Selective Binding of the Factor VIII/von Willebrand Factor Protein to Human Platelets

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The factor VIII/von Willebrand factor protein was radiolabeled after modification by galactose oxidase and reduction with tritiated potassium borohydride. This mild efficient method for labeling resulted in retention of over 90% of the biologic activities of the factor VIII/von Willebrand factor protein. Binding of this protein to platelets was found to be specific, and binding sites could be saturated in the presence of ristocetin. However, binding was highly dependent on ristocetin concentration, as the number of human factor VIII/von Willebrand factor molecules bound per platelet was a function of the ristocetin concentration. At a ristocetin concentration of 0.55 mg/ml, each platelet binds approximately 11,000 factor VIII/von Willebrand factor molecules per platelet. Scatchard analysis of the concentration-dependent binding sites yielded a hyperbolic plot that appeared to be related to the existence of two classes of binding sites. The higher affinity class had a $K_d$ of $3.7 \times 10^{-10} M$, 3500 sites/platelet, whereas the lower affinity class had a $K_d$ of $2.35 \times 10^{-9} M$ and a capacity of 7500 sites/platelet. As with ristocetin-induced platelet agglutination, the carbohydrate content plays a significant role in the binding of the factor VIII/von Willebrand factor protein to the platelet.

The human factor VIII/von Willebrand factor (fVIII/vWF) protein has two biologic activities: (1) procoagulant activity—the ability to correct the long clotting time of hemophilic plasma, and (2) von Willebrand factor (vWF) activity—the ability to cause platelet retention in a glass-bead column or to agglutinate normal human platelets in the presence of ristocetin. In von Willebrand disease (vWD), this protein is either reduced in amount or is quantitatively normal but qualitatively abnormal; in either instance, the vWF activity is reduced as measured by the ristocetin-induced platelet aggregation assay. Understanding of the interaction of the vWF protein with platelets might increase our knowledge concerning the defect in vWD and of platelet–plasma protein interaction(s) in other disease states.

Several investigators have shown that the interaction of the fVIII/vWF protein with platelets requires the antibiotic, ristocetin.1-5 The mechanism of the ristocetin agglutination reaction has been suggested to be due to the positively charged ristocetin decreasing the negative charges on the platelet and thus decreasing the electrostatic repulsion between platelets: this allows the macromolecular fVIII/vWF protein to act as an effective bridge to interconnect platelets.4,6-12 Some investigators have suggested that in the interaction with platelets, only a subpopulation of the fVIII/vWF protein is bound to the platelet. Others have suggested a direct effect of ristocetin on the fVIII/vWF protein rather than the platelet.13

Binding of the fVIII/vWF protein to the platelet has been investigated;1-5 however, no quantitative data are available concerning the types and avidity of the binding sites or the quantitation of binding sites on the platelet surface for the fVIII/vWF protein. In this article we demonstrate that there is a specific receptor on the platelet surface for the fVIII/vWF protein, which can be saturated. Two classes of binding sites, low and high affinity receptor sites, are present, and the binding of the fVIII/vWF protein to the receptor is highly dependent on ristocetin concentration, time of incubation, and the carbohydrate content of the fVIII/vWF protein.

MATERIALS AND METHODS

Factor VIII/von Willebrand Factor Protein

The fVIII/vWF protein was purified as previously described.14 In brief, intermediate purity fVIII concentration from the National American Red Cross was subjected to Sepharose 4B gel chromatography after polyethylene glycol precipitation. The void volume fraction was pooled, concentrated, and used in all the experiments described.

Labeling of the Factor VIII/von Willebrand Factor Protein

The fVIII/vWF protein 0.75–1.0 mg/ml was labeled with tritiated potassium borohydride (Amersham Searle Corp., Arlington Heights, Ill.) after the modification of terminal galactose by galactose oxidase.15 Galactose oxidase at a final concentration of 5 U/mg fVIII/vWF protein and horseradish peroxidase at 15 mg/mg of protein (final concentration) were incubated together for 4 hr at 37°C. Then 8–10 mCi of tritiated potassium borohydride were added to 2–3 mg of the fVIII/vWF protein at 23°C and incubated for 30 min. The unbound tritiated potassium borohydride was removed by dialysis at 4°C against 0.05 Tris, 0.1 M NaCl, pH 7.35. This material was subjected to polyacrylamide gel electrophoresis in the presence of SDS with and without reducing agents.16 Duplicate gels were electrophoresed; one was stained with Coomasie blue, while the other was sliced and radioactivity was measured. All binding studies were performed within 72 hr after termination of dialysis.
Binding Assay

