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Research Article

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Interaction of Factor VIII Antigen in Hemophilic Plasmas with Human Antibodies to Factor VIII

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ABSTRACT By utilizing a simple modification of previous immunological assays, we have demonstrated that most, if not all, hemophilic plasmas contain antigen reactive with human antibodies directed against Factor VIII procoagulant activity (VIII_c). Antibodies developing in a nonhemophiliac patient and in a hemophiliac patient gave similar results. The VIII_c antigen so identified was removed from hemophilic plasmas with immobilized rabbit antibody which reacted with normal VIII_c and von Willebrand's disease antigen. These data suggest that there are greater antigenic similarities between normal and hemophilic Factor VIII than previously thought.

INTRODUCTION

Antibodies have contributed considerably to our understanding of the molecular biology of diseases associated with abnormalities of Factor VIII procoagulant activity. Heterologous antibodies raised in rabbits and goats have served to identify a protein, variably referred to as the Factor VIII-related antigen, or von Willebrand's disease antigen (vW_{Ag}),¹ which is present in

classic hemophilia but is either decreased or virtually absent in most cases of von Willebrand's disease (1, 2). Human anti-VIII_c antibody has also been used to explore the nature of the molecular defect in hemophilia. Human anti-VIII_c antibodies inactivate Factor VIII procoagulant activity (VIII_c) but, unlike heterologous antibodies, do not precipitate vW_{Ag}. Such antibodies develop in the hemophiliac after Factor VIII infusions, or more rarely, in non-hemophilic individuals without prior transfusion therapy. With one exception (3), previous studies utilizing human anti-VIII_c antibodies have detected VIII_c antigens in only about 10% of hemophilic plasmas (4–10). We now report that such antigens can be detected in all hemophilic plasmas tested; and that these VIII_c antigens can be removed from hemophilic plasmas by immobilized rabbit antibodies to normal human VIII_c and vW_{Ag}.

METHODS

Preparation of immobilized rabbit antibody to VIII_c antigen and vW_{Ag}. Rabbit antiserum to VIII_c and vW_{Ag} was prepared and adsorbed with contaminating antigens as described elsewhere (11, 15). After absorption the antiserum gave only one precipitin line against cryoprecipitates of normal plasma, but did not precipitate with concentrates of plasmas from individuals with severe von Willebrand's disease. Immobilized antibody was prepared by coupling ammonium sulfate precipitated globulin fractions of this antiserum (15) to Sepharose 2B agarose beads (Pharmacia Fine Chemicals Inc., Piscataway, N. J.) with cyanogen bromide as described by others (16a). These beads removed both vW_{Ag} and VIII_c activity from normal plasma but not in a stoichiometric or 1:1 ratio (11). Control beads were prepared by similarly coupling the globulin fractions of a pool of three normal rabbit sera to Sepharose 2B beads.

Preparation of normal human vW_{Ag} antigen free of VIII_c activity. Plasma (acid citrate dextrose or citrate phosphate dextrose) was initially absorbed with aluminum hydroxide (Rehsorptar, Armour Pharmaceuticals Co., Chicago, Ill.) by mixing 1 ml of aluminum hydroxide to

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Abbreviations used in this paper: VIII_c activity, Factor VIII procoagulant activity; VIII_c antigen, Antigen which interacts with human antibody to Factor VIII (anti-VIII_c) as shown by its ability to block the VIII_c inactivating capacity of human anti-VIII antibodies. Heterologous antibodies directed against these antigens will also inactivate VIII_c activity; von Willebrand's disease antigen (Factor VIII-related antigen; vW_{Ag}). An antigen which precipitates with heterologous antibodies and which is decreased or absent in the plasma of most individuals with von Willebrand's disease. Though it co-purifies with VIII_c activity and VIII_c antigen under most conditions, its precise relationship to the molecule(s) expressing VIII_c activity and/or VIII_c antigen is a subject of considerable debate at present (11–14).

