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Nature of von Willebrand Factor: A New Assay and a Specific Inhibitor

(platelet aggregating factor/macromolecules and hemostasis/factor VIII inhibitors/antihemophilic factor/hemophilia A)

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ABSTRACT **Platelet-active "von Willebrand factor" is a poorly characterized activity of a plasma-protein macromolecular complex. A new simple assay for von Willebrand factor is based on the dose response relation of the factor and the ristocetin platelet aggregation time. This assay uses the "snowstorm" macroscopic endpoint. A multiply transfused subject with von Willebrand's disease was observed to have a circulating inhibitor that blocks normal ristocetin aggregation of platelets, but not ADP- , epinephrine- , or collagen-induced aggregation. The inhibitor was not adsorbed by normal platelets, and was stable to heating at 56° for 30 min and to repeated freezings and thawings. This inhibitor also prevents action on human platelets by platelet-aggregating factor of bovine plasma, indicating this bovine factor activity is a function of von Willebrand factor. Three inhibitors were compared: (*1*) the von Willebrand factor inhibitor specifically blocked von Willebrand factor activity, (*2*) a human antibody from a hemophiliac inhibited only antihemophilic factor activity, and (*3*) a rabbit antiserum to a preparation of human antihemophilic factor inhibited both activities. The active site for von Willebrand factor on the macromolecular complex appears to be spaced some distance from the antihemophilic factor site.**

In hemophilia A there is a deficiency of a procoagulant activity, antihemophilic factor (AHF, factor VIII), needed for hemostasis and fibrin clotting. In von Willebrand's disease, there is a deficiency in plasma not only of AHF but also of a platelet-active factor, so-called "von Willebrand factor" (vWF), needed for "cellular clotting" and hemostatic plug formation. In bleeder disease of swine, similar in most respects to human von Willebrand's disease, there is a plasma deficiency not only of AHF and vWF but also of platelet aggregating factor (PAF), which causes aggregation of human but not homologous platelets. These three biological activities of plasma are functions of high-molecular-weight plasma protein(s) found in the exclusion volume on agarose gel chromatography (1–4). There has been much speculation whether these three activities are functions of one or of several large proteins contained in the AHF–vWF–PAF complex.

Assays for AHF, particularly the partial thromboplastin time procedure (5), are well standardized. An assay for PAF in bovine and porcine plasmas was recently described (4, 6). This procedure uses a macroscopic platelet aggregation test and makes possible relatively precise quantitation of PAF. Until recently, there have been only qualitative tests for vWF, based on procedures either *in vivo* or *in vitro*, such as correction of the prolonged bleeding time of affected subjects by transfusion or correction of impaired platelet "adhesion" tests. With the demonstration that ristocetin requires vWF for the aggregation of platelets, the possibilities for developing a quantitative assay for vWF have become apparent (7, 8).

Specific neutralizing but nonprecipitating antibodies to AHF (anti-AHF) have been identified in the plasma of some multiply transfused subjects with hemophilia A and are often referred to as AHF inhibitors. These antibodies have been useful in the characterization of the antihemophilic factor and have been used as a test of identity of AHF procoagulant activity to distinguish it from other clotting activities. A specific vWF inhibitor occurring in von Willebrand's disease, similar to AHF inhibitors in hemophilia, has not been recognized. However, heterologous precipitating antibodies raised with human AHF concentrates as antigens (9) neutralize both AHF and vWF activity in plasma.

An assay for vWF using ristocetin and the macroscopic platelet aggregation procedure (10) has been developed. The assay is comparable in methodology and sensitivity to the recently described assay for PAF (4). With this new assay, a specific inhibitor of vWF, analogous to the human AHF inhibitor, has been identified in the plasma of a multiply transfused subject with severe von Willebrand's disease. This vWF inhibitor is described and partially characterized. These findings have allowed a re-examination of certain aspects of the AHF–vWF–PAF complex. Further support is provided for the idea that PAF is identical to vWF.

MATERIALS AND METHODS

Plasma Preparations. Human plasma was obtained from normal healthy adult donors, both male and female, and from subjects with severe von Willebrand's disease (2 men, 1 woman), hemophilia A (1 subject), and hemophilia A with high titer (1:32) circulating anticoagulant (1 subject). By history, donors had taken no drugs for at least two weeks before blood collection. Plasma salicylate levels (11) were determined on selected samples as a check on drug histories. Blood (8 parts) was collected in 3.2% (w/v) trisodium citrate (1 part) by the two-syringe technique. Siliconized glassware was used. For platelet-rich plasma (PRP), the blood was centrifuged at 320 × *g* for 8 min (22°) and used promptly in the experiments. For plasma poor in platelets, centrifugation was at 1600 × *g* for 10 min; if not used promptly, the plasma was frozen (−20°) in aliquots of 2 ml. In some experiments, heated plasmas (56°, 30 min) were used. The bovine plasma was prepared as described (4).

Abbreviations: AHF, antihemophilic factor; PAF, platelet aggregating factor; vWF, "von Willebrand factor".

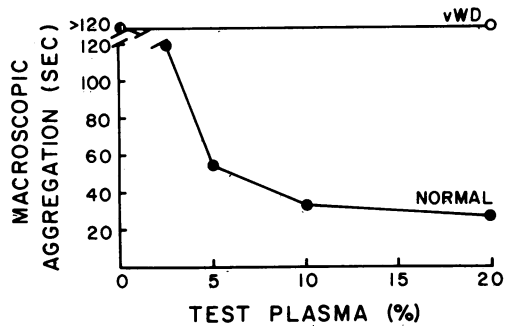

FIG. 1. The effect of normal or von Willebrand's disease plasma in the macroscopic test for ristocetin-induced platelet aggregation. The per cent test plasma refers to the concentration of plasma in the final test mixture. Normal plasma (●); von Willebrand's disease (*vWD*) plasma (○).

Platelet Suspensions. The platelets were separated from plasma by column chromatography (12), with a Tris–saline–dextrose buffer (5 mM Tris·HCl, 0.15 M NaCl, 0.1% (w/v) dextrose, pH 7.4). The platelet suspensions from the column were then centrifuged (1600 × *g*, 10 min, 26°) and washed twice, using the buffer as a diluent for the stock EDTA solution (10). The final suspension contained 1,000,000/mm³ of platelets in buffer.

For the plasma adsorption studies with normal platelets, aliquots of washed platelet suspensions containing 8×10^8 platelets were sedimented and suspended in 2 ml of citrated plasma of different types: normal, von Willebrand's disease, or vWF inhibitor. These suspensions were incubated at 22° for 10 min, sedimented again, and washed once before final suspension to give 1×10^6 platelets per mm³.

Rabbit Antiserum to Human AHF was obtained commercially (Nordic Pharmaceuticals and Diagnostics, Tilburg). Each lyophilized ampule was reconstituted with 1 ml of distilled water.

Platelet Aggregation Tests with Ristocetin. Two separate tests of ristocetin-induced platelet aggregation were used. In one test, platelet aggregation time was determined macroscopically (10). In the other test, platelet aggregation was judged by light transmission using an aggregometer (Payton Associates, Buffalo, N.Y.). Each test was performed at 26° with a final concentration in the mixture of 1.2 mg of ristocetin (Abbott, Chicago) per ml.

The macroscopic test for ristocetin-induced platelet aggregation was performed as follows: To a mixture of 0.1 ml of plasma, whole or diluted with buffer, 0.1 ml of buffer, and 0.1 ml of test material or buffer were added 0.1 ml of platelet suspension and then 0.1 ml of ristocetin. The time in seconds from the addition of ristocetin to the appearance of 4+ platelet aggregates ("snowstorm effect") was recorded if aggregation occurred within 120 sec (10).

In the aggregometer test for ristocetin-induced platelet aggregation, 0.1 ml of ristocetin was added to a mixture of 0.6 ml of platelet-rich plasma and 0.3 ml of test plasma, whole or diluted with buffer. Platelet-poor plasmas were brought to pH 7.3 with 0.1 M acetic acid before they were mixed. Plasmas were stirred (900 rpm) in the aggregometer at 22° for 1 min for a base-line tracing before addition of ristocetin.

Bovine PAF Neutralization Test. The procedure was a modification of the PAF test (4) and was performed as follows: To a mixture consisting of 0.1 ml of 12.5% (v/v) bovine plasma and 0.3 ml of test material diluted with buffer was added 0.1 ml of human platelet suspension. Macroscopic aggregation times were recorded in seconds.

Platelet Aggregation with Other Agents. All tests were done in the aggregometer at 37° and stirred at 900 rpm. The aggregating agents used were adenosine diphosphate (ADP) (Sigma, St. Louis) with a final concentration of 5 μM, adrenalin (Parke-Davies, Detroit) in a final concentration of 0.1 mg/ml, and a microcrystalline collagen suspension (Avicon, Fort Worth) (13).

AHF Activity. AHF was assayed by the partial thromboplastin time method (5) by a kaolin-activated system. Substrate plasma was from human hemophilia A donors for human plasma assays, and from the Chapel Hill colony of hemophilia A dogs for bovine plasma assays. AHF neutralization was tested by incubation (28°, 30 min) of equal volumes of normal plasma and test material, followed by determination of residual AHF.

RESULTS

Macroscopic Test for vWF Activity. The time required for macroscopic platelet aggregation induced by ristocetin was a function of the concentration of normal plasma. Fig. 1 demonstrates that aggregation times in the range of 30–120 sec correspond to normal plasma concentrations in the range of 20–2.5% and indicates the sensitivity of the test. In separate experiments with seven different normal subjects the mean aggregation time with 20% normal plasma was 29 sec. Replicate values in the same experiment were within the range of ±10%. On the other hand, plasmas from three subjects with von Willebrand's disease were inactive. It thus appears that the aggregation time is an indicator of the relative vWF activity of plasma.

Evidence for an Inhibitor of vWF Occurring in a Subject with von Willebrand's Disease. An inhibitor for vWF activity was revealed in one of three subjects with von Willebrand's disease when their plasmas were tested in the presence of normal plasma. The subject with inhibitor had been multiply transfused for bleeding episodes over a period of years. The results are shown in Fig. 2. Plasmas from two subjects with

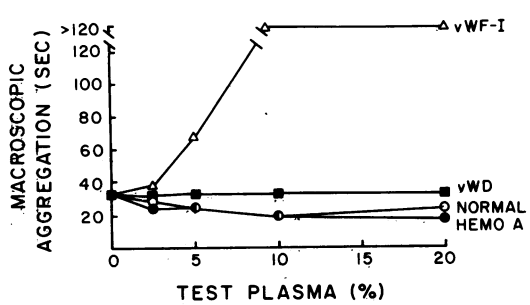

FIG. 2. The effect of various plasmas on vWF activity, measured by the macroscopic ristocetin aggregation test. Each final test mixture contained 20% normal plasma and various concentrations of hemophilic, von Willebrand, or additional normal plasma. Added normal plasma (○); severe hemophilia A (*Hemo A*) (●); severe von Willebrand's disease (*vWD*) plasma (■); plasma of subject with von Willebrand's disease and an inhibitor of von Willebrand factor (*vWF-I*) (△). Results with vWF inhibitor, heated or subjected to freezing and thawing, were similar to those with untreated vWF inhibitor.

von Willebrand's disease (1 man, 1 woman) did not influence the macroscopic aggregation times. On the other hand, plasma from the third subject with the disease (vWF inhibitor) caused increasingly prolonged aggregation times as the amount of plasma was increased. When this von Willebrand plasma was mixed with normal plasma in equal amounts, no aggregation was detected either macroscopically or microscopically. In contrast to the results with von Willebrand plasmas, hemophilia A plasma caused some further shortening of the platelet aggregation time, comparable to parallel observations with similar increments of normal plasma.

Another test of platelet aggregation was used to study the inhibitor plasma. Fig. 3 shows aggregometer tracings of ristocetin-induced aggregation using mixtures of normal platelet-rich plasma and normal, von Willebrand, or vWF inhibitor platelet-poor plasma. Tracings for normal plasma, buffer control, and von Willebrand plasma show similar rapid aggregation after addition of ristocetin. The tracing with the vWF inhibitor plasma shows inhibition of aggregation which is dose-dependent (Fig. 3, curves *d–h*).

The inhibitor plasma and normal plasma were incubated up to 1 hr to determine if the extent of neutralization of vWF activity increased with time. The amount of inhibitor plasma used was sufficient to cause a lengthening of aggregation times without incubation in the range of 15–20 sec. Immediate but not progressive neutralization of vWF activity with incubation was observed.

It is possible that the inhibitor activity is directed either toward vWF or toward platelet receptor(s) for vWF. Therefore, the ability of normal platelets to adsorb the inhibitor was tested. Normal platelets were incubated with vWF inhibitor, von Willebrand, or normal plasma and then tested for aggregability in the macroscopic test as in Fig. 1. There was no prolongation of aggregation times of platelets treated with vWF inhibitor as compared to the controls, and the times in all tests were comparable to those indicated in Fig. 1 with 5, 10, and 20% normal plasmas. The ratio of vWF inhibitor plasma to normal platelets was at least 5 times greater than that required to completely inhibit aggregation in the test system illustrated in Fig. 2. The vWF inhibitor plasma treated with normal platelets was assayed for inhibitor titer in the test system illustrated in Fig. 2. There was no decrease in the inhibitor titer. These experiments indicate that the normal platelets did not adsorb vWF inhibitor.

The effect of vWF inhibitor on other types of platelet aggregation was studied also. ADP, epinephrine, and collagen were used as aggregating agents with platelet-rich plasma in an aggregometer. One set of experiments was done with normal platelets, the other set with platelets from the subject with the vWF inhibitor. With normal platelets, in experiments similar to those illustrated in Fig. 3, but with platelet-rich plasma and vWF inhibitor plasma being present in a ratio of 2:1, the aggregometer recordings with the different aggregating agents were the same, whether or not vWF inhibitor plasma was present. With platelets from the subject with vWF inhibitor, the results with ADP and epinephrine were the same as with normal platelets; with collagen, the amplitude of the recordings was somewhat reduced even though large aggregates formed.

Effect of vWF Inhibitor on Bovine PAF. The effect of the vWF inhibitor plasma on bovine plasma PAF activity in the macroscopic aggregation test was compared with that of

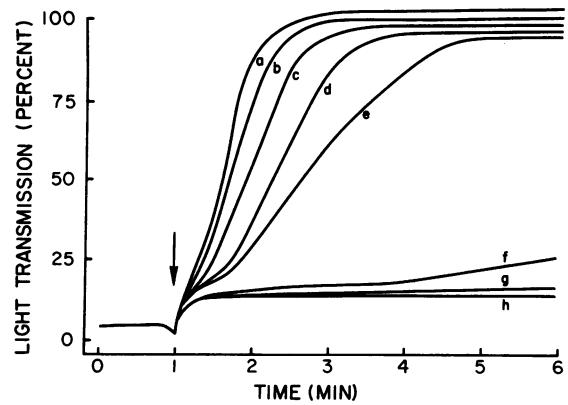

FIG. 3. Tracings of aggregometer recordings showing the effect of addition of various plasmas on ristocetin-induced platelet aggregation in normal platelet-rich plasma. Ristocetin was added (*arrow*) to mixtures of normal platelet-rich plasma (435,000 platelets per mm^3) and platelet-poor plasmas. The platelet-poor plasmas used were as follows: (*a*) 30% (final concentration) additional normal plasma; (*b*) buffer control; (*c*) 30% von Willebrand's disease plasma; (*d*) 10% vWF inhibitor plasma; (*e*) 13% vWF inhibitor plasma; (*f*) 15% vWF inhibitor plasma; (*g*) 20% vWF inhibitor plasma; (*h*) 30% vWF inhibitor plasma.

normal human plasma and of uncomplicated von Willebrand human plasma (Fig. 4). The von Willebrand plasma had no effect on aggregation time, while normal plasma caused shortening from 33 sec to 20 sec. In contrast, the vWF inhibitor plasma prolonged the aggregation time to 120 sec. This inhibitory effect was dose dependent. When the concentration of vWF inhibitor plasma was raised to 60% (not indicated in Fig. 4), no aggregation occurred. This specific effect of the vWF inhibitor plasma on the bovine PAF would indicate that bovine PAF is indeed vWF.

Comparison of vWF Inhibitor with Two Antibody Inhibitors of AHF. Two sets of experiments were done. In the first set, the inhibitory effect of a rabbit anti-AHF on human vWF and bovine PAF was determined (Fig. 5). The aggregation times became progressively longer as the concentration of antiserum was increased, comparable to those observed with vWF inhibitor (Figs. 2 and 4).

In the second set of experiments, a comparison of the effect of vWF inhibitor with that of rabbit "anti-AHF" and a human anti-AHF was made (Table 1). Neutralization of both

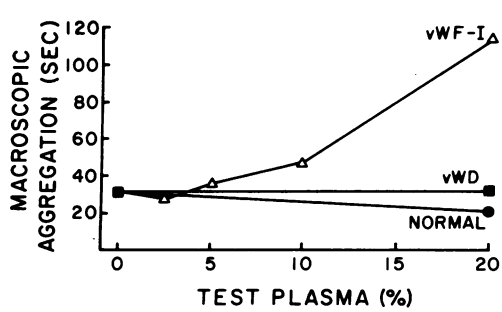

FIG. 4. The effect of normal, von Willebrand, and von Willebrand inhibitor human plasmas on bovine PAF activity, measured by the macroscopic aggregation test. The concentration of test plasma in the final mixture of bovine plasma, test plasma, and human platelet suspension is indicated on the abscissa. Normal human plasma (●); von Willebrand (*vWD*) plasma (■); vWF inhibitor plasma (△).

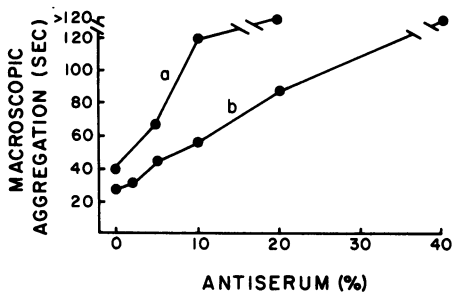

FIG. 5. Neutralization of vWF and PAF by a rabbit antiserum to an AHF preparation. Mixtures of antiserum and either human or bovine plasma were incubated at 22° for 10 min before testing. (*a*) Bovine PAF neutralization test, using bovine plasma (2.5% final concentration), dilutions of rabbit antiserum, and human platelets. (*b*) Human vWF neutralization test, using normal human plasma (20% final concentration), dilutions of rabbit antiserum, and human platelets plus ristocetin.

AHF activity and vWF activity was determined. The vWF inhibitor completely neutralized vWF activity but did not neutralize AHF procoagulant activity. Conversely, the hemophilia A anticoagulant neutralized the AHF activity but was without effect on vWF activity. In contrast, the rabbit "anti-AHF" neutralized both the AHF and vWF.

DISCUSSION

The platelet-active von Willebrand factor activity of plasma, deficient in patients with von Willebrand's disease, is poorly characterized and heretofore has been difficult to quantitate. The discovery that ristocetin-induced aggregation of platelets requires vWF has provided a means for studying human vWF (7, 8). It was demonstrated many years ago that suspensions of isolated platelets can be used for quantitation of aggregating reactions of platelets with different agents (10). Platelet aggregation can be readily observed macroscopically. If large platelet aggregates are formed rapidly, they produce a so-called "snowstorm" effect, which provides a sharp endpoint. We have used this endpoint in the development of an assay for vWF with ristocetin. The test is relatively simple to perform and the skill required to accurately determine the endpoint is not difficult to attain. The platelets used in the test are gel filtered and then washed, with pH controlled. The wash is needed because the vWF activity, like AHF activity, is excluded along with the platelets from the agarose column (14). This washing procedure and the control of pH are important steps in making the assay sensitive to small variations (±5–10%) in concentration of vWF. This procedure appears to have advantages over the use of an aggregometer. Availability of this assay led to the recognition of a specific inhibitor of the vWF.

TABLE 1. *Comparison of two naturally occurring human inhibitors with a rabbit antiserum to a purified human AHF preparation*

	AHF neutralization		vWF neutralization	
Inhibitor	Dilution	% Residual activity	Dilution	% Residual activity
Human anti-AHF	1.4	<5	1:5	100
	1:16	25	1:10	100
	1:64	45	1:20	100
	1:256	100	1:50	100
vWF inhibitor	1:1	80	1:5	0*
	1:2	100	1:10	20
	1:4	80	1:20	40
	1:8	100	1:50	100
Rabbit "anti-AHF"	1:1	10	1:2.5	<2.5†
	1:2	35	1:5	20
	1:4	40	1:10	25
	1:8	70	1:20	40
Buffer control	—	100	—	100

* Also no platelet aggregates microscopically.

† 4+ platelet aggregates after 2 min; estimated as <2.5% vWF.

Evidence that the inhibitor in a subject with von Willebrand's disease is specifically directed against the von Willebrand factor was obtained along several lines. First, the extent of the inhibition of ristocetin-induced platelet aggregation was dependent on the amount of inhibitor added. Further, the inhibitor prevented aggregation completely if present in sufficient concentration. A second line of evidence was that the inhibitor did not interfere with normal platelet aggregation due to adenosine diphosphate, adrenalin, or collagen. The same was true with aggregation of platelet-rich plasma obtained from the subject with the vWF inhibitor except that the degree of collagen-induced aggregation was somewhat reduced, as judged by aggregometer recordings. This finding suggests that the vWF might facilitate collagen-induced platelet aggregation but that it is not essential for the reaction. The third line of evidence had to do with reactivity of the platelets after exposure to the inhibitor. Such platelets, after washing, were fully active in the macroscopic aggregating test with ristocetin. This finding would be incompatible with the alternate hypothesis that the inhibitor reacted with the platelet membrane receptor for the vWF rather than vWF itself. Further, it was found that the plasma inhibitor titer was not reduced by platelet "adsorption." From these data it was concluded that the inhibitor did not react with the platelets but reacted directly in the plasma phase with the vWF.

The action of the specific inhibitor of vWF on bovine PAF was similar to its neutralizing action on human vWF (Figs. 2 and 4). The presence of PAF and AHF in the void volume after agarose gel chromatography of bovine plasma, similar to the presence of vWF and AHF in the void volume of human plasma, along with the finding that AHF could be dissociated from PAF and vWF with a high salt concentration, had previously led to the suggestion that PAF and platelet-active vWF may be manifestations of the same basic plasma activity (4). Our findings with the specific inhibitor would indicate that bovine PAF is, in fact, the vWF. Recently the porcine PAF was found to be deficient in bleeder swine, and it was likewise concluded that the porcine PAF is vWF (6). Our data also show that the human vWF inhibitor is not species specific, since it inhibits not only the vWF in human plasma but also the vWF in bovine plasma.

The activity of vWF inhibitor was compared with that of two other inhibitors, both antibodies (Table 1). One of these was a relatively high-titer human anti-AHF. The other was an antibody raised in rabbits to an AHF plasma concentrate that presumably also possessed vWF activity. The inhibitor to vWF was distinct from each of the other two inhibitors as it

alone inhibited only vWF and not AHF. The circulating anticoagulant, human anti-AHF, inhibited only AHF and not vWF. Also, it was shown previously that this inhibitor did not neutralize bovine PAF, although it did neutralize bovine AHF (4, 15). The rabbit antibody neutralized not only AHF, but also vWF and PAF (Fig. 5). The difference in neutralizing titers suggests that the rabbit antiserum may have possessed more anti-vWF activity than anti-AHF activity. The vWF inhibitor, like the human anti-AHF, is heat stable at 56° for 30 min and is stable to repeated freezing and thawing steps. Although more data are needed before its antibody nature can be established, the data do demonstrate that the specificity of the inhibitor is for only vWF (PAF). In this respect the vWF inhibitor is unique and appears to be concerned with only a single type of platelet aggregation. The availability of a specific inhibitor of vWF provides a test of identity of this platelet aggregating factor in both qualitative and quantitative studies, to distinguish it from other platelet aggregating activities.

The molecular basis of the AHF–vWF–PAF complex has not been fully elucidated. In addition to its macromolecular and subunit characteristics, it is known that the AHF procoagulant activity can be dissociated from the macromolecule to yield a small molecular weight entity ("little piece"). Unlike the subunits obtained after reduction of disulfide bonds, which are biologically inactive (16), the little piece retains its activity (17). The availability of the vWF inhibitor provides another probe for characterizing this complex. Our data would indicate that the active site(s) for vWF and PAF on the macromolecular complex are identical. These data would also suggest that the vWF/PAF receptor on the human platelet is a single site. The comparative neutralization data with inhibitors demonstrate that it is possible to block only the AHF site with the AHF inhibitor, the vWF site with the vWF inhibitor, and both sites with the heterologous antibody. The covering of both sites by the rabbit antiserum suggests that the antiserum contains at least two separate and specific antibodies. Also, if the two active sites are similar and are on a single macromolecule, they would be spaced some distance apart. Unlike the AHF inhibitor, which requires time to express its full AHF neutralization capacity (18), the vWF inhibitor activity is immediately expressed. If the procoagulant activity of AHF is first dissociated from the macromolecule, the AHF inhibitor is quantitatively much more effective in neutralizing AHF (17, 19). One possibility suggested by these data is that the vWF site on the macromolecule is readily available to the inhibitor, unlike the AHF site, in which steric hindrance may account for the unusual kinetics of the macromolecular AHF neutralization reaction.

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