Quantitative Assay of a Plasma Factor Deficient in von Willebrand's Disease that is Necessary for Platelet Aggregation

RELATIONSHIP TO FACTOR VIII PROCOAGULANT ACTIVITY AND ANTIGEN CONTENT

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ABSTRACT In a previous paper, we showed that the abnormality of ristocetin-induced platelet aggregation in platelet-rich plasma in 10 patients with von Willebrand's disease could be corrected by a factor in normal plasma that was present in the same fractions as factor VIII procoagulant activity (antihemophilic factor, AHF, $VIII_{AHF}$) when prepared by chromatography on Bio-Gel 5 M (Bio-Rad Laboratories, Richmond, Calif.). This observation suggests that patients with this disorder are deficient in a plasma factor, associated with the factor VIII molecule, that is necessary for normal platelet function. In the present paper, we describe, an assay for this factor, the von Willebrand factor ($VIII_{vWF}$), based on the observation that a log-log relationship exists between the amount of ristocetin-induced aggregation of *washed, normal* platelets and the concentration of normal plasma present in the test system. We assayed the activity of $VIII_{vWF}$ as well as antihemophilic factor procoagulant activity ($VIII_{AHF}$) and factor VIII antigen ($VIII_{AGN}$) in 15 patients with von Willebrand's disease and 20 normal subjects. A highly significant correlation ($r \sim 0.80$) between $VIII_{vWF}$ and both $VIII_{AGN}$ and $VIII_{AHF}$ was found in normal subjects and in patients with von Willebrand's disease. This finding, in addition to the observation that agarose gel chromatography fractions that have $VIII_{AHF}$ procoagulant activity also have $VIII_{vWF}$ activity, strongly suggests that the von Willebrand factor is associated with the factor VIII molecule. $VIII_{vWF}$ in normal plasma was not inhibited by human anti-VIII, and $VIII_{vWF}$ levels were normal in hemophilic plasma. Thus, the $VIII_{vWF}$ site on the factor VIII molecule appears to be different from that determining $VIII_{AHF}$. Finally, the activity of $VIII_{vWF}$ appeared to correlate better with the bleeding time than either $VIII_{AHF}$ or $VIII_{AGN}$. This suggests that $VIII_{vWF}$ assayed in this study may be the "anti-bleeding factor" that is deficient in von Willebrand's disease. These findings are consistent with a decreased synthesis of the factor VIII molecule in von Willebrand's disease and suggest the possibility of additional abnormalities of the site on the molecule that determines the activity of $VIII_{vWF}$.

INTRODUCTION

We have shown in an accompanying paper that ristocetin-induced platelet aggregation was decreased in patients with von Willebrand's disease and to a degree which correlated in general with the decreased antihemophilic factor (AHF,[1] $VIII_{AHF}$) procoagulant activity and prolonged bleeding time that are also features of this

This work was presented, in part, at the 15th Annual Meeting of the American Society of Hematology, Hollywood, Fla., 5 December 1972.

Received for publication 22 March 1973 and in revised form 18 June 1973.

[1] *Abbreviations used in this paper:* AHF, antihemophilic factor; PRP, platelet-rich plasma; $VIII_{AGN}$, factor VIII antigen; $VIII_{AHF}$, AHF procoagulant activity; $VIII_{vWF}$, von Willebrand factor associated with factor VIII molecule.

disorder (1). In addition, this abnormality in ristocetin-induced platelet aggregation was corrected by a factor in normal plasma that eluted with $VIII_{AHF}$ activity after chromatography on Bio-Gel 5 M (Bio-Rad Laboratories, Richmond, Calif.). This corrective effect was inhibited by a rabbit antibody to human factor VIII (1). These findings suggest that the prolonged bleeding time in von Willebrand's disease may be owing to the deficiency of a factor associated with the factor VIII molecule that is necessary for the formation of a hemostatic platelet aggregate. The findings in the accompanying paper (1) as well as those in previous studies that used platelet retention methods as the test system (2–4) suggest that this von Willebrand factor ($VIII_{vWF}$) may be located at a different site on the factor VIII molecule than that determining $VIII_{AHF}$ procoagulant activity. We have developed a quantitative assay for $VIII_{vWF}$ activity, based on the observation that ristocetin aggregates *washed* normal platelets, but only in the presence of plasma or semi-purified factor VIII. We have assayed $VIII_{vWF}$ in the plasma of 15 patients with von Willebrand's disease, 20 normal subjects, and 14 patients with hemophilia. This paper reports the quantitative relationships that we found between $VIII_{vWF}$ activity, $VIII_{AHF}$ procoagulant activity and the concentration of the antigen identified by rabbit anti-VIII, $VIII_{AGN}$, in these three groups.

METHODS

Terminology. In the present paper, antihemophilic factor procoagulant activity will be referred to as $VIII_{AHF}$. The factor in plasma that is necessary for ristocetin-induced platelet aggregation and that, as will be shown, is deficient in von Willebrand's disease, is referred to as $VIII_{vWF}$, the von Willebrand factor. The antigens on the factor VIII molecule that are identified by rabbit anti-VIII are designated as $VIII_{AGN}$. The term "factor VIII" will be used nonspecifically to refer to the presumed molecular species in normal plasma on which the three activities cited above are located.

Patients. 15 unrelated patients with von Willebrand's disease were studied. The typical findings of a decreased $VIII_{AHF}$ level, prolonged bleeding time, and decreased retention of platelets in glass bead filters (5) were obtained in all cases. Most of the patients have been the subjects of previous studies (1, 3–5). 10 patients with classical severe hemophilia had $VIII_{AHF}$ levels of less than 1 U/100 ml, and a patient with congenital afibrinogenemia was also studied. Control subjects were normal hospital personnel, ages 19–45.

Buffers. Tris[2]-buffered saline, pH 7.3, was prepared by mixing one part of 0.25 M Tris, pH 7.3 with two parts of 0.85% NaCl (Tris-saline). Tris-buffered EDTA contained 1% Na_2 EDTA[3] in Tris-saline, pH 7.3.

Platelet suspensions. Venous blood was mixed with 1/10 volume of 1% Na_2 EDTA and centrifuged at 20°C to obtain platelet-rich plasma (PRP) as previously described (1). The PRP was centrifuged at 2,400 *g* and 20°C for 30 min, and the platelets were resuspended, in Tris-buffered EDTA. The platelets were then sedimented at 1,900 *g* and 20°C for 20 min, resuspended in Tris-EDTA, and then sedimented for a third time in a similar manner and suspended in Tris-saline. After a fourth and final centrifugation in a graduated McNaught tube, they were suspended in a final concentration of 1% (vol/vol) in Tris-saline. This suspension contained about 300,000 platelets/mm^3 and was kept at room temperature prior to its use in the platelet aggregation studies. All glassware was silicone-coated (General Electric SC-87 Dri-Film, 15% in toluene).

Platelet aggregation. Platelet aggregation in the platelet suspension described above was studied in the aggregometer (1). The test system consisted of 1.0 ml of the 1% platelet suspension + 0.6 ml of Tris-saline + 0.4 ml of either plasma, dilutions of plasma, semipurified factor VIII or Tris-saline + 0.1 ml of a solution of ristocetin (Abbott Laboratories, North Chicago, Ill., 25 mg/ml in Tris-saline). The degree of platelet aggregation was expressed as percent change in OD (1).

Assay of $VIII_{AHF}$ and $VIII_{AGN}$. $VIII_{AHF}$ was assayed in duplicate on freshly drawn plasma by a one-stage kaolin-activated partial thromboplastin time method, as previously described (6). Activity was expressed as units per 100 ml (1). The plasmas were then frozen in aliquots at −60°C in plastic containers for subsequent assay of $VIII_{vWF}$ and $VIII_{AGN}$. $VIII_{AGN}$ was measured using a radioimmunoassay that has been previously described (7). The method for assaying $VIII_{vWF}$ activity is described under Results.

Reference plasmas. Reference plasma for the $VIII_{AHF}$ assays consisted of a pool of freshly drawn plasmas from four to seven healthy, male laboratory and professional personnel previously found to have $VIII_{AHF}$ activities of 90–115 U/100 ml when assayed against a pool of 20 normal donors. The pooled reference plasma was designated to have an $VIII_{AHF}$ activity of 100 U/100 ml. Reference plasma for the $VIII_{vWF}$ assays was obtained by pooling the plasmas of these seven normal subjects in aliquots at −60°C. The activity of the $VIII_{vWF}$ appeared to be quite stable in plasmas stored at −60°C. Specimens of plasma from four normal subjects that had been frozen at −60°C for 1 yr had $VIII_{vWF}$ activities that were within 10% of those in freshly drawn plasmas from the same subjects. A reference plasma for determination of $VIII_{AGN}$ content was prepared by pooling plasmas from 10 normal donors (different from those used for the reference plasma in the $VIII_{AHF}$ and $VIII_{vWF}$ assays) in aliquots stored at −70°C for no longer than 2 mo (7). The pooled plasma of the seven normal subjects used for the $VIII_{AHF}$ and $VIII_{vWF}$ assays contained 115 $VIII_{AGN}$ U/100 ml. 1 U of $VIII_{AGN}$ is defined as the content present in one ml of pooled, normal human plasma (7).

Agarose gel chromatography. Cryoprecipitate obtained from 40 ml of plasma was chromatographed on Bio-Gel 5 M as previously described (1).

Antibodies to factor VIII. The monospecific rabbit antibody, prepared by immunizations with purified human factor VIII (8) and the plasma from a hemophiliac with a circulating antibody to $VIII_{AHF}$ (human anti-VIII) were the same as in a previous study (1). Incubation of 0.9 ml of normal human plasma with 0.1 ml of rabbit or human anti-VIII for 30 min at 37°C reduced the $VIII_{AHF}$ activity from 110 U/100 ml to 7 and 2.5 U/100 ml, respectively.

Statistical methods. Comparison between groups for each of the three variables $VIII_{AHF}$, $VIII_{AGN}$, and $VIII_{vWF}$ was

[2] Tris-(hydroxymethyl) iminomethane.

[3] Na_2 ethylene diaminetetraacetate.

performed by the Student's t test (9). Equations of the regression lines for pairs of the three variables were obtained by the method of least squares (9) and segments of these lines were drawn within the limits of the experimental data. The slopes were compared with each other by the use of F tests (10). Correlation coefficients for pairs of the variables were compared by Fisher's Z transformation (11).

RESULTS

Aggregation of washed platelets

Washed normal platelets, studied immediately after their preparation, showed little or no (1–5%) aggregation after addition of ristocetin in a final concentration of 1.25 mg/ml. In platelet suspensions that were allowed to remain at room temperature for several hours during the day, aggregation by ristocetin sometimes occurred. Occasionally this amounted to about 20% aggregation and could be completely eliminated by addition of a rabbit antibody to factor VIII. Ristocetin-induced aggregation never occurred in washed platelets from patients with von Willebrand's disease that had been kept at room temperature for a similar period of time. These findings suggest that factor VIII, which is necessary for ristocetin-induced aggregation (see below), is removed from the platelets by repeated washing but that some remains with the platelet and is subsequently made available.

Plasma requirement for ristocetin-induced aggregation

Requirement for a factor present in plasma. Ristocetin only aggregated freshly prepared washed platelets in the presence of plasma. To determine the relationship between the degree of aggregation and the amount of plasma, we studied ristocetin-induced aggregation in the presence of various dilutions of plasma. The platelet aggregation curves obtained for a typical experiment are depicted in Fig. 1 and demonstrate that the degree of aggregation decreased with increasing dilutions of plasma. A plot of the amount of platelet aggregation vs. plasma dilution consistently gave a straight line on log-log paper (Fig. 2).

Evidence that the plasma factor is associated with factor VIII. Fractions that eluted with $VIII_{AHF}$ after chromatography of normal cryoprecipitate on Bio-Gel 5 M promoted ristocetin-induced aggregation of washed, normal platelets (Table I). The ability of this semipurified factor VIII or of plasma to support ristocetin-induced aggregation was inhibited by incubation with a rabbit antibody to factor VIII, but was not abolished by a human anti-VIII that inhibits $VIII_{AHF}$ procoagulant activity (Table I). As indicated in Methods, this factor in normal plasma that is necessary for ristocetin-induced aggregation of washed normal platelets will be referred to as the von Willebrand factor $VIII_{VWF}$.

Assay of the von Willebrand factor ($VIII_{VWF}$)

Pooled normal plasma (see Methods) was designated as having 100 U/100 ml $VIII_{VWF}$ activity, and serial dilutions of this plasma were used to obtain a line on log-log paper that related plasma concentrations of $VIII_{VWF}$ to the degree of ristocetin-induced platelet aggregation (see Fig. 2). Test plasmas were assayed in duplicate in a similar manner, and the activity, in units per 100 ml, that corresponded to the degree of aggregation was read from the standard graph. Thus, 1 U of $VIII_{VWF}$ is the amount of activity in 1 ml of pooled normal plasma. Replicate determinations of $VIII_{VWF}$ on the same plasma gave values that differed from the mean value by 3–5%, while plasma samples from the same subject obtained on different days gave values that differed from the mean value by 7–10%. The washed platelets used in the assay were obtained from any one of five normal subjects, and

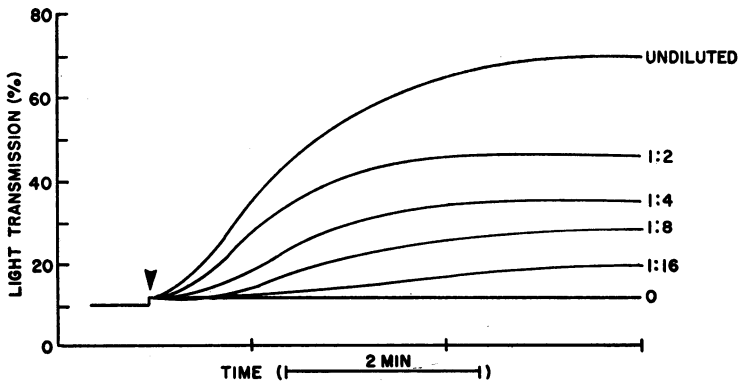

FIGURE 1 Ristocetin-induced aggregation of washed platelets. A mixture containing 1.0 ml of washed normal platelets + 0.6 ml Tris-saline + 0.4 ml of serial dilutions of plasma was stirred in the aggregometer. Changes in light transmission shown are diagrammatic.

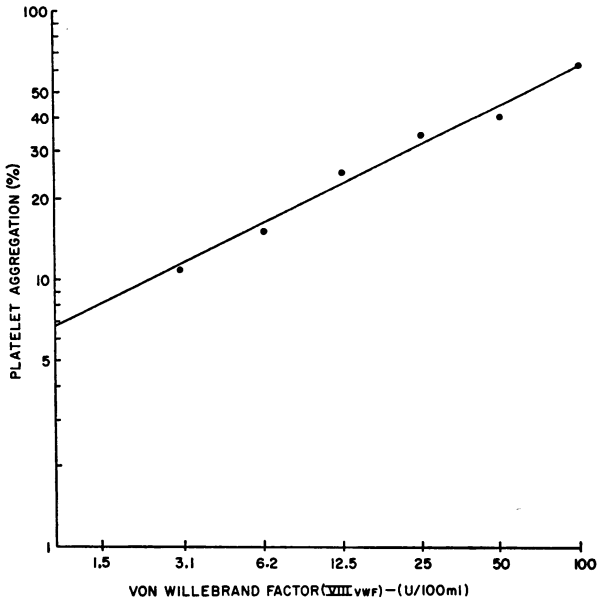

FIGURE 2 Assay of the von Willebrand factor ($VIII_{vWF}$). Ristocetin-induced aggregation of washed platelets was determined in the presence of serial dilutions of pooled normal plasma, as in Fig. 1. Undiluted plasma was arbitrarily designated as having a $VIII_{vWF}$ activity of 100 U/100 ml and the relationship between platelet aggregation (percent change in OD) and the activity of $VIII_{vWF}$ is shown.

a new standard curve was determined on each day that the assay was performed.

Assays of $VIII_{vWF}$, $VIII_{AHF}$, and $VIII_{AGN}$ in normal subjects, patients with von Willebrand's disease and hemophilic patients (Table II)

The activity of $VIII_{vWF}$ was decreased in all patients with von Willebrand's disease, and the average $VIII_{vWF}$ level in the patients with von Willebrand's disease was significantly decreased (18.6 U/100 ml) compared with the mean value obtained in normal subjects (94.7 U/100 ml) ($P < 0.001$). As expected, $VIII_{AGN}$ and $VIII_{AHF}$ were also significantly decreased in these patients. Both $VIII_{vWF}$ and $VIII_{AGN}$ were normal or slightly increased in 14 patients with hemophilia. These were also normal in one patient with congenital afibrinogenemia.

Interrelationships between the three variables, $VIII_{vWF}$, $VIII_{AHF}$, and $VIII_{AGN}$

The correlation matrices for the three groups of subjects are presented in Table III. In the normal subjects, there was a high degree of correlation between all three pairs of variables ($r \sim 0.80$). A similar and somewhat higher correlation coefficient was found in the patients with von Willebrand's disease ($r \sim 0.90$). The correlation coefficient between $VIII_{AGN}$ and $VIII_{vWF}$ in the hemophiliacs was smaller ($r = 0.58$), but not significantly different from those in the other two groups. The χ_2^2 for this comparison was 6.68 ($P > 0.05$). All r values, tested by Fisher's Z transformation, differed significantly from 0. Regression lines for pairs of the variables are shown in Figs. 3–5. Comparison of the slopes indicated that for all comparisons there was no significant deviation from parallelism. Patients J. L. and A. S., whose bleeding time values were 40 and 24 min, had $VIII_{vWF}$ values of 6 and 8 U/100 ml which appeared to be disproportionately low, compared with their $VIII_{AHF}$ values of 30 and 23 U/100 ml and, in patient J. L., an $VIII_{AGN}$ value of 19 U/100 ml.

Relation between the three variables and the bleeding time

Because in five patients the bleeding time values were greater than 60 min, it was not possible to compute regression curves relating the bleeding time to each of the three factor VIII variables. The three sets of relationships between these variables are depicted in Fig. 6. The data suggests but does not prove that the $VIII_{vWF}$ activity correlated better with the bleeding time than did either of the other two values.

DISCUSSION

The cause of the prolonged bleeding time in von Willebrand's disease is still not clear. Transfusion of normal plasma (12–14) or cryoprecipitate (4, 15) shortens the

TABLE I
Factor VIII Requirements for Ristocetin-Aggregation of Washed Platelets

Source of test material	Treatment	Ristocetin-induced platelet aggregation*
		%
Saline	—	2
Plasma	—	66
Plasma	Saline	64
Plasma	Rabbit anti-VIII	24
Plasma	Human anti-VIII	63
Factor VIII‡	—	73
Factor VIII	Saline	70
Factor VIII	Rabbit anti-VIII	17
Factor VIII	Human anti-VIII	72

* Pooled, normal plasma or factor VIII‡ was treated by incubation with $\frac{1}{10}$ volume of substances shown for 30 min at 37°C. Test system consisted of 1.0 ml of washed normal platelets +0.6 ml of Tris-saline +0.4 ml of treated test substance +0.1 ml of ristocetin.

‡ Tube containing the maximal OD_{280} reading of the fractions eluted in the void volume after chromatography of normal cryoprecipitate on Bio-Gel 5M. This tube also showed the maximal $VIII_{AHF}$ procoagulant activity.

TABLE II*A*

Values Obtained for $VIII_{VWF}$, $VIII_{AHF}$, and $VIII_{AGN}$ in Plasma

	$VIII_{VWF}$	$VIII_{AHF}$	$VIII_{AGN}$
	U/100 ml	*U/100 ml*	*U/100 ml*
Normal subjects			
1	102	103	128
2	115	105	133
3	125	125	160
4	95	85	111
5	103	113	124
6	75	90	80
7	98	104	114
8	104	85	82
9	95	75	107
10	60	65	83
11	55	60	70
12	82	95	71
13	90	80	104
14	100	75	101
15	75	75	63
16	85	105	122
17	110	92	101
18	130	120	170
19	75	80	85
20	120	100	121
Mean	94.7	91.6	106.5
SD	20.4	17.8	28.8
Geometric mean			
SD (in log)	92.4	90.0	102.8
	0.10	0.09	0.12

$VIII_{VWF}$, von Willebrand factor; $VIII_{AHF}$, antihemophilic factor (AHF) procoagulant activity; $VIII_{AGN}$, factor VIII antigen.

TABLE II*B*

Values Obtained for $VIII_{VWF}$, $VIII_{AHF}$, and $VIII_{AGN}$ in Plasma

von Willebrand's disease	$VIII_{VWF}$	$VIII_{AHF}$	$VIII_{AGN}$
	U/100 ml	*U/100 ml*	*U/100 ml*
D. R.	2	4	0.3
J. H.	3	4	0.4
C. M.	4	3	0.3
I. C.	30	41	40
J. L.	6	30	19
A. S.	8	23	7
D. G.	50	52	56
M. L.	40	49	60
J. W.	12	13	11
P. H.	9	9	4
A. V.	10	20	18
E. S.	52	35	49
D. P.	36	38	45
H. S.	10	18	24
J. N.	8	6	10
Mean	18.6	23.0	22.9
SD	17.7	16.9	21.4
Geometric mean	11.7	16.0	7.9
SD	0.45	0.43	0.80

TABLE II*C*

Values Obtained for $VIII_{VWF}$, $VIII_{AHF}$, and $VIII_{AGN}$ in Plasma

Hemophiliacs	$VIII_{VWF}$	$VIII_{AHF}$	$VIII_{AGN}$
	U/100 ml	*U/100 ml*	*U/100 ml*
1	84	<1	141
2	155	<1	279
3	72	<1	74
4	175	<1	163
5	92	<1	133
6	92	<1	190
7	60	<1	58
8	104	<1	166
9	130	<1	164
10	120	<1	100
11	120	<1	117
12	65	<1	149
13	108	<1	153
14	100	<1	105
Mean	105.5	—	142.3
SD	31.6	—	54.2
Geometric mean	100.9	—	132.8
SD (in log)	0.13	—	0.17

bleeding time, suggesting that patients with this disease are deficient in an "anti-bleeding" factor. Patients with von Willebrand's disease also show a decreased retention of platelets in glass bead filters (5, 16) and an abnormality in ristocetin-induced platelet aggregation (1, 17) and both of these in vitro defects are also corrected by plasma or cryoprecipitate (1, 4). Taken together, the above findings suggest that the formation of a hemostatic platelet aggregate during the primary arrest of bleeding may require the presence of this "anti-bleeding" factor and that a deficiency of this factor accounts for the prolonged bleeding time in von Willebrand's disease. In the present study, we have shown that ristocetin-induced platelet aggregation of washed normal platelets requires the presence of plasma and that under appropriate conditions, a log-log relationship exists between the plasma concentration and platelet aggregation. Using the same principle as that employed in assaying plasma coagulation factors, we developed an assay for the factor in plasma necessary for ristocetin-induced platelet aggregation. This factor was decreased in all 15 patients with von Willebrand's disease who were studied. More-

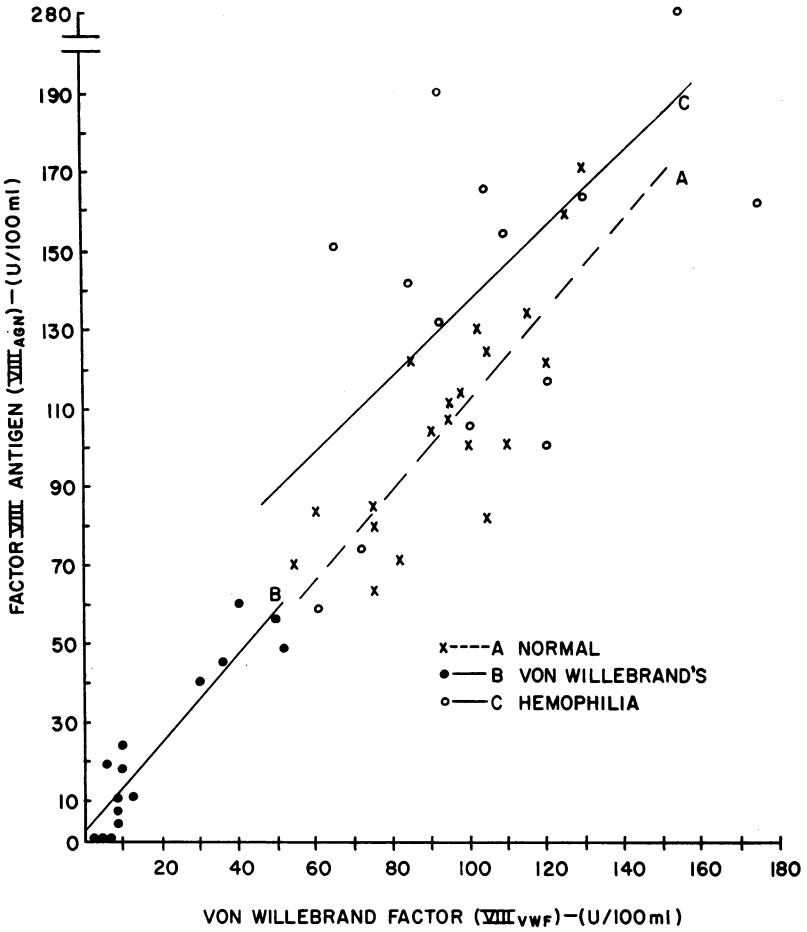

FIGURE 3 Relationship between factor VIII antigen ($VIII_{AGN}$) and the von Willebrand factor ($VIII_{VWF}$). Equations of the regression lines were: normal subjects (A), $y = 1.15\ x - 2.7$; von Willebrand's disease (B), $y = 1.14\ x + 1.7$; hemophiliacs (C), $y = 0.95\ x + 41.9$. Comparison of the three slopes showed an $F_{2,43} = 0.17$, $P > 0.50$.

over, the level of this factor correlated well with the degree of their hemostatic defect; the longer the bleeding time, the lower was the level of this "von Willebrand" factor ($VIII_{VWF}$). The correlation of $VIII_{VWF}$ activity with the bleeding time appeared to be better than that of the other two factor VIII variables studied (Fig. 6). It is entirely plausible that this factor that is necessary for ristocetin-induced platelet aggregation in vitro may also be the anti-bleeding factor that is deficient in von Willebrand's disease. The assay described herein may therefore provide a method for quantitatively measuring the amount of "anti-bleeding factor" in plasma and be useful for studying patients with bleeding disorders.

Since patients with von Willebrand's disease are also deficient in both the antihemophilic factor procoagulant activity ($VIII_{AHF}$) and factor VIII antigen ($VIII_{AGN}$) (7, 18–20), the presence of the von Willebrand factor on the same molecule as these other two activities should be considered. Recent studies indicate that factor VIII is a glycoprotein whose molecular weight as determined in vitro is in excess of 2×10^6 (21–24). In an accompanying paper, we showed that the abnormal ristocetin-induced aggregation in von Willebrand's disease was corrected by a fraction of plasma that eluted with $VIII_{AHF}$ after chromatography on a gel that excluded molecules larger than 5×10^6 (1). This corrective effect was specifically inhibited by a rabbit antibody to factor VIII. In the present study, with similar methods, we found

TABLE III
Pearson Correlation Coefficients (r)

	Normal subjects		von Willebrand's		Hemophilia
	$VIII_{VWF}$	$VIII_{AGN}$	$VIII_{VWF}$	$VIII_{AGN}$	$VIII_{AGN}$
$VIII_{AHF}$	0.77	0.81	0.86	0.94	—
$VIII_{VWF}$	—	0.82	—	0.94	0.58

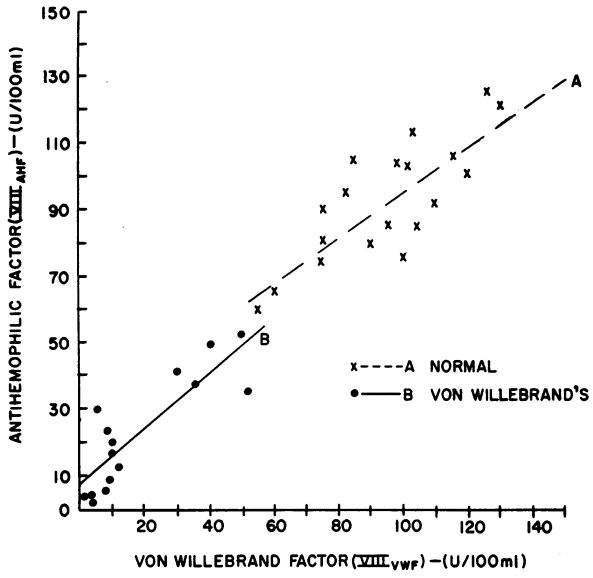

FIGURE 4 Relationship between antihemophilic factor activity ($VIII_{AHF}$) and von Willebrand factor activity ($VIII_{VWF}$). Equations of the regression lines are: normals (*A*), $y = 0.67\ x + 28.3$; von Willebrand's (*B*), $y = 0.82\ x + 7.7$. Comparison of the two slopes showed an $F_{1,31} = 0.57$, $P > 0.50$.

that semipurified factor VIII supported the aggregation of washed, normal platelets by ristocetin. The findings in the two studies strongly suggest that the von Willebrand factor activity is associated with the factor VIII molecule ($VIII_{VWF}$). In further support of this possibility was the high degree of correlation between the activity of the von Willebrand factor ($VIII_{VWF}$) and both $VIII_{AHF}$ and $VIII_{AGN}$ that we found in normal subjects and in patients with von Willebrand's disease, as well as the close relationship of $VIII_{VWF}$ and $VIII_{AGN}$ in hemophilic plasmas.

These findings, as well as those using platelet retention methods as the test system (2, 3), indicate that the $VIII_{VWF}$ activity is located on the factor VIII molecule. It is apparent, however, that the site on the molecule that determines $VIII_{VWF}$ activity is probably different from that responsible for $VIII_{AHF}$ procoagulant activity. Thus, $VIII_{VWF}$ activity was normal in hemophiliacs, despite the absence of $VIII_{AHF}$ activity in these patients. In addition, the $VIII_{VWF}$ activity of normal plasma was not inhibited by a potent human antibody that inactivated $VIII_{AHF}$ activity. In an accompanying paper, we also showed that a factor in hemophilic plasma that is eluted in the same position as factor VIII on agarose gel chromatography corrects the abnormality of ristocetin-induced platelet aggregation in patients with von Willebrand's disease (1).

The nature of the molecular defect in von Willebrand's disease remains to be determined, but the results of this and previous studies permit some speculations. Zimmerman, Ratnoff, and Powell (18), Stites, Hershgold, Perlman, and Fuenberg (19), Hoyer (7), and Meyer, Lavergne, Larrieu, and Josso (20) have shown that hemophilic plasma contains normal amounts of $VIII_{AGN}$, despite a deficiency of $VIII_{AHF}$, while patients

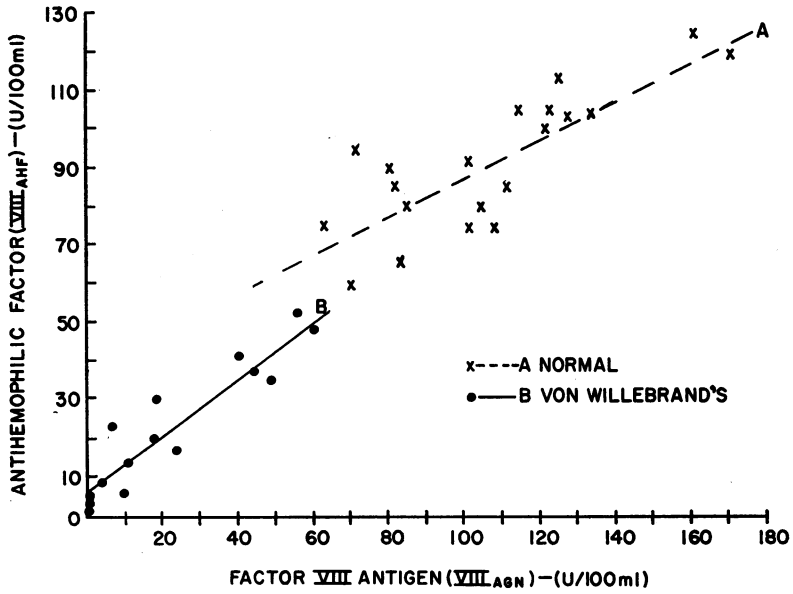

FIGURE 5 Relationship between antihemophilic factor ($VIII_{AHF}$) activity and factor VIII antigen ($VIII_{AGN}$). Equations of the regression lines are: normals (*A*), $y = 0.50\ x + 38.2$; von Willebrand's (*B*), $y = 0.74\ x + 5.9$. Comparison of the two slopes showed an $F_{1,31} = 3.31$, $P > 0.05$.

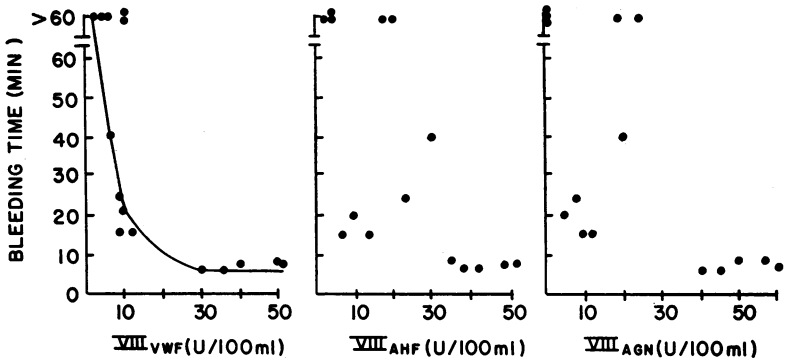

FIGURE 6 Relationship between bleeding time and $VIII_{VWF}$, $VIII_{AHF}$, and $VIII_{AGN}$ in patients with von Willebrand's disease. Because bleeding time values were truncated at 60 min, regression curves could not be calculated. Among the three sets of data shown, the best curve which could be drawn visually appeared to be one depicting a relationship between the bleeding time and $VIII_{VWF}$ activity.

with von Willebrand's disease are deficient in both activities. These findings suggest that patients with hemophilia produce an incomplete or dysfunctional molecule, while the defect in von Willebrand's disease may be an impaired synthesis of the entire factor VIII molecule. The reduction in still a third activity, $VIII_{VWF}$, in von Willebrand's disease is consistent with this hypothesis. In the simplest model, a decreased synthesis of the factor VIII molecule would result in a proportional decrease in $VIII_{AHF}$, $VIII_{AGN}$, and $VIII_{VWF}$. As we know very little about the synthesis of factor VIII or the molecular configuration that determines its biological and antigenic properties, it is entirely possible that some bleeding syndromes may be owing to a decreased synthesis of a factor VIII molecule that has, in addition, functional abnormalities of the sites that determine $VIII_{AGN}$, $VIII_{AHF}$, or $VIII_{VWF}$. This would be recognized as nonproportional reduction of one or more properties. The relatively greater decrease in $VIII_{VWF}$ activity in patients J. L. and A. S. may represent this kind of molecular change. Holmberg and Nilsson have recently reported that some patients thought to have von Willebrand's disease have reduced $VIII_{AHF}$ levels but normal amounts of $VIII_{AGN}$ (25). As these patients have a prolonged bleeding time, this syndrome may represent a dysfunction of $VIII_{AHF}$ *and* the von Willebrand factor even though normal amounts of the molecule are produced. Assay of $VIII_{VWF}$ activity in these patients would be of interest. Another dissociation of the properties of the factor VIII molecule is recognized in patients with von Willebrand's disease who have been transfused with plasma. Bennett, Ratnoff, and Levin have reported that the delayed increase in $VIII_{AHF}$ activity in these patients is not associated with a parallel increase in $VIII_{AGN}$ (26). A more satisfactory interpretation of the findings in von Willebrand's disease awaits a better understanding of the structure and function of factor VIII.

Recent studies have shown that under conditions of high salt concentration, $VIII_{AHF}$ activity is detected with proteins that have a much lower molecular weight than plasma $VIII_{AHF}$ (6, 27, 28), while $VIII_{AGN}$ remains with proteins that have a molecular weight in excess of 2×10^6 (29). Although there is evidence that $VIII_{VWF}$ activity is located on a site of the factor VIII molecule different from that of $VIII_{AHF}$, it is not certain that it remains with the $VIII_{AGN}$ fragment after dissociation in high salt concentrations. Studies to resolve this question are in progress.

Addendum. We have subsequently shown that $VIII_{VWF}$ activity does, in fact, remain with the $VIII_{AGN}$ fragment under these conditions (30).

ACKNOWLEDGMENTS

The authors thank Mrs. Carol Church and Mrs. Margaret Van Why for their expert technical assistance.

This work was supported in part by U. S. Public Health Service Grant HL 14595 from the National Heart and Lung Institute and by a Veterans Administration Research Grant.

REFERENCES

1. Weiss, H. J., J. Rogers, and H. Brand. 1973. Defective ristocetin-induced platelet aggregation in von Willebrand's disease and its correction by factor VIII. *J. Clin. Invest.* **52**: 2697.
2. Bouma, B. N., Y. Wiegerinck, J. J. Sixma, J. A. Van Mourik, and I. A. Mochtar. 1972. Immunological characterization of purified antihaemophilic factor A (factor VIII) which corrects abnormal platelet retention in von Willebrand's disease. *Nat. New Biol.* **236**: 104.
3. Weiss, H. J., J. Rogers, and H. Brand. 1973. Properties of the platelet retention (von Willebrand) factor and its similarity to the anti-hemophilic factor (AHF). *Blood.* **41**: 809.
4. Weiss, H. J., and J. Rogers. 1972. Correction of the platelet abnormality in von Willebrand's disease by cryoprecipitate. *Am. J. Med.* **53**: 734.

5. Weiss, H. J. 1968. von Willebrand's-disease diagnostic criteria. *Blood.* **32**: 668.
6. Weiss, H. J., and S. Kochwa. 1970. Molecular forms of antihaemophilic globulin in plasma, cryoprecipitate and after thrombin activation. *Br. J. Haematol.* **18**: 89.
7. Hoyer, L. W. 1972. Immunologic studies of antihemophilic factor (AHF, factor VIII). IV. Radioimmunoassay of AHF antigen. *J. Lab. Clin. Med.* **80**: 822.
8. Hoyer, L. W. 1972. Immunologic studies of antihemophilic factor (AHF, factor VIII). III. Comparative binding properties of human and rabbit anti-AHF. *Blood.* **39**: 481.
9. Snedecor, G. W., and W. G. Cochran. 1967. Statistical Methods. Iowa State University Press, Ames. 6th edition.
10. Graybill, F. A. 1961. An Introduction to Linear Statistical Models. Vol. I. McGraw-Hill, New York.
11. Fisher, R. A. 1958. Statistical Methods for Research Workers. Hafner Publishing Co., Inc., New York.
12. Cornu, P., M. J. Larrieu, J. Caen, and J. Bernard. 1963. Transfusions studies in von Willebrand's disease: Effect on bleeding time and factor VIII. *Br. J. Haematol.* **9**: 189.
13. Nilsson, I. M., M. Blomback, and I. von Francken. 1957. On an inherited autosomal hemorrhagic diathesis with antihemophilic globulin (AHG) deficiency and prolonged bleeding time. *Acta Med. Scand.* **159**: 35.
14. Cornu, P., M. J. Larrieu, J. Caen, and J. Bernard. 1963. Transfusion studies in von Willebrand's disease. Effect on bleeding time and factor VIII. *Br. J. Haematol.* **9**: 189.
15. Perkins, H. A. 1967. Correction of the hemostatic defects in von Willebrand's disease. *Blood.* **30**: 375.
16. Salzman, E. W. 1963. Measurement of platelet adhesiveness. A simple in vitro technique demonstrating an abnormality in von Willebrand's disease. *J. Lab. Clin. Med.* **62**: 724.
17. Howard, M. A., and B. G. Firkin. 1971. Ristocetin—a new tool in the investigation of platelet aggregation. *Thromb. Diath. Haemorrh.* **26**: 362.
18. Zimmerman, T. S., O. D. Ratnoff, and A. E. Powell. 1971. Immunologic differentiation of classic hemophilia (factor VIII deficiency) and von Willebrand's disease. *J. Clin. Invest.* **50**: 244.
19. Stites, D. P., E. J. Hershgold, J. D. Perlman, and H. H. Fudenberg. 1971. Factor VIII detection by hemagglutination inhibition: hemophilia A and von Willebrand's disease. *Science (Wash. D. C.).* **171**: 196.
20. Meyer, D., J. M. Lavergne, M. J. Larrieu, and F. Josso. 1972. Cross-reacting material in congenital factor VIII deficiencies (haemophilia A and von Willebrand's disease). *Thromb. Res.* **1**: 183.
21. Kass, L., O. D. Ratnoff, and M. A. Leon. 1969. Studies on the purification of antihemophilic factor (factor VIII). I. Precipitation of antihemophilic factor by concanavalin A. *J. Clin. Invest.* **48**: 351.
22. Hershgold, E. J., A. M. Davison, and M. E. Janszen. 1971. Isolation and some chemical properties of human factor VIII (antihemophilic factor). *J. Lab. Clin. Med.* **77**: 185.
23. Schmer, G., E. P. Kirby, D. C. Teller, and E. W. Davie. 1972. The isolation and characterization of bovine factor VIII (antihemophilic factor). *J. Biol. Chem.* **247**: 2512.
24. Marchesi, S. L., N. R. Shulman, and H. R. Gralnick. Studies on the purification and characterization of human factor VIII. *J. Clin. Invest.* **51**: 2151.
25. Holmberg, L., and I. M. Nilsson. 1972. Genetic variants of von Willebrand's disease. *Br. Med. J.* **3**: 317.
26. Bennett, B., O. D. Ratnoff, and J. Levin. 1972. Immunologic studies in von Willebrand's disease. Evidence that the antihemophilic factor (AHF) produced after transfusions lacks an antigen associated with normal AHF and the inactive material produced by patients with classic hemophilia. *J. Clin. Invest.* **51**: 2597.
27. Weiss, H. J., L. L. Phillips, and W. Rosner. 1972. Separation of subunits of antihemophilic factor (AHF) by agarose gel chromatography. *Thromb. Diath. Haemorrh.* **27**: 212.
28. Thelin, G. M., and R. H. Wagner. 1961. Sedimentation of plasma and antihemophilic factor. *Arch. Biochem. Biophys.* **95**: 70.
29. Rick, M. E., and L. W. Hoyer. 1973. Immunologic studies of antihemophilic factor (AHF, factor VIII). V. Immunologic properties of AHF subunits produced by salt dissociation. *Blood.* In press.
30. Weiss, H. J., and L. W. Hoyer. 1973. Dissociation of antihemophilic factor procoagulant activity from the von Willebrand factor. *Science (Wash. D. C.).* In press.