10 ml of plasma and stirring for 15 min at 22–25°C, and then removing the aluminum hydroxide by centrifugation at 15,826 *g* for 10 min. 1 vol of absorbed plasma was mixed for 1 h with 10 vol of packed QAE-Sephadex (G-50) which had been equilibrated with 0.01 M phosphate buffer, pH 7.2, adjusted to a conductance of 10 mmho with sodium chloride. The QAE-Sephadex was sedimented at 2,500 *g* for 10 min and washed twice in 10 times the volume of buffer with a conductance of 20 mmho. VIII_c and vW_{Ag} were eluted from the beads by two successive washes with a volume of buffer equal to that of the QAE-Sephadex and with a conductance of 35 mmho. Between 20 and 45% of VIII_c and vW_{Ag} were recovered. This material was then rendered free of VIII_c activity, though not of VIII_c antigen or vW_{Ag}, in the following manner. The preparations from 200 ml of plasma were concentrated to 10 ml in dialysis tubing against Aquacide I-A (Calbiochem, San Diego, Calif.) and were subjected to chromatography on a 2.5 × 90 cm column of 10% agarose, 200–400 mesh (Bio-Rad A-0.5 M, Bio-Rad Laboratories, Richmond, Va.) in 0.8 M sodium chloride (13), 0.05 M Tris (pH 7.3), 0.01 M phenylmethylsulfonylfluoride. Over 90% of the VIII_c was retarded by the column whereas over 90% of the vW_{Ag} eluted in the void volume. All of the preceding manipulations were carried out at 22–25°C. The void volume fractions were concentrated and rechromatographed over the same column and then stored at –20° with repeated freeze-thawing (usually six times) until no residual VIII_c activity could be detected.

Removal of VIII_c antigen and vW_{Ag} from hemophilic plasmas by heterologous antibody beads. Hemophilic plasmas with vW_{Ag} concentrations greater than 1.5 U/ml were incubated with an equal vol (0.4 ml) of packed antibody beads, or control beads, for 4 h at 22–25°C with gentle rocking. The beads were removed by centrifugation at 7,000 *g* for 5 min in a Fisher centrifuge (Fisher Scientific Co., Pittsburgh, Pa.) and an equal volume of beads added to the supernatant plasma as before and incubated overnight with gentle rocking. The beads were again removed by centrifugation. Less than 0.03 vW_{Ag} U/ml remained in the plasma after treatment by antibody beads. vW_{Ag} concentrations in the same plasmas treated with control beads were decreased 20–40% but were still greater than 1.0 U/ml (Table V). In some of the experiments VIII_c-free vW_{Ag} isolated from normal plasma was then added to depleted hemophilic plasma to restore the vW_{Ag} content.

Assays of vW_{Ag} and VIII_c activity. vW_{Ag} was measured by a quantitative immunoelectrophoretic technique (15). 1 U of vW_{Ag} was defined as that amount present in 1 ml of a pool of plasma from 20 normal individuals (15). VIII_c activity was determined by a partial thromboplastin time technique with plasma from individuals with severe hemophilia A (less than 1% VIII_c) as substrate (17).

Human antibodies to VIII_c. Two antibodies were used. One had developed in a nonhemophilic woman; the other in a severe classic hemophiliac. For most experiments, the antibodies were not isolated from the plasma before use, though they were diluted 20-fold or more in barbital buffered saline (2.76 g diethyl barbitaluric acid, 7.3 g sodium chloride, and 2.07 g sodium barbital/liter, pH 7.4–7.6). Neither antibody plasma contained detectable VIII_c and at the dilution used contained less than 0.05 U/vW_{Ag}. The first antibody was also used after it had been heated to 56°C for 60 min and isolated from plasma by ammonium sulfate precipitation (15). The hemophilic and nonhemophilic antibody preparations were used at sufficient dilution so that they introduced less than 5 or 0.5% respectively, of total vW_{Ag} to the incubation mixtures. Neither antibody inactivated ristocetin cofactor activity.

Blocking assay for VIII_c antigens with human anti-VIII_c

Hemophilic plasmas were from unrelated donors and contained less than 1% VIII_c. All were obtained at least 2 wk after Factor VIII concentrate infusion and in some this interval was considerably greater (Table III).