Platelets were prepared by two methods. Both methods have been previously described. The first method utilized a modified washed human platelet system. The second method employed a washed formalin-fixed platelet system. All binding assays were performed under identical conditions, i.e., to 0.4 ml platelets with a platelet count of 200,000 or 300,000/μl, 0.05 ml of labeled FVIII/vWF protein containing approximately 25–50 μg/ml (final concentration 2.75–5.5 μg/ml) and 5 μl of ristocetin concentration 50 mg/ml (final concentration 0.55 mg/ml) was added. The binding studies were performed by incubating the 3 components together for 10 min at room temperature. After centrifugation at 1200 g for 3 min, the supernatant was removed and counted. Phase microscopy examination of the supernatant did not reveal any platelets to be present.

The cpm in a supernatant aliquot of the labeled FVIII/vWF protein and platelets without ristocetin was considered 100% of the total cpm. The cpm in the supernatant of the labeled material, platelet, and ristocetin incubation was subtracted from the total cpm and then divided by the total cpm. The results are expressed as percentage of the counts bound.

Dissociation of the 1H-FVIII/vWF from the platelet was studied at room temperature. After the labeled FVIII/vWF (final concentration 1.5–3.0 μg/ml) had reached equilibrium with the platelets (200,000/μl) and ristocetin (0.275 mg/ml), cold FVIII/vWF (1.4–11 μg/ml final concentration) and ristocetin (0.275 mg/ml) were added to the incubation mixture and specific binding was determined at timed intervals. No unlabelled FVIII/vWF was added to the control tubes; instead, buffer and ristocetin were used to keep the volume and ristocetin concentrations constant.

In some experiments, the supernatant was concentrated by either dialysis against Ficol (Pharmacia, Piscataway, N.J.) or by lyophilization. In addition, the amount of binding of the FVIII/vWF protein during thrombin-induced washed platelet aggregation was determined. Washed platelets (0.4 ml) incubated with radiolabeled FVIII/vWF protein (0.05 ml) and buffer (0.05 ml) were aggregated by the addition of highly purified human thrombin (770 U/mg) to a final concentration of 0.05–0.30 U/ml. In some experiments, 0.05 ml of plasma from a patient with vWD (antigen level <3%, procoagulant activity 2%) was substituted for the buffer solution. The antigen content of these concentrated supernatants was determined by counterimmunoelectrophoresis or by the Laurel immunoassay. All determinations of radioactivity were performed in a Nuclear Chicago Scintillation counter.

Modification of the Binding Experiments

Several modifications of the basic binding experiments were utilized to evaluate the influence of each of the three components of the ristocetin-induced platelet agglutination. (1) The platelet count was tested at 25,000, 50,000, 75,000, 100,000, 150,000, and 200,000/μl with the ristocetin and FVIII/vWF protein concentration remaining constant. (2) The ristocetin was added at final concentrations of 0.11 mg/ml–1.1 mg/ml to 0.4 ml of platelets (200,000/μl) and 0.05 ml FVIII/vWF protein (50 μg/μl). (3) The platelet count was maintained at 200,000/μl and the ristocetin concentration at 0.55 mg/ml; the FVIII/vWF protein varied in the reaction from 5 μg/ml to 500 μg/ml in a constant volume of 0.05 ml.

Carbohydrate Analysis

Neuraminidase treatment and measurement of free and bound sialic acid of the FVIII/vWF protein were performed as previously described. Galactose oxidase treatment of the FVIII/vWF protein was performed prior to and after neuraminidase treatment of the FVIII/vWF protein as previously described. Galactose was determined by utilizing galactose oxidase according to the method of Roth et al. Galactose was also measured after treatment of the intact or asialo FVIII/vWF protein with β-galactosidase (5.5 U/ml) purified from Streptococcus pneumoniae. The enzyme was a gift of Dr. Gilbert Ashwell and was stabilized with 3 mg/ml of bovine serum albumin. These enzymes did not demonstrate proteolytic activity when tested as described previously. The FVIII/vWF protein was incubated with β-galactosidase (0.02–0.05 μl/mg FVIII/vWF protein) for periods ranging from 2 hr to 24 hr. The free galactose was measured by the method of Finch, utilizing galactose dehydrogenase (Boehringer Mannheim, Indianapolis, Ind.). Three measurements of galactose were obtained: (1) terminal galactose by the treatment of the intact FVIII/vWF protein with galactose oxidase; (2) total galactose (terminal and penultimate) by treatment of the asialo FVIII/vWF protein with galactose oxidase; and (3) penultimate galactose measured by the release of galactose from the asialo FVIII/vWF protein by β-galactosidase.

Platelets prepared as described above were used with intact and carbohydrate-modified FVIII/vWF protein in the presence of ristocetin to measure ristocetin cofactor (vWF) activity. The washed platelet system assay was monitored in an aggregometer, and the washed formalin-fixed platelet assay was determined by flocculation and in the aggregometer.

Intact FVIII/vWF protein binding was considered 100%. This was compared to the asialo FVIII/vWF protein and the asialoagalacto FVIII/vWF protein. In some studies, the FVIII/vWF protein was separated from the enzyme(s) by chromatography over a 1.5 × 10 cm Sepharose 4B column. The void volume fractions were pooled, concentrated, and used in the binding assays.

D-Galactose, at concentrations of 50 and 100 mM, was incubated with the platelets, ristocetin, and FVIII/vWF protein.

RESULTS

The FVIII/vWF protein was considered of high purity by its appearance on polyacrylamide gel electrophoresis, as previously described, and by its lack of reactivity with antifibrinogen antibody and anticold insoluble globulin antibody in immunodiffusion and immunoelectrophoresis. The FVIII/vWF protein had a mean specific activity of 41.4 U/mg and procoagulant activity of 28.9 U/mg (pooled normal plasma equals 1 U/ml). The mean value of sialic acid of the FVIII/vWF protein was 109.6 ± 9.0 nmole/mg (mean ± 1 SD, n = 17).

Treatment of the intact FVIII/vWF protein with β-galactosidase did not cause release of free galactose. However, oxidation of the intact FVIII/vWF protein

<p>| Table 1. Galactose Content of the FVIII/vWF Protein |
|-----------------------------------|-------------------|----------------|--------------------|</p>
<table>
<thead>
<tr>
<th>Protein</th>
<th>Enzyme</th>
<th>nmole/mg</th>
<th>mole/230,000 MW</th>
</tr>
</thead>
<tbody>
<tr>
<td>A.</td>
<td>Intact</td>
<td>73</td>
<td>17</td>
</tr>
<tr>
<td>B.</td>
<td>Intact</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C.</td>
<td>Asialo*</td>
<td>173</td>
<td>40</td>
</tr>
<tr>
<td>D.</td>
<td>Asialo*</td>
<td>111</td>
<td>26</td>
</tr>
<tr>
<td>E.</td>
<td>C-A</td>
<td>100</td>
<td>23</td>
</tr>
</tbody>
</table>

*Sialic acid content of the FVIII preparations is 110 nmole/mg or 25 mole/230,000 MW subunit.
with galactose oxidase revealed 73.2 ± 6.1 n mole galactose/mg fVIII/vWF protein \((n = 7)\). When the asialo fVIII/vWF protein was treated with β-galactosidase, \(111 ± 9.2\) n mole galactose/mg fVIII were liberated \((n = 5)\). When the total galactose was measured on the asialo fVIII/vWF protein by galactose oxidase, \(173 ± 10.4\) n mole galactose/mg protein was found \((n = 7)\) (Table 1). Thus, by the β-galactosidase method, no terminal galactose was detected, but 111 n mole/mg of penultimate galactose were released, while with the galactose oxidase method, 73 nM/mg were terminal and \(100 ± 8.1\) n mole/mg galactose were penultimate \((n = 6)\).

When the intact fVIII/vWF protein was treated with galactose oxidase in the presence of horseradish peroxidase and the 6-aldehyde intermediate was reduced with trinitiated potassium borohydride \((\text{KBH}_3)\), the material retained 84%–96% of the procoagulant and vWF activity compared to the unlabelled material and was labeled to \(10^5\) cpm/μg or 0.1 μCi/μg of the fVIII/vWF protein. Addition of trinitiated KBH3 to the fVIII/vWF protein that had not been treated with galactose oxidase resulted in incorporation of less than 5% of the counts of the enzyme-treated material. When the enzyme-treated and labeled material was compared with the unlabelled material on polyacrylamide gel electrophoresis, the gels were identical. When the unstained gel was sliced and the radioactivity determined, it was found that, in the unreduced state, 98% of the radioactivity was in the first 2 mm of the gel, and after reduction, the relative mobility of over 96% of the radioactivity was the same as the 230,000 molecular weight subunit. Column chromatography on Sepharose 4B of radiolabeled material combined with unlabeled material revealed that the radiolabeled material eluted in the identical position as the unlabeled material. Since the labeled material had the same biologic and physicochemical activities as did the unlabeled material, it was then used for binding studies with human platelets.

Binding of the radiolabeled fVIII/vWF protein was done with both formalin-fixed platelets and fresh platelets, both at 200,000–300,000/μl. The results indicate that there is minimal binding without ristocetin to both the fresh and the formalin-fixed platelet, 4% ± 1% and 8% ± 2% \((\text{mean} ± 1 \text{ SD}, n = 14)\), respectively. Similar binding was observed with thrombin-induced platelet aggregation (5%). In the presence of ristocetin, the binding with the formalin-fixed platelets is 58% ± 4%, while with the fresh platelets, it is 87% ± 6%, \(n = 4\). The time courses of binding both with formalin-fixed and fresh platelets show that the binding was near maximum at 10 min, and incubations up to 60 min only increased binding 5%–7% (Fig. 1). Thus, with all subsequent experiments, we used the 10-min period as the time in which binding studies were completed.

Measurements of the antigen remaining in the supernatant with and without the addition of ristocetin showed a proportionate loss of cpm and antigen from the supernatant. Without the addition of ristocetin, the cpm was reduced 4%–8% and the antigen was reduced approximately 5%–10%, while after the addition of ristocetin, the cpm in the supernatant was reduced 87% and the antigen was reduced 82%.

The binding of the fVIII/vWF protein to the platelet could be inhibited by unlabeled fVIII/vWF but was not inhibited by fibrinogen (which was free of cold insoluble globulin and fVIII-related antigen). The addition of cold fVIII/vWF protein inhibited binding from 9% to 92%, depending on the amount of cold fVIII/vWF added. The binding was not affected by the addition of α-galactose at 50 or 100 nM concentrations.

The binding of the \(^3\)H-fVIII/vWF to the platelet in the presence of ristocetin was reversible. Dissociation of \(^3\)H-fVIII/vWF from the platelet surface after equilibrium by unlabeled fVIII/vWF (111 μg/ml final concentration) was 50% in 30 min, 64% in 60 min, 75% in 90 min, and 82% in 120 min. Lower concentrations of unlabeled fVIII/vWF (55.5, 6.9, and 1.4 μg/ml) were relatively slower in dissociating \(^3\)H-fVIII/vWF from the platelet.

In other studies, the influence of ristocetin concentration on the binding of the fVIII/vWF protein to the
human platelet was determined (Fig. 2). At a ristocetin concentration of 10 mg/ml (final concentration 0.11 mg/ml), binding was 7%, at 50 mg/ml (final concentration 0.55 mg/ml), binding increased to 87%, and at 100 mg/ml (final concentration 1.1 mg/ml), binding rose to 91%.

We determined the number of binding sites for the fVIII/vWF protein per platelet by two techniques. In one technique, a constant amount of fVIII/vWF protein was added to platelets varying in concentration between 25,000 and 200,000/μl or 10^7 and 8 × 10^7 in 0.4 ml. The number of fVIII/vWF molecules bound (Table 2) averaged 9700 molecules/platelet. In a second study, the platelets were kept constant at 200,000/μl or 8 × 10^7 platelets in 0.4 ml, and the fVIII/vWF protein concentration was varied between 25 and 200 μg/ml. In this study, 9800 fVIII/vWF molecules were bound per platelet.

Scatchard analysis of the concentration-dependent aspects of binding of the fVIII/vWF binding was complex and revealed a hyperbolic curve (Fig. 3). These analyses estimated 11,000 binding sites; 7500 low affinity sites with a K_d of 2.75 × 10^-9 and 3500 high affinity binding sites with a K_d of 3.90 × 10^-10.

Carbohydrate modification of the fVIII/vWF protein did not affect binding to the platelet in the absence of ristocetin. With the removal of sialic acid from the fVIII/vWF protein, the binding was 95% (range 91%-103%, n = 4) compared to the intact protein. Removal of greater than 95% of the sialic acid with neuraminidase and over 90% of the penultimate galactose by β-galactosidase resulted in only 25% (range 16%-39%, n = 4) binding of the radiolabeled material to the platelet. These carbohydrate-modified proteins were also tested for their ability to support agglutination of washed platelets and washed formaldehyde platelets. The asialo fVIII/vWF protein retained its ability to agglutinate platelets (93%, range 91%-100%, n = 6), while the asialo galacto fVIII/vWF protein lost most of its activity (16%, range 12%-22%, n = 7).

**DISCUSSION**

In this article we describe a simple and specific labeling procedure for the fVIII/vWF protein. Studies with the labeled protein have revealed its retention of biologic activities. There are no significant alterations in the protein by immunologic and physicochemical (i.e., polyacrylamide gel electrophoresis and agarose gel chromatography) criteria. Labeling of the terminal galactose is advantageous, since from prior studies it does not appear that terminal galactose is important in the interaction of the fVIII/vWF protein with platelets and ristocetin. We have previously shown that the treatment of intact proteins with galactose oxidase did not reduce the procoagulant or the vWF (ristocetin cofactor) activities.

There was minimal binding to either washed normal platelets or washed and formalized normal platelets without the addition of ristocetin, i.e., 4% and 8%, respectively. However, after the addition of ristocetin, there was binding of the labeled protein to platelets which was dependent on time, fVIII/vWF protein concentration, ristocetin concentration, and platelet number. This binding was not inhibited by fibrinogen,
but was competitively inhibited by the addition of unlabeled fVIII/vWF protein. We found that the time course of binding of the fVIII/vWF protein was near maximum at 10 min, with small increments in percent binding up to 60 min. The ristocetin concentration had a profound effect on the binding of the fVIII/vWF protein to the platelet. For these studies, we did all the studies at a final ristocetin concentration of 0.55 mg/ml.

The effect of varying the platelet count and varying the fVIII/vWF protein concentration was independently utilized to determine the number of binding sites per platelet. The number of binding sites, determined by varying the platelet number, was 9700 sites/platelet, while studies in which the fVIII/vWF protein was varied had 9800 binding sites/platelet. Scatchard analysis revealed a hyperbolic plot of binding data with an estimated 11,000 binding sites/platelet. Of these 11,000 sites, 3500 were high affinity with a $K_d$ of $3.90 \times 10^{-10} M$ and 7500 binding sites of low affinity with a $K_d$ of $2.75 \times 10^{-9} M$ at a ristocetin concentration of 0.55 mg/ml.

When ristocetin was added to the purified fVIII/vWF protein for periods of time ranging from 1 to 5 min prior to the addition of platelets, we found no augmentation or decrease in the binding to the platelet. Likewise, thrombin-induced platelet aggregation did not cause binding of the labeled fVIII/vWF protein to the platelet (5%; concomitant incubation of the fVIII/vWF protein and platelets without ristocetin also had 5% binding).

Removal of sialic did not affect binding or decrease ristocetin-induced platelet aggregation, while removal of the penultimate galactose reduced the binding and the agglutination reaction to 20%–25% and 16% of the control, respectively. This would suggest that the agglutination reaction is directly related to the binding of the fVIII/vWF protein to the platelet surface, and this is modulated in part by the carbohydrate content of the fVIII/vWF protein.

Analysis of the galactose content of the fVIII/vWF protein revealed that 73 nmole galactose/mg of protein are ultimate, and these are not released by $\beta$-galactosidase. Penultimate galactose determined by galactose oxidase was 100 nmole/mg and by $\beta$-galactosidase was 111 nmole/mg. The terminal galactose residues that were not released by $\beta$-galactosidase may be related to the type of linkage of the galactose, in that the $\beta$-galactosidase from Streptococcus pneumoniae only hydrolyzes galactose in a $\beta$ 1–4 linkage. Thus, a $\beta$ 1–3 linkage would not be susceptible to hydrolysis by this enzyme. Likewise, $\alpha$-galactose linkage or substituted galactose derivatives would not be susceptible to the $\beta$-galactosidase. This last possibility,
that some of the terminal galactose is substituted derivatives, cannot be excluded by the assay method that was used for measurement of terminal galactose, i.e., galactose oxidase method, since this will interact with galactose as well as with substituted galactose moieties. Our values are in general agreement with those of Sodetz et al., although we obtained higher values for galactose content of the fVIII/vWF protein.

Several investigators have reported studies concerning the interaction of the fVIII/vWF protein and platelets with and without ristocetin. These studies, in general, have been done with fresh or formalinized platelets, with normal plasma as the source of the fVIII/vWF protein. These studies have brought forth various hypotheses for ristocetin-induced platelet agglutination and have examined what activities are not bound to the platelet (activities measured in the supernatant). Studies by Zucker and colleagues and Jenkins and coworkers demonstrated that with the ristocetin-induced platelet agglutination there was an enhanced removal of vWF activity compared to antigen or procoagulant activity, while no activities were bound in the absence of ristocetin.

Green and Potter studied the binding of plasma fVIII/vWF to vWd platelets, which did not react with a fluorescein isothiocyanate-conjugated anti-fVIII/vWF antibody. In these studies they demonstrated that when nonreactive vWd platelets were incubated with normal platelet-poor plasma and ristocetin, they underwent an agglutination reaction and these platelets then stained brightly for the fVIII/vWF protein on their surface (as did the normal platelets both before and after treatment with ristocetin). They interpreted their observations to suggest that the ristocetin caused the binding of the fVIII/vWF protein to platelets as well as inducing the platelet-to-platelet adhesion. Subsequent studies by this group suggested that there was also an effect of ristocetin causing aggregation on the fVIII/vWF protein. The studies were performed in an ultracentrifuge, and at a ristocetin concentration of 1 mg/ml, the effect on the fVIII/vWF protein was minimal, while at large doses of ristocetin (5 mg/ml), the effect was more marked. The only study in which the human fVIII/vWF protein has been found to bind to platelets without ristocetin is that by Koutts and Zimmerman using a membrane preparation of platelets. Kirby and Tang reported binding of bovine fVIII/vWF protein to human platelets without ristocetin. Interestingly, this binding was not affected by high levels of human fVIII/vWF protein. Work by Doucet de Bruine et al. and Koutts and Zimmerman has examined the subpopulation of molecules binding to the platelets utilizing crossed antigen–antibody electrophoresis. This work and ours (Gralnick and Williams, unpublished observations), are in agreement when normal plasma is the source of the fVIII/vWF protein. The larger molecular weight forms, i.e., least anodic, are absent from the supernatant plasma after ristocetin-induced platelet agglutination.

Coller recently demonstrated that ristocetin reduced electrophoretic mobility of platelets, and the addition of plasma containing the fVIII/vWF protein had an additive effect on the reduced mobility. He suggested that there is some interaction of ristocetin with platelets and then the fVIII/vWF protein binds to the platelet at a site different from where ristocetin acts.

Several studies have shown that platelet-rich plasma of patients with Bernard-Soulier syndrome do not agglutinate with ristocetin, although they have normal levels of fVIII/vWF. It has also been shown that a platelet glycoprotein is necessary for the fVIII–ristocetin agglutination to take place. Trypsinization of normal platelets has decreased membrane glycoproteins, and these platelets have had reduced agglutination with the fVIII/vWF protein and ristocetin. The glycoprotein thought to be involved in the ristocetin-induced platelet agglutination has been called GPI. An antibody to GPI (observed in a patient with Bernard-Soulier syndrome) inhibited agglutination of normal platelet-rich plasma by ristocetin. Nurden et al. and Jenkins et al. found decreased amounts of GPI from the platelets of patients with Bernard-Soulier syndrome. Thus, it seems likely that the platelet has a specific membrane glycoprotein that may act as a receptor for the fVIII/vWF protein. Recent reports by Kao et al. and Moake et al. have demonstrated that binding of purified human fVIII/vWF protein to platelets requires ristocetin. These studies are in general agreement with the ones we have reported here.

In this study we have demonstrated that there is a specific platelet receptor for the fVIII/vWF protein, that the protein binds rapidly to this receptor, the receptor sites can be saturated by the fVIII/vWF protein, and that this binding is reversible. This binding requires ristocetin, and in these studies it appears that the ristocetin has its primary action on the platelet, since prior incubation of the purified fVIII/vWF and ristocetin did not augment binding or ristocetin-induced platelet agglutination. The rate and degree of binding are dependent on the ristocetin concentration. There appears to be a limited number of binding sites (~ 11,000) per platelet, of which 3500 are a higher affinity class and 7500 are a lower affinity class. An alternative explanation of the hyperbolic curve of the
Scatchard plot may be that negative cooperativity is involved in the binding of the FVIII/vWF protein to platelets. Our studies have shown that carbohydrate plays an important role in the specific binding of the FVIII/vWF protein to platelets and in the ability of the FVIII/vWF protein to agglutinate human platelets. Although ristocetin-induced platelet agglutination is a nonphysiologic phenomenon, investigation of the binding of the protein may prove to be a useful probe in understanding early events in hemostasis and the abnormality in vWD and other disorders of platelet–plasma protein interactions.

ACKNOWLEDGMENT

The authors wish to thank Eddie Cregger, Georgia Jackson, and Sybil Williams for excellent technical assistance and Lynda Ray for invaluable secretarial services.

REFERENCES