was corrected at each point by subtracting the value obtained for an incubation mixture which contained $30 \mu\text{g}$ of unlabeled FVIII/vWF before the chase quantity of FVIII/vWF was added.

RESULTS

After reduction, the purified FVIII/vWF showed the usual single, $\approx 200,000$ -dalton protein band by sodium dodecyl sulfate gel electrophoresis. Only one immunoprecipitin line was seen when $3 \mu\text{g}$ of purified FVIII/vWF was diffused against $5 \mu\text{l}$ of unadsorbed rabbit anti-FVIII/vWF on 1% agarose slides. After radioiodination and reduction, sodium dodecyl sulfate gel analyses of the ^{125}I -FVIII/vWF showed a single protein band which corresponded to a single peak of radioactivity when sliced and counted. No detectable loss of platelet-aggregating activity of ^{125}I -FVIII/vWF was observed when assayed by the ristocetin-induced platelet aggregation method with unlabeled FVIII/vWF as a standard.

Effect of incubation time, numbers of platelets, and ristocetin concentration on binding. The time-course of ^{125}I -FVIII/vWF binding to human platelets at different concentrations of ristocetin was studied.

Fig. 1 shows that 2 h of incubation time were required for the binding to reach equilibrium when 3×10^6 platelets per incubation were used, regardless of the concentration of ristocetin. Binding reached equilibrium at 100 min when 5×10^6 platelets per incubation were used, whereas equilibrium was attained at 40 min when 8×10^6 platelets per incubation were present. These results suggest that the time required to reach equilibrium depends upon platelet concentration, but not upon ristocetin concentration.

The effect of ristocetin concentration on the binding of ^{125}I -FVIII/vWF when incubated with different concentrations of platelets is shown in Fig. 2. The results clearly demonstrate that if the period of incubation is now held constant, the amount of ^{125}I -FVIII/vWF which becomes bound to platelets depends upon the concentration of ristocetin in the incubation mixture. Binding increases in a cooperative manner as the concentration of ristocetin increases, being linear between ristocetin concentrations of 0.2 and 1.0 mg/ml . At ristocetin concentrations above 1 mg/ml , the amount of binding gradually approaches a plateau. This phenomenon was most obvious at the higher platelet concentration, 8×10^6 cells per incubation, in which case binding clearly leveled off after 1.0 mg/ml of ristocetin.

Kinetic study of ^{125}I -FVIII/vWF binding to platelets. The above studies indicate that the binding of ^{125}I -FVIII/vWF to platelets is ristocetin-dependent. To study how ristocetin might promote FVIII/vWF binding, the following experiments were performed. A constant number of platelets (5×10^6 cells per mixture)

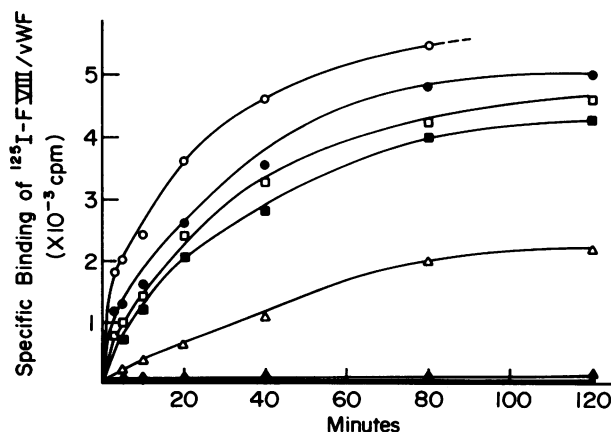


FIGURE 1 Time-course of ^{125}I -FVIII/vWF binding to human platelets at different concentrations of ristocetin. Platelets (3×10^6 cells) were incubated with $0.1 \mu\text{g}$ of ^{125}I -FVIII/vWF ($\approx 15,000 \text{ cpm}$) at specified concentrations of ristocetin. The key to the concentration of ristocetin used for each incubation is as follows: (\circ), 1.5 mg/ml ; (\bullet), 1.0 mg/ml ; (\square), 0.8 mg/ml ; (\blacksquare), 0.6 mg/ml ; (\triangle), 0.3 mg/ml ; (\blacktriangle), 0.1 mg/ml ; ($-$), 0 mg/ml .

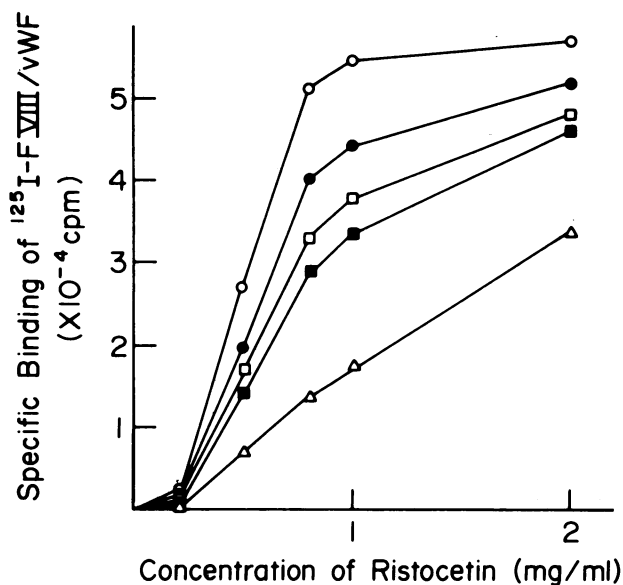


FIGURE 2 Effect of different concentrations of platelets and ristocetin on the specific binding of ^{125}I -FVIII/vWF. The specific binding of 8×10^6 platelets per incubation (\circ); 6×10^6 platelets per incubation (\bullet); 5×10^6 platelets per incubation (\square); 4×10^6 platelets per incubation (\blacksquare); or 2×10^6 platelets per incubation (\triangle) was examined at the different concentrations of ristocetin shown on the abscissa. All incubations were done at 24°C for 120 min. The ^{125}I -FVIII/vWF added in each incubation is $0.1 \mu\text{g}$ ($\approx 65,000$ cpm).

was incubated with increasing amounts of ^{125}I -FVIII/vWF at two concentrations of ristocetin (1 or 0.5 mg/ml). The results are illustrated in Fig. 3. The specific binding of ^{125}I -FVIII/vWF initially increased as the concentration of ^{125}I -FVIII/vWF increased, but eventually a plateau was reached. This result indicates that the binding of ^{125}I -FVIII/vWF to platelets is a saturable process, and that the specific binding at 1 mg/ml of ristocetin is greater than at 0.5 mg/ml of ristocetin. Nonspecific binding, which represents non-displaceable, bound ^{125}I -FVIII/vWF, was determined by adding excessive amounts of unlabeled FVIII/vWF (300 times the concentration of ^{125}I -FVIII/vWF). Fig. 3 shows that nonspecific binding increased linearly with increasing concentrations of ^{125}I -FVIII/vWF and was therefore nonsaturable. It is noteworthy that nonspecific binding is the same at either concentration of ristocetin. This result suggests that nonspecific binding is not affected by different concentrations of ristocetin, at least up to 1 mg/ml.

The binding data were also analyzed by Scatchard plot (18) to determine the dissociation constant of binding and the total number of binding sites. The Scatchard analysis in Fig. 4A shows that the apparent dissociation constant (K_d) is 0.46 and 0.5 nM at concentrations of ristocetin of 1.0 and 0.5 mg/ml, respectively. The total concentrations of binding sites deter-

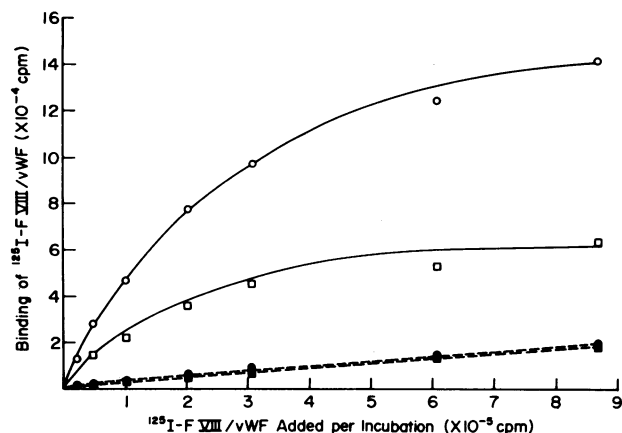


FIGURE 3 Saturable binding of ^{125}I -FVIII/vWF to washed, human platelets. Incubations were performed at 24°C with a constant amount of platelets (5×10^6 cells per incubation) and increasing concentrations of ^{125}I -FVIII/vWF at two different concentrations of ristocetin: (\circ), 1 mg/ml of ristocetin and (\square), 0.5 mg/ml of ristocetin. The solid lines represent specific binding; the broken lines represent nonspecific binding.

mined from the intercepts of the abscissa were 0.52 nM (31,000 sites per platelet) and 0.23 nM (13,700 sites per platelet), respectively; these results are calculated assuming univalency for the FVIII/vWF molecule of 1.1×10^6 daltons. In Fig. 4B, these values for the binding affinity and receptor concentration were validated by Scatchard analysis of the data obtained from the competition study illustrated in Fig. 5. These results confirmed that a significantly greater number of total specific binding sites are present at the higher ristocetin concentration, but that the binding affinities are about the same at either concentration of ristocetin.

Specificity of the ^{125}I -FVIII/vWF binding to platelets. The specificity of FVIII/vWF binding sites on platelets was studied by incubating $0.1 \mu\text{g}$ of ^{125}I -FVIII/vWF with various concentrations of unlabeled human FVIII/vWF, thrombin, fibrinogen, α_2 -macroglobulin, bovine FVIII/vWF, equine collagen, or RC-60. Fig. 5 shows that only unlabeled human or bovine FVIII/vWF can compete with ^{125}I -FVIII/vWF for the binding, and that bovine FVIII/vWF is somewhat less potent in competing for binding than human FVIII/vWF. Human thrombin, fibrinogen, α_2 -macroglobulin, equine collagen, and RC-60, even at very high concentrations (at least ≥ 800 -fold molar excess of ^{125}I -FVIII/vWF added), could not compete, despite the fact that most of these proteins are either known or believed to interact with platelets; some induce aggregation and/or release cytoplasmic granules (20, 21).

The binding of ^{125}I -FVIII/vWF to human RBC or PMN in the presence of ristocetin was also tested. Table I shows that only insignificant amounts of ^{125}I -FVIII/vWF can bind to these two types of blood

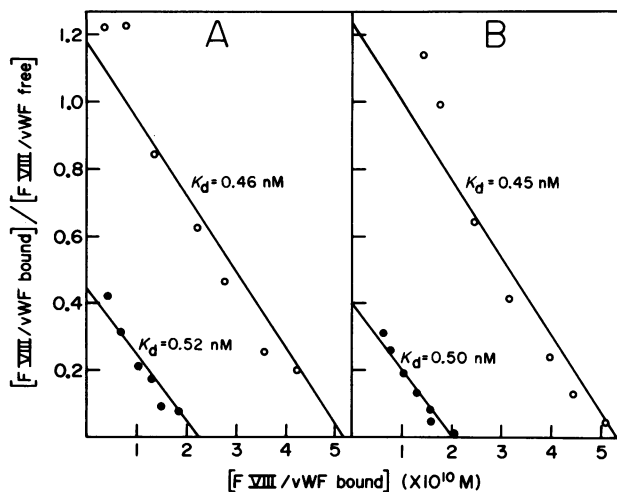


FIGURE 4 Scatchard plots of ^{125}I -FVIII/vWF binding to receptors at two concentrations of ristocetin: (○), 1 mg/ml or (●), 0.5 mg/ml. (A) Scatchard plot of the data from Fig. 3. The concentration of free FVIII/vWF is the molar concentration of free ^{125}I -FVIII/vWF in each incubation. K_d is the apparent dissociation constant. (B) Scatchard plots of the data from Fig. 5. [FVIII/vWF bound] is the molar concentration of total specifically bound FVIII/vWF, including both iodinated and noniodinated species in each incubation. [FVIII/vWF free] is the molar concentration of total free FVIII/vWF in each incubation. All of the Scatchard plots were drawn using the least squares method (19). The assumption of a one-to-one binding ratio between FVIII/vWF molecule and receptor is made for the Scatchard analysis. The molecular weight of FVIII/vWF is taken as 1.1×10^6 .

cells. We found that small numbers of platelets contaminated the preparations of RBC and PMN (2.5 and 10.6%, respectively). When the number of washes for the RBC was increased from 5 to 12 times, the amount of specifically bound ^{125}I -FVIII/vWF was reduced to 2.5%. Increasing the number of times the platelets were washed had no effect on the amount of FVIII/vWF which became bound to them. Hence, it is most likely that the ^{125}I -FVIII/vWF which bound to the RBC or PMN resulted from the contamination of these cells by small numbers of platelets. The sum of these studies supports the notion that the binding sites for FVIII/vWF are unique to platelets.

Effect of Mg^{++} , Ca^{++} , EDTA, EGTA, and nucleotides on the binding of ^{125}I -FVIII/vWF to platelets. The importance of Mg^{++} , Ca^{++} , and different nucleotides on the binding of drug and hormone receptors is well known (22–24). The results shown in Table II indicate that Ca^{++} and Mg^{++} are not important for the binding of ^{125}I -FVIII/vWF to platelets. Some inhibitory effects were observed at relatively high concentrations (20 mM) of Ca^{++} , Mg^{++} , or EDTA; however, no effect on binding was observed when 2 or 20 μM of ATP, ADP, AMP, adenosine, guanosine triphosphate, guanosine diphosphate, or guanosine monophosphate

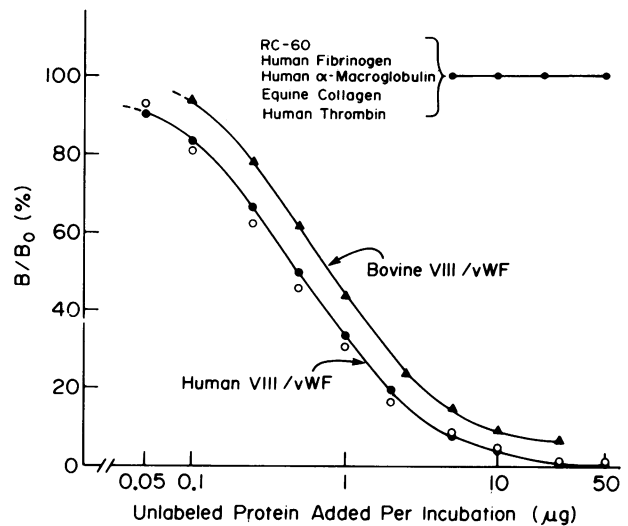


FIGURE 5 Competition curve of ^{125}I -FVIII/vWF binding to washed, human platelets. The experiments were performed by incubating a constant amount of platelets (5×10^6) and ^{125}I -FVIII/vWF (0.1 μg /incubation) with specified concentrations of unlabeled proteins (0–50 μg); B defines the amount of ^{125}I -FVIII/vWF which specifically binds to platelets and cannot be displaced by amounts of unlabeled FVIII/vWF as specified on the abscissa. B_0 is the total specific binding which is the difference in counts per minute between platelet pellets from incubations with and without 50 μg of unlabeled FVIII/vWF. B/B_0 gives the ratio of total specific binding which remains. Two concentrations of ristocetin were used: (○), 0.5 mg/ml and (●, ▲), 1.0 mg/ml.

were used. In contrast, EGTA was highly inhibitory. At a concentration of 2 mM, EGTA inhibited 86% of ^{125}I -FVIII/vWF binding. The inhibitory effect of EGTA cannot be attributed to its Ca^{++} chelating ability because EDTA, Ca^{++} , and Mg^{++} do not affect binding. Thus, the inhibitory effect of EGTA must be a result of other of its physiochemical properties.

Effect of pH on the binding of ^{125}I -FVIII/vWF to platelets. This study was performed in 0.025 M Tris-maleate-NaOH, 0.15 M NaCl buffers ranging from pH

TABLE I
Binding of ^{125}I -FVIII/vWF to Platelets, RBC, and PMN

Type of cells	Specific binding
	cpm/5 $\times 10^6$ cells
Platelet	36,058
RBC	2,736
PMN	5,165

^{125}I -FVIII/vWF (0.1 μg) and 5×10^6 cells (platelets, RBC, or PMN) were incubated with and without 30 μg of unlabeled FVIII/vWF for 100 min at 24°C , and the specific binding was then calculated as described in Methods. Each value is the mean of duplicate experiments and has a variation of <4%.

TABLE II
Effects of Ca^{++} , Mg^{++} , EDTA, and EGTA on the Binding of ^{125}I -FVIII/vWF to Washed, Human Platelets

	Final concentration in incubate	Specific binding	Percentage of control
	mM	cpm/5 $\times 10^6$ cells	%
Control	—	33,154	100
CaCl_2	2	31,052	94
	10	26,831	81
	20	22,603	68
MgCl_2	2	32,059	97
	10	30,173	91
	20	25,279	76
EDTA	2	32,044	97
	10	30,735	93
	20	31,647	87
EGTA	2	4,771	14
	10	1,679	5
	20	1,463	4.4

The technique for determining specific binding is described in Methods. The pH of all incubations was 7.4. Each specific-binding value is the mean of duplicate experiments, the variation between duplicates is <4%.

5.3 to pH 9.0. Fig. 6 shows that maximum binding occurs between pH 7.0 and pH 7.5. On either side of this range, binding is reduced drastically. These values of optimal pH for the binding of ^{125}I -FVIII/vWF to platelets in the presence of ristocetin are essentially the same as reported for FVIII/vWF-dependent, ristocetin-induced platelet aggregation (25, 26).

Dissociation of specifically bound ^{125}I -FVIII/vWF from platelets. The dissociation of specifically bound ^{125}I -FVIII/vWF from platelets was investigated at room temperature by adding a chase quantity of unlabeled FVIII/vWF into incubations in which binding had reached equilibrium. Fig. 7 shows that the dissociation of bound ^{125}I -FVIII/vWF is very slow. Initially a relatively rapid dissociation is observed, but then the dissociation gradually ceases at longer incubation times. Because of the loss of reversibility of the binding after long-term incubation, kinetic analysis of the dissociation of bound ^{125}I -FVIII/vWF was not practical. Similar phenomena have been observed previously with peptide hormone receptors for prolactin (27) and gonadotropin (28). Fig. 7 shows that if the ristocetin concentration is reduced to 0.1 mg/ml at zero time, the amount of ^{125}I -FVIII/vWF becoming dissociated is increased. At 6 h only 45% of that bound at zero time remained on fresh, washed platelets; beyond 6 h, no further dissociation occurred. Fig. 7 also shows that if formalin-fixed platelets are used and the ristocetin concentration is reduced to 0.1

mg/ml at zero time, $\approx 90\%$ of the bound ^{125}I -FVIII/vWF becomes dissociated by 6 h.

Binding of ^{125}I -FVIII/vWF to platelets treated with chymotrypsin. It has been demonstrated that platelet membrane proteins are hydrolyzed during incubation with trypsin (29) or chymotrypsin (30). The binding sites of FVIII/vWF are presumed to be on the membrane of platelets because of the high molecular weight of FVIII/vWF. If the membrane binding sites for FVIII/vWF are protein, they might be susceptible to enzymatic digestion and, therefore, the specific binding of ^{125}I -FVIII/vWF should become diminished. This hypothesis is supported by this study. Table III shows that the specific binding of ^{125}I -FVIII/vWF to platelets did decrease progressively as platelets were preincubated with increasing concentrations of α -chymotrypsin.

DISCUSSION

Our studies demonstrate that high affinity specific binding sites for FVIII/vWF exist on human plate-

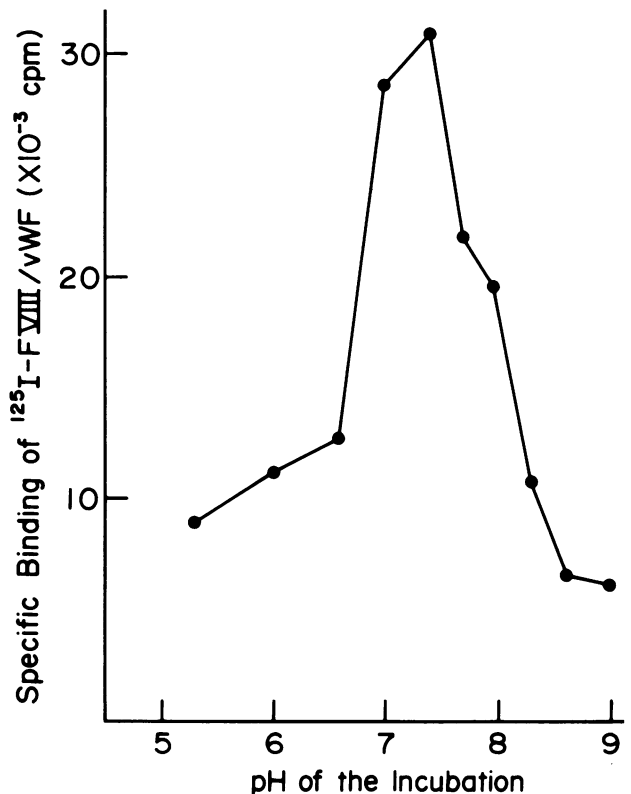


FIGURE 6 Effect of pH on binding of ^{125}I -FVIII/vWF to washed human platelets; 0.025 M Tris-maleate-NaOH, 0.15 M NaCl buffer ranging in pH from 5.3 to 9.0 was used for these experiments. Specific binding was determined as described in Methods. The pH at each point is the final pH of incubation.

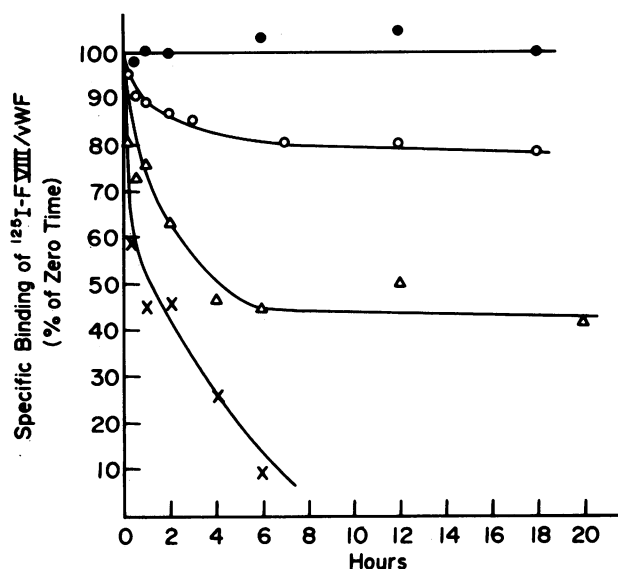


FIGURE 7 Dissociation of specifically bound ^{125}I -FVIII/vWF from washed (\bullet , \circ , Δ) and formalin-fixed (\times) platelets. The closed circles give the control curve, and the open circles give the amount of dissociation of ^{125}I -FVIII/vWF in the presence of 1.0 mg/ml of ristocetin. The triangles represent the degree of dissociation when the ristocetin is diluted to 0.1 mg/ml. The curve given by the (\times) indicates the amount of dissociation which occurs when formalin-fixed platelets are used and the ristocetin concentration is reduced to 0.1 mg/ml.

lets. Uniquely, ristocetin, an antibiotic with a mol wt $\approx 5,000$ (31), is essential for the binding of FVIII/vWF to the platelet membranes. Two possible mechanisms, either of which could account for the promotion of specific binding by ristocetin, were considered in our studies: (a) ristocetin enhances the binding affinity and therefore shifts the equilibrium toward binding; and (b) ristocetin somehow increases the number of accessible binding sites for FVIII/vWF on platelets. Our results clearly demonstrate that ristocetin increases the number of accessible binding sites on platelets; however, the exact mechanism by which this is accomplished is still unclear. By using highly negatively charged molecules (11, 25) or chemically modified

ristocetin and electrometric titration (32), other investigators have suggested that ristocetin neutralizes negative charges on the platelet surface and that this effect somehow promotes the interaction of platelets with FVIII/vWF and, subsequently, platelet aggregation. This hypothesis is also supported by the report that the electrophoretic mobility of platelets becomes altered when exposed to ristocetin, presumably by ristocetin neutralizing the surface negative charges of platelets (33). Yet, Baugh et al. (34) could not show that ^{125}I -labeled ristocetin became bound to platelets during ristocetin-induced platelet aggregation, suggesting perhaps that charge-neutralizing effects may not be the fundamental explanation for the role of ristocetin as a cofactor for von Willebrand activity.

It is significant that bovine FVIII/vWF aggregates and adsorbs to human platelets without ristocetin (11). Such data may indicate that bovine FVIII/vWF itself has a ristocetin-like activity in addition to the other activities usually assigned to FVIII/vWF. This possibility is supported by the recent finding that bovine FVIII/vWF neutralizes the surface negative charges of platelets in the absence of ristocetin (33). Our studies definitely demonstrate that bovine FVIII/vWF competes with ^{125}I -labeled human FVIII/vWF for the same binding site. When considered collectively, the findings suggest that ristocetin neutralizes negative charges on the platelet membrane, thereby forming or making the FVIII/vWF binding site accessible.

Recently Jenkins et al. (30) reported that membrane glycoprotein I of human platelets is involved in FVIII/vWF-dependent, ristocetin-induced platelet aggregation. Furthermore, Nachman et al. (35) demonstrated that ristocetin-induced platelet aggregation was inhibited by rabbit antiserum to glycoprotein I. Okumura and Jamieson (36) reported that another glycoprotein (glycocalicin), which functions as a thrombin receptor in the exterior coat of platelets, inhibited ristocetin-induced platelet aggregation. These authors further demonstrated that glycocalicin is immunologically and electrophoretically similar to membrane glycoprotein I of platelets (37, 38). Thus the possibility that FVIII/vWF shares the same receptor

TABLE III
Binding of ^{125}I -FVIII/vWF to α -Chymotrypsin-Treated Human Platelets

Concentration of chymotrypsin, $\mu\text{g/ml}$	0	10	50	100
Specific binding, $\text{cpm}/5 \times 10^6 \text{ cells}$	$15,374 \pm 353$	$14,171 \pm 88$	$11,060 \pm 135$	$7,372 \pm 282$

3 ml of washed, human platelets were prepared from 5 ml of venous blood and incubated with the specified concentrations of α -chymotrypsin for 15 min at 37°C . The platelets were then collected by centrifugation, washed twice, and diluted to an appropriate concentration for the assay. Each specific-binding value is the mean \pm SEM for three determinations at each chymotrypsin concentration.

with thrombin was proposed. Results of our study, however, demonstrate that human thrombin does not compete with FVIII/vWF for binding sites. We also observed that 1 mg/ml of ristocetin did not inhibit platelet aggregation by human thrombin (0.05 NIH U/ml).² Thus the possibility that ristocetin inhibits thrombin action is unlikely. Hence separate receptors appear to exist for the binding of FVIII/vWF and thrombin. Although no direct experiments were performed to correlate binding of FVIII/vWF to the platelets with platelet-aggregating activity, we found that the optimal pH (7.0–7.5) for binding does correspond to the values of optimal pH (7.0–7.5) for ristocetin-induced platelet aggregation (25, 26). This correspondence suggests a parallel relationship between the degree of binding and aggregating activity. Weiss et al. have also shown that the extent and rate of platelet aggregation are related to the concentration of ristocetin (39). Our results coupled with their observations suggest that binding of FVIII/vWF can be correlated with ristocetin-induced platelet aggregation.

In conclusion, by applying competitive protein binding assay techniques with ¹²⁵I-labeled FVIII/vWF as a tracer, we have identified a class of FVIII/vWF binding sites on human platelets. The binding characteristics fulfill the criteria for receptors in terms of high affinity, saturability, and specificity; however, the functional correlation of binding and biological effect, i.e., platelet aggregation, is not included here. The fact that binding depends upon the presence of ristocetin, a glycopeptide antibiotic, is an unique feature which has not been observed before in other systems. The ristocetin dependency of binding suggests the existence of such a mechanism in vivo by which platelet aggregation reactions are regulated to allow an innocuous coexistence of FVIII/vWF and platelets in circulating blood. Finally, the establishment of a FVIII/vWF binding assay provides a new approach to the accurate measurement of FVIII/vWF protein concentration in human plasma as well as a method for studying the biochemical interactions between FVIII/vWF and platelets.

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² Unpublished observation. The washed platelet suspension was used for the experiment.

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