Blocking step. One part of blocking material (either hemophilic plasma, hemophilic plasma depleted of vW_{Ag} by rabbit antibody beads, VIII_c-free vW_{Ag} prepared from normal plasma, or buffer as a control) was mixed with one part of human anti-VIII_c. The anti-VIII_c, which had been diluted to approximately 0.75 U/ml (3) so that it would inactivate only about 75–80% of the active VIII_c, was supplied in the second step

TABLE I
Blocking of Human, Nonhemophilic Anti-VIII_c
with Hemophilic Plasmas

Subject no.	vW _{Ag}	Decrease in clotting time	Increase in VIII activity
	U/ml	s	U/ml
1	1.27	–15.0	+0.40
2	1.77	–19.1	+0.62
3	0.58	–13.8	+0.35
4	1.11	–15.5	+0.52
5	1.66	–17.0	+0.64
6	0.84	–16.6	+0.47
7	0.68	–5.7	+0.21
8	1.50	–16.7	+0.45
9	0.88	–6.9	+0.19
10	1.41	–8.1	+0.22
11	0.78	–9.6	+0.29
12	1.61	–6.9	+0.21
13	1.34	–15.7	+0.39
14	0.75	–8.8	+0.28
15	1.18	–11.7	+0.32
16	1.25	–14.7	+0.29
M ± 6	1.16 ± 0.38	12.6 ± 4.3	0.37 ± 0.14

Nonhemophilic human antibody to Factor VIII was incubated, in the first (blocking) step, with hemophilic plasma or with buffer. In the second (indicator) step, pooled diluted normal plasma was then added as a source of VIII_c. After a second incubation, the VIII_c remaining was assayed. The degree of blocking by hemophilic plasma was assessed by comparing the VIII_c remaining when hemophilic plasma was used in the blocking step with the lesser amount of VIII_c remaining when buffer was used. In the third column, this difference is expressed as the decrease in clotting time in the VIII_c assay. In the fourth column, this difference is expressed as the increase in VIII_c activity that this decrease in clotting time represents. Increase in VIII_c activity did not bear a constant relationship to decrease in clotting time because the assays were performed on different days utilizing different VIII_c-deficient substrate plasmas with different calibration curves. Hemophilic plasmas used in these experiments were obtained at least 2 wk after the last infusion of Factor VIII concentrate. The plasma samples from subjects no. 15 and 16 were tested after incubation with the globulin fraction of normal rabbit sera coupled to agarose beads (see Table V).

TABLE II
Blocking of Hemophilic Anti-VIII_c with
Hemophilic Plasmas

Subject no.	vW _{AS}	Clotting time	VIII _c
	U/ml	s	U/ml
10	1.41	-13.5	+0.25
13	1.36	-11.3	+0.19
15	1.18	-19.6	+0.47

The ability of hemophilic plasma to block anti-VIII_c arising in a hemophiliac was assessed as in Table I.

(see below). This was 1/200 for the nonhemophilic anti-VIII_c when used in the plasma form and 1/500 after ammonium sulfate fractionation. The hemophilic antibody was diluted 1/20. The mixture was incubated for 2 h at 37°C. The ability of the blocking material to block the diluted anti-VIII_c was then assessed by adding active VIII_c and observing the degree of subsequent inactivation.

Indicator step (inactivation of normal VIII_c). Active VIII_c was supplied in the form of a pool of 20 normal plasmas stored at -70°C. It was diluted 1-15 parts in barbital buffered saline and added to an equal volume of incubation mixture from the blocking step. As a control and also to provide an appropriate quantity of VIII_c for the calibration curve, the plasma pool (diluted 1/15) was also added to an equal volume of barbital buffered saline. These mixtures were then incubated for an additional 2 h. VIII_c remaining after this second 2-h incubation was assayed in triplicate or quadruplicate in the partial thromboplastin time VIII_c assay (17) without further dilution. The normal plasma pool, previously diluted 1/15 and incubated with an equal volume of barbital buffered saline for 2 h, was also assayed undiluted. The VIII_c present was assigned the value of 1 U/ml. Serial dilutions of this standard were used to construct the VIII_c calibration curve. The blocking assay was performed in duplicate for each plasma tested and the results averaged. Deviation between duplicates was routinely less than 1-3 s. If the discrepancy was greater, the assay was

TABLE III
Blocking of Human, Nonhemophilic Anti-VIII_c
with Hemophilic Plasmas Obtained after
Prolonged Infusion-Free Periods

Subject no.	Days postinfusion	vW _{AS}	Decrease in clotting time	Increase in VIII activity
		U/ml	s	U/ml
12	56	1.61	-5.5	+0.20
12	63	1.61	-6.9	+0.21
13	28	1.95	-13.7	+0.24
13	58	1.32	-11.9	+0.16
13	72	1.79	-13.1	+0.28
13	79	1.36	-15.7	+0.39
14	78	0.75	-8.8	+0.28

Plasma samples were obtained at the indicated intervals following infusion of Factor VIII concentrates. The degree of blocking was assessed as for Table I.

repeated. The degree of blocking was assessed by comparing the quantities of VIII_c remaining at the end of the second incubation. If a hemophilic plasma (or other blocking material) had blocked the anti-VIII_c, then more VIII_c remained after the final incubation than if buffer had been used instead of blocking material. This increase in residual VIII_c seen when blocking occurred is reported as both the decrease in clotting time (seconds) observed in the VIII_c assay and the increase in units/milliliter of VIII_c that this decrease in clotting time represents (see Tables).

RESULTS

Each of the 16 hemophilic plasmas tested blocked the capacity of nonhemophilic human anti-VIII_c antibody to inactivate VIII_c of normal plasma (Table I). Three of these plasmas were also tested with human antibody which had arisen in a hemophiliac and similar results were obtained (Table II). All of the plasmas tested were obtained at least 2 wk after the last infusion of Factor VIII concentrate. However, some were obtained after infusion-free periods of up to 79 days. Based on the t-½ of 20-40 h of vW_{AS} observed by Bennett and Ratnoff (18) less than 5.4 × 10⁻¹³% of the material infused would have been remaining after this time. Yet, no significant difference in blocking by plasmas obtained after these prolonged infusion-free periods was seen as compared to plasmas obtained after much shorter infusion-free periods (Table III). Thus, it is unlikely that infused Factor VIII can account for the blocking. Antibody separated from plasma by ammonium sulfate precipitation gave results similar to those obtained when antibody in diluted plasma was used (Table IV).

Blocking in this system is determined by assaying the VIII_c activity remaining at the end of the indicator step incubation. If VIII_c antigen is present in the blocking step then less antibody is free to inactivate the VIII_c of the pooled normal plasma supplied in the indicator step. This is reflected as a shortened clotting

TABLE IV
Comparison of Blocking of Plasma Nonhemophilic
Anti-VIII_c and Ammonium Sulfate
Precipitated Anti-VIII_c

Subject no.	vW _{AS}	Decrease in clotting time	Increase in VIII _c activity
	U/ml	s	U/ml
Plasma anti-VIII _c (diluted 1:200)			
14	0.78	-9.2	+0.23
Ammonium sulfate precipitated anti-VIII _c (diluted 1:500)			
14	0.78	-10.6	+0.28

Blocking of these two different preparations of the same antibody was assessed as for Table I.

TABLE V
Depletion of vW_{Ag} from Hemophilic Plasmas: Effect on
Blocking of Nonhemophilic Anti-VIII_c

Subject no.	vW _{Ag}	Clotting time	VIII activity
	U/ml	s	U/ml
15 (control)	1.18	-11.7	+0.32
15 (depleted)	<0.03	-1.9	+0.03
16 (control)	1.25	-14.7	+0.29
16 (depleted)	<0.03	-1.3	+0.02

Hemophilic plasmas were depleted of vW_{Ag} by incubation with rabbit anti-VIII_c-anti-vW_{Ag} which had been coupled to agarose beads (see Methods). As a control, the hemophilic plasma was incubated with the globulin fraction of normal rabbit serum which had been similarly coupled to agarose beads. vW_{Ag} concentration indicated is that after incubation with control or antibody beads. The ability of these plasmas to block human nonhemophilic anti-VIII_c was then evaluated as for Table I.

time in the VIII_c partial thromboplastin time assay. To rule out nonspecific clotting time shortening due to the relatively concentrated hemophilic plasma used in the first step (hemophilic plasma:normal plasma pool ratio = 7.5:1) three additional experiments were performed. First, hemophilic plasma was incubated with immobilized rabbit antibody to vW_{Ag} and VIII_c (Table V). This removed most of the vW_{Ag} (residual concentrations less than 0.03 U/ml). The capacity of the hemophilic plasma to block human anti-VIII_c was also removed. However, nonimmune immobilized rabbit globulin did not remove the capacity of these same hemophilic plasmas to block. Thus the shortened clotting time in the VIII_c assay of the indicator step is directly related to material removed by the rabbit antibody and not to a non-specific quality of the hemophilic plasma used for blocking. In addition, repletion of the hemophilic plasma with vW_{Ag} isolated from normal human plasma—but rendered free of VIII_c activity—restored the ability of the vW_{Ag} depleted hemophilic plasma to block the human antibody (Figs. 1 and 2). This antigenic material blocked the human antibody comparably to hemophilic plasmas containing similar amounts of vW_{Ag}.

Secondly, the hemophilic plasmas to be tested were mixed with an equal amount of buffer instead of antibody, incubated for 2 h, then incubated for 2 h with an equal volume of buffer instead of diluted plasma pool. This mixture was then assayed in the partial thromboplastin time VIII_c assay to see if the hemophilic plasmas alone could shorten the clotting time of the substrate plasma and thus give a false impression of blocking. Most hemophilic plasma tested had no effect on the assay clotting time as compared to that seen when buffer alone was assayed. Some plasmas

lengthened the clotting time up to 5–8 s. An occasional plasma shortened the clotting time up to 5 s. Shortening of 5 s in this portion of the VIII_c calibration curve amounted to less than 0.01 U/ml VIII_c activity. Though this was considerably less than the average of 0.37 U/ml increase in VIII_c activity seen in the blocking experiments, these plasmas were nevertheless excluded from the study.

Thirdly, hemophilic plasma was mixed with an equal volume of antibody, incubated 2 h, and then mixed with an equal volume of buffer instead of diluted normal plasma. This mixture was then incubated an additional 2 h and assayed in the partial thromboplastin time assay. The clotting time was unchanged for that seen when buffer alone was assayed in the partial thromboplastin time assay.

The reproducibility of the blocking assay was assessed by re-assaying samples of plasma from patient no. 10 on eight occasions over a 2-mo period. Blocking, as judged by a decrease in clotting time in the VIII_c assay averaged 8.1 s with a SD of 2.3 s and a range of 5.5–12 s. The corresponding increase in VIII_c activity averaged 0.22 U/ml with a SD of 0.06 U/ml and a range of 0.13–0.33 U/ml.

DISCUSSION

These studies demonstrate that most, if not all, hemophilic plasmas contain antigens capable of interacting with and neutralizing human antibodies to VIII_c. These findings contrast with previous studies (4–10) in which such antigens could only be detected in approximately 10% of hemophilic plasmas. In one previous study—that of Biggs—VIII_c antigens could be detected in all hemophilic plasmas tested but only at

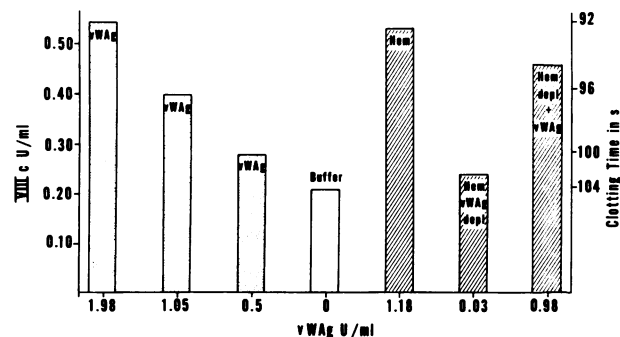


FIGURE 1 Comparison of blocking capacity of vW_{Ag} antigen isolated from normal human plasma (and freed of VIII_c activity—see Methods) with hemophilic plasma, hemophilic plasma depleted of vW_{Ag} by immobilized antibody, and depleted hemophilic plasma repleted with vW_{Ag} antigen isolated from normal human plasma. Factor VIII activity remaining in the indicator step of the assay is indicated on the left vertical axis and the corresponding clotting times on the right vertical axis. The vW_{Ag} content of blocking materials is indicated on the horizontal axis.

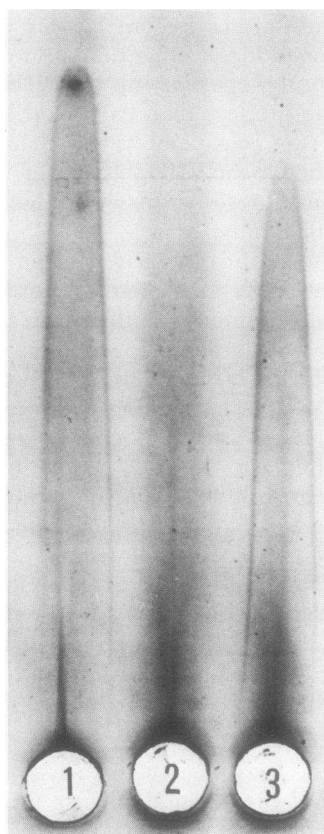


FIGURE 2 Quantitative immunoelectrophoretic measurement of vW_{Ag} in hemophilic plasