Antibody nature of circulating inhibitor of plasma von Willebrand factor

(factor VIII inhibitor/platelet-aggregating factor/transfusion response)

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Contributed by K. M. Brinkhous, July 17, 1975

ABSTRACT   A circulating plasma inhibitor of the "von Willebrand factor" was observed in a multiply transfused subject with severe von Willebrand's disease. The platelet-active von Willebrand factor is associated with a plasma protein macromolecular complex that is deficient in the disease. The inhibitor appears to be an IgG antibody, kappa type, based on neutralization tests with goat antisera to specific human immunoglobulins. The IgG and inhibitor separated out together in plasma fractions obtained by "salting-out" and chromatographic procedures. Two separate inhibitor neutralization tests for the platelet-active factor, one with human plasma and ristocetin, the other with bovine plasma, gave similar results, based on the macroscopic aggregation time test of fixed human platelets. With cryoprecipitate transfusions the inhibitor was transiently neutralized with the temporary appearance of von Willebrand factor, factor VIII, and factor VIII-like antigen in the plasma. The plasma inhibitor level increased after transfusion, suggesting an anamnestic response. Lower titer inhibitor plasmas neutralized only the platelet activity. Highest titer plasma also neutralized human factor VIII, but only in part; it did not neutralize either bovine factor VIII or the human small active factor VIII fragment. The anti-factor VIII activity of the von Willebrand factor inhibitor may be due to steric hindrance, dependent on the spatial relationships of factor VIII sites on the macromolecular complex.

The high-molecular-weight plasma protein of human plasma, termed "von Willebrand factor" (vWF), is a platelet-aggregating agent which is deficient in the bleeder disorder, von Willebrand's disease (vWD). Human platelets are aggregated by vWF in the presence of the antibiotic ristocetin (1). A hemorrhagic disorder analogous to human vWD exists in swine (2) and is characterized by a deficiency of a platelet-aggregating factor or PAF (3). PAF in normal porcine plasma as well as bovine plasma will aggregate human platelets directly without ristocetin (3, 4). While the vWF in human plasma and the PAF in animal plasma are tested for in a different manner, the activities appear to be basically the same (3–6) and the abbreviation, vWF/PAF, will be used to refer to them collectively. Platelets are aggregated by many other biological materials besides vWF/PAF, such as collagen, thrombin, and adenosine diphosphate. Recently it was demonstrated that if human platelets are fixed with formaldehyde, they retain their ability to aggregate with vWF/PAF, but will not react with other physiological aggregating agents (7). This finding has been utilized in a recently described simple quantitative test for vWF/PAF, based on the observation that the macroscopic platelet aggregating time is dependent on the concentration of vWF/

PAF (3–5, 7). The use of fixed washed platelets instead of fresh washed platelets has increased the specificity and simplicity of this procedure for the assay of vWF/PAF (8).

Recently several of us with Sarji (5) described a circulating inhibitor to vWF. This inhibitor is designated vWF-I, or simply as the inhibitor. This inhibitor was observed in a multitransfused patient with severe von Willebrand's disease. The inhibitor was heat stable at 60° and was not absorbed by normal human platelets. The inhibitor caused immediate neutralization of the vWF in human plasma and the PAF in bovine plasma. The neutralization of the platelet-aggregating action by the inhibitor appears to provide a test of identity of vWF/PAF in distinction to other platelet-aggregating agents. The vWF/PAF activity of plasma is contained in the void volume on 4% agarose chromatography (4, 9) and is associated with the antihemophilic factor (AHF, factor VIII) activity and the AHF-like antigen of plasma in a macromolecular complex. Small active factor VIII can be dissociated from the complex by buffered 0.25 M $CaCl_2$ (9). Certain subjects with severe hemophilia A develop a circulating anticoagulant which neutralizes factor VIII or AHF (AHF-I). Unlike vWF-I, AHF-I is time dependent in its action and requires incubation with normal plasma or plasma concentrates for demonstration of its full neutralizing capacity. The circulating inhibitor against factor VIII has been shown to be an antibody (10). In this paper studies relating to the antibody nature of the circulating vWF inhibitor, along with a description of the response of the inhibitor vWD subject to transfused cryoprecipitate containing vWF are reported.

MATERIALS AND METHODS

Plasma Preparations. Human plasmas were obtained from normal subjects and subjects with hemophilia, vWD, hemophilia with inhibitor and vWD with inhibitor. They were prepared as described previously (5).

Assay of Human von Willebrand Factor. Assays were done utilizing the macroscopic platelet aggregating test with ristocetin (5) as modified by Brinkhous *et al.* (3). Fixed washed human platelets were used (7).

Assay of Inhibitor. Two separate assays were used, both based on measurements of residual vWF/PAF after incubation of plasmas with serial dilutions of inhibitor plasma (5). One assay was the ristocetin cofactor inhibitor test, in which human plasma was the source of vWF. The test was performed as follows: to a mixture of 0.1 ml of test plasma (whole or diluted) and 0.1 ml of normal plasma (diluted 1:2 with sodium phosphate buffer, pH 7.3) were added 0.1 ml of fixed washed platelet suspension ($600,000/mm^3$) and then 0.1 ml of ristocetin (2.4 mg/ml in phosphate buffer). The

Table 1. Bovine PAF neutralization by inhibitor plasma absorbed with specific antisera

Antiserum	Macroscopic aggregation (sec)*	
	Inhibitor plasma	vWD control plasma
Buffer	>60	31
	>60	30
Anti-IgG	28	24
	29	25
Anti-IgA	>60	28
	>60	27
Anti-kappa	24	32
	25	25
Anti-lambda	52	30
	>60	35

* Buffer control with bovine plasma and normal plasma gave times of 26 and 28 sec; bovine plasma alone gave times of 23 and 23 sec.

time from the addition of ristocetin to the appearance of platelet aggregates was the macroscopic platelet aggregation time. To determine relative inhibitor levels in a plasma sample, a reference curve was constructed, plotting aggregating times against the log of the concentration of a reference inhibitor plasma. The inhibitor reference plasma was obtained from the vWD inhibitor subject in April, 1973 and was arbitrarily assigned a value of 100% inhibitor content. This same plasma or other plasmas with similar inhibitor titer were used for antibody studies (Tables 1 and 2 and Fig. 1).

The other assay was the bovine PAF inhibition test. The procedure was a modification of that previously described (5), and was performed as follows: to a mixture consisting of 0.1 ml 10% (v/v) bovine plasma and 0.2 ml test material diluted with Tris-buffered saline was added 0.1 ml suspension of fixed washed human platelets (600,000/mm³). The test material consisted of human plasmas or plasma fractions from the vWD inhibitor patient and normal subjects for controls. A reference curve similar to that used with the ristocetin cofactor neutralization test was constructed.

Absorption of Plasma with Specific Antisera. Goat antihuman-IgG, antihuman-IgA, antihuman-kappa, and antihuman-lambda antisera (Meloy Laboratories, Springfield, Va.) were concentrated by Amicon pressure dialysis to ¼ volume. The concentrated antisera were added to plasma in four steps to give a final antisera to plasma ratio of 3:1. Each step included a 2 hr incubation at 4° followed by centrifugation to remove the precipitate. Double immunodiffusion of the

Table 2. Inhibitor distribution after fractionation of inhibitor plasma with saturated ammonium sulfate

Inhibitor plasma fraction	Macroscopic aggregation (sec)*	
	Undiluted	1:2 Dilution
0–25% Precipitate	27	24
25–33% Precipitate	>120†	110
33–50% Precipitate	40	28
50% Supernatant	20	20
Buffer	16	—

* In the bovine PAF inhibition test
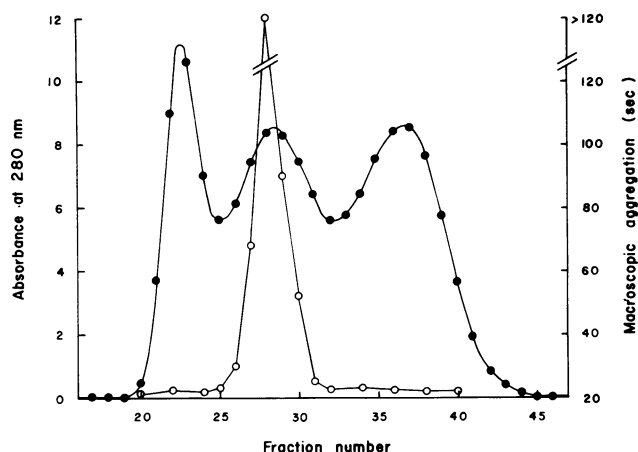† No aggregation microscopically

FIG. 1. Gel filtration of 5 ml of inhibitor plasma on Sephadex G-200 (2.5 × 38 cm) in 0.05 M Tris, 0.15 M NaCl, pH 6.8. Fractions of 3.3 ml were collected at a flow rate of 25 ml/hr. Absorbance at 280 nm was monitored (●). Fractions were tested for inhibition in the bovine PAF inhibition test (O).

absorbed plasma samples against the antisera and the unabsorbed plasma samples was carried out. Absorbed plasma samples were tested for inhibitor in the PAF inhibition test.

Immunodiffusion Studies. Double immunodiffusion was done on absorbed plasma samples or chromatographed plasma fractions to demonstrate presence of antigen (IgG, IgA, kappa, or lambda) using specific goat antisera. Wells were cut in the gel (0.9% agarose, 0.1% N₃ in Tris-buffered saline, pH 6.8) and 10 μl aliquots of sample, untreated plasma, and antiserum were added to wells 6 mm apart and allowed to diffuse overnight at room temperature.

Single radial immunodiffusion was done to determine the concentration of IgG in chromatographed plasma fractions. A molten agarose gel, prepared as above, was cooled to 50°, mixed with a goat antihuman-IgG antiserum to give a 1% solution, and then poured into a mold to give a 1 mm thick slab. After cooling, wells were cut at 3 cm intervals and 10 μl samples were added to the wells. After 72 hr incubation at room temperature the diameter of the immunoprecipitates was measured. Photographs of immunoprecipitates were made with a Cordis immunodiffusion camera.

Ammonium Sulfate Fractionation. To three parts plasma was added one part saturated ammonium sulfate solution (22°) to give a 25% saturated solution. After centrifugation at 1600 × g for 10 min the supernatant was brought to 33% saturation, centrifuged, then brought to 50% saturation. The resulting precipitates were dissolved in saline. The samples were concentrated to ½ original plasma volume by dialysis against 10% polyethylene glycol (20,000 daltons), then dialyzed against saline and tested for inhibitor.

Chromatography. For DEAE-Sephadex ion exchange chromatography, plasma samples were dialyzed against the initial buffer (0.1 M sodium phosphate, pH 8.0), centrifuged, and chromatographed in a 0.9 × 20 cm column, using 1 g of DEAE-Sephadex gel per 20 ml of plasma. Chromatograms were developed with a 200 ml linear gradient. The limit buffer was 1.0 M NaCl. Fractions were monitored at 280 nm and peaks were pooled and dialyzed against Tris-buffered saline and then tested for inhibitor and IgG with double immunodiffusion.

For agarose gel filtration chromatography, fractions (1 ml) from the ion exchange column were chromatographed on a 0.9 × 18 cm column of Bio-Gel A-15m with Tris-buff-

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Table 3. Inhibitor levels following infusion of
cryoprecipitate

Date of infusion	Amount of cryoprecipitate (units)	Time from infusion to collection of samples (months)	Ristocetin cofactor (% I)*	Bovine PAF (% I)*	Anti-AHF activity
			vWF/PAF neutralization tests		
Jan. 1972	40	17	100	100	Neg.
May 1973	120	9	135	170	Neg.
Oct. 1974	160	3	650	640	+

* I = vWF-I

Table 4. Factor VIII neutralization tests with vWD plasma
having high titer vWF-I and with hemophilia A
inhibitor plasma

Factor VIII source	vWD inhibitor plasma* residual factor VIII, %	Hemophilia A inhibitor plasma residual factor VIII, %
	Factor VIII neutralization test	
Human plasma	30†	<5‡
Bovine plasma	100	<5‡
Human small fragment	100	<5

* Same plasma as in Table 3, line 3, undiluted
† Same value immediately and after one hr incubation
‡ Delayed action

ered saline, pH 6.9, at 9 ml/hr. Fractions were monitored at 280 nm and were tested for inhibitor and IgG.

For Sephadex G-200 gel filtration chromatography, samples were chromatographed on a 2.5 × 38 cm column of Sephadex G-200 A50 with Tris-buffered saline, pH 6.8. Fractions were monitored at 280 nm and tested for inhibitor.

AHF-like Antigen. Antigen levels were determined by electroimmunoassay with a rabbit antihuman factor VIII antiserum (Nordic Pharmaceutical and Diagnostics, Tilburg), using the method of Zimmerman *et al.* (11).

Assay of Human Factor VIII. Factor VIII assays were done by the partial thromboplastin time method (12), using a kaolin-activated system and human hemophilia A donors for plasma substrate.

Factor VIII Neutralization Test. Factor VIII neutralization was tested by mixing the test plasma, serially diluted with buffer, with human factor VIII (normal plasma, undiluted or diluted with Tris buffer, or small active factor VIII "little piece" fragment) (9). Assay of residual factor VIII was done immediately and after 1 hr incubation at 28°.

Posttransfusion Studies. Assays for inhibitor, vWF, factor VIII, and AHF-like antigen were done after transfusions of the vWF-I subject. The plasma samples for study were obtained incidental to those collected for monitoring the status of the vWF-I subject after transfusion therapy for hemorrhagic episodes. The vWF-I subject was a member of a well-studied vWD kindred and was apparently homozygous for the disorder (13). He was a 38-year-old white man, weighing approximately 100 kg, with a lifelong history of bleeding, mainly from mucous membranes, with over 70 bleeding episodes. He was treated with repeated transfusions of plasma and whole blood, and since the late 1960's with cryoprecipitate only. There was no detectable vWF. He consistently had a greatly prolonged Ivy bleeding time and low plasma factor VIII. The cryoprecipitate used in the infusion studies was furnished by the Blood Bank, North Carolina Memorial Hospital.

RESULTS

Comparison of the two neutralization tests for assay of inhibitor of vWF-I

The two tests for determining inhibitor levels were compared by performing simultaneous assays on separate samples of inhibitor plasma collected at different times over a period of about 2 years. The results were similar. Thus, the PAF test was used as the standard, with periodic comparisons with the ristocetin cofactor test.

Evidence for the antibody nature of the vWF/PAF inhibitor

The evidence includes the presence of the inhibitor after absorption of specific immunoglobulin classes, after preparation of plasma fractions by a salting out procedure, and after ion exchange and gel filtration chromatography. Also, data were obtained showing the rise of inhibitor level following infusion of cryoprecipitate into the vWF-I subject.

Inhibitor plasma was absorbed with goat antihuman-IgG and anti-IgA, anti-kappa or anti-lambda antisera to remove the specific immunoglobulins as determined by double immunodiffusion. The absorbed plasmas were then tested for inhibitor activity. The results are shown in Table 1. The inhibitor plasma, unlike control vWD plasma, prevented platelet aggregation by bovine plasma. The removal of IgG also removed the inhibitor. The platelet aggregating times after absorption of IgG were 28 and 29 sec, comparable to those obtained with normal human plasma control (26 and 28 sec). On the other hand, the removal of IgA did not remove the inhibitor. The aggregation times remained greater than 60 sec. Similarly, removal of kappa but not lambda immunoglobulins removed the inhibitor. The anti-IgA and anti-lambda antisera did not cause nonspecific inhibition of platelet aggregation when mixed with the vWD control plasma. The goat antisera had no detectable PAF, whereas normal goat plasma did. Thus the loss of inhibitor activity was not due to neutralization by PAF, since the antiserum was free of this factor. The removal of inhibitor was not by nonspecific adsorption to the antigen–antibody precipitate, as evidenced by lack of removal of inhibitor by the precipitation of IgA. It thus appears that the inhibitor is an IgG, kappa antibody.

Inhibitor plasma was fractionated by precipitation of proteins with increasing concentrations of ammonium sulfate to determine if the inhibitor behaves similarly to IgG. The results are shown in Table 2. The bulk of the inhibitor precipitated in the "cut" between one-fourth and one-third ammonium sulfate saturation of the plasma, similar to the known behavior of IgG.

In ion exchange chromatographic studies the inhibitor came through the DEAE-Sephadex column with the first peak, in the initial buffer, as did IgG. Gel filtration chromatography of this first peak on agarose gave a small void volume peak with no inhibitor activity, and a large second peak containing the IgG and the inhibitor.

Gel filtration chromatography of inhibitor plasma on Sephadex G-200 is shown in Fig. 1. The inhibitor was found in fractions 27–30, the pool of which was found by double immunodiffusion to contain IgG. Chromatography of normal plasma on Sephadex G-200 gave similar separation, but there was no inhibitor in any fraction. When the pooled fractions containing inhibitor were rechromatographed on Sephadex G-200, the inhibitor again appeared at the same dilution volume and coincided with the peak of IgG detected by radial immunodiffusion. Thus, in all the chromatographic studies, the inhibitor and the IgG eluted together.

Levels of inhibitor following repeated and widely spaced transfusions

Table 3 shows the plasma inhibitor levels following cryoprecipitate infusions. The shorter the time the sample was obtained after infusion, the higher the inhibitor level. The inhibitor plasma had no AHF-I activity in the first two samples listed in Table 3, as had been observed previously (5). However, in the third sample with the high inhibitor titer, factor VIII neutralization was manifest. Unlike the AHF-I occurring in hemophilia A patients, the action of which is time dependent, the neutralization activity for factor VIII expressed itself immediately.

Factor VIII neutralization studies

In view of the discordant behavior of different vWF-I plasmas against factor VIII, additional testing was done. vWF-I was compared to a high titer AHF-I from an inhibitor hemophilia A patient. The high titer vWF-I was tested to determine if it, like classical AHF-I, would neutralize factor VIII in bovine plasma and a small active human factor VIII preparation obtained by calcium dissociation of the macromolecule. The results are shown in Table 4. The vWF-I plasma did not neutralize either bovine factor VIII or the human low molecular weight factor VIII.

Infusion of cryoprecipitate into vWF inhibitor subject

Cryoprecipitate containing both vWF and factor VIII was infused into the subject with the circulating vWF inhibitor, as indicated in Fig. 2. After each infusion, increased levels of vWF, factor VIII, and AHF-like antigen were observed briefly with a concomitant fall in inhibitor level. After each infusion vWF rose, but returned to the base line value within 1 hr. The factor VIII level rose from less than 1% to 74% (94% predicted) and fell off without a secondary rise of AHF. Following the second transfusion, the factor VIII rose to 58% and fell off again without a secondary rise in AHF activity. The AHF-like antigen was undetectable prior to infusion. In the first postinfusion study, the following series of values were obtained: 30 min, 77%; 60 min, 52%; and 3 hr, 19%. No antigen was detected in subsequent samples collected between 3 and 23 hr. In the second postinfusion study, antigen was detected in only the 30 min sample and was 62% of normal. The inhibitor decreased abruptly after each infusion to undetectable levels, but returned to higher than starting values in about 48 hr. Bleeding time determinations were also determined repeatedly throughout the course of the study. The bleeding time consistently remained greater than 20 min.

DISCUSSION

The vWF-I was previously shown to neutralize both human vWF and bovine PAF, while not interfering with ADP-, epinephrine-, or collagen-induced platelet aggregation (5). Evi-
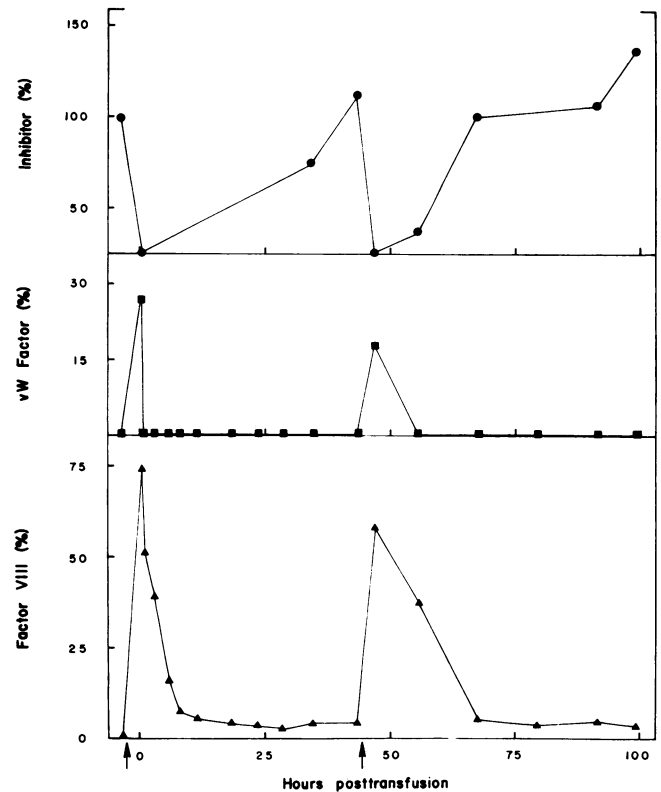

FIG. 2. Factor VIII, von Willebrand factor, and inhibitor levels following infusions of cryoprecipitate into patient with inhibitor. In the first infusion (first arrow) 80 units of cryoprecipitate were given; at 44 hr (second arrow) an additional 40 units were administered. The value of von Willebrand factor on the base line is <5%. The initial level of inhibitor activity determined by the bovine PAF neutralization method was assigned 100%; base line is <25%. ●, inhibitor levels; ■, von Willebrand factor levels; ▲, factor VIII levels.

dence that neutralization tests with either bovine PAF or human vWF could be used to measure the inhibitor level was demonstrated by the close correlation of the inhibitor levels measured by either test on three separate inhibitor samples (Table 3). This finding further supports the hypothesis (3, 5) that active site(s) for vWF and PAF on the macromolecular complex are identical. Perhaps the non-eponymic term PAF, rather than von Willebrand factor, should be used for this platelet-active factor in plasma, regardless of the method used for its detection or the species of plasma used. The limited supply of ristocetin made the PAF neutralization test the desirable one for most vWF-I determinations.

The absorption of the vWF-I by antisera specific for human IgG and kappa light chains but not by anti-IgA and anti-lambda antisera is evidence that the inhibitor is an IgG, kappa antibody (Table 1). Control tests demonstrated that the inhibitor was not absorbed nonspecifically to immunoprecipitates and that the antisera used contained no goat PAF which could have neutralized the inhibitor. The plasma used in these studies had only vWF-I activity.

The inhibitor was salted out in the 25–35% saturated fraction by $(NH_4)_2SO_4$ (Table 2). This fractionation pattern is characteristic of IgG but not of large-molecular-weight factor VIII, which is precipitated in the 25% saturated fraction. The chromatographic results of the inhibitor with ion exchange and gel filtration are all consistent with the hypothesis that it is an IgG.

The rise in the level of the inhibitor following infusion of plasma concentrates suggests an anamnestic response after each infusion, followed by declining levels with time (Table 3). The findings are reminiscent of the changing titers of the anti-AHF antibody in inhibitor hemophilia, where there is often a rapid posttransfusion rise of AHF-inhibitor levels to a maximum, followed by a slow steady decline in levels until the next transfusion (14). The lowest vWF-I level of inhibitor plasma, observed 17 months posttransfusion, was arbitrarily selected as the 100% or "base line" inhibitor level for comparative studies of inhibitor titer.

The ability of the high titer vWF-inhibitor but not the lower titer plasma to neutralize the factor VIII activity of human plasma is of interest (Tables 3 and 4). Unlike the time-dependent neutralization of factor VIII with classical anti-AHF, the vWF-inhibitor plasma acted immediately. Also, the factor VIII activity was only partially quenched by the vWF-I plasma, compared to essentially complete neutralization with inhibitor hemophilia plasma. There was no action by the vWF-inhibitor on the factor VIII activity of bovine plasma. Further, the inhibitor had no action on the factor VIII activity of low molecular weight factor VIII. This unique set of circumstances suggests that one is dealing with a single antibody specific for vWF/PAF and not two antibodies which developed sequentially, the first directed against vWF/PAF, the second against AHF. If this is correct, the anti-AHF action of the vWF-I could be due to a close spacing of the vWF and AHF sites on the human macromolecular complex, with steric hindrance of antibody bound to the vWF preventing full expression of human factor VIII activity. Somewhat analogous findings have been observed with the circulating anticoagulant from hemophilia A patients. Some high titer anti-AHF plasmas may partially neutralize vWF, while lower-titer plasmas do not (15). The vWF-inhibitor could be of monoclonal origin.

The biologic half-life of transfused vWF in human subjects is not known with certainty. In vWD swine, the half-disappearance time of infused porcine PAF is approximately 5–6 hr (3). In the inhibitor subject, there was only a transient posttransfusion rise in vWF. After the first infusion it reached only 27%, lower than the expected level of 100%. The inhibitor temporarily disappeared, apparently neutralized by infused vWF. The initial factor VIII rise after transfusion was somewhat less than expected, 75% versus 97% following the first infusion. Although the factor VIII half-disappearance time was short, it persisted longer than did vWF activity. More striking was the complete absence of a secondary rise in factor VIII following transfusion, unlike the response in uncomplicated vWD. This suggests that while vWF may be needed for the sustenance of factor VIII production, transient vWF availability does not provide a sufficient stimulus for factor VIII response in the inhibitor subject. It has previously been observed in swine vWD that bleeding times became normal when PAF levels were greater than 25–35% (3, 16). In this study the vWF levels only reached this range briefly and the bleeding time always remained prolonged. The AHF-like antigen levels were not parallel to vWF levels in the first transfusion experiment, as might be expected. It could be that the antigen in the circulating antigen–antibody complex is detectable in the immunoassay, even though the platelet aggregating activity is neutralized. The quick return of the inhibitor to the circulation suggests transfer from extravascular to the vascular compartment. There are many points of similarity of this inhibitor with the hemophilia anti-AHF antibody following transfusion, including temporary neutralization of inhibitor, its quick reappearance after transfusion, and an anamnestic response after a time. The AHF-I antibody is also an IgG, with most having kappa light chains (10). Both inhibitors are neutralizing but nonprecipitating in character.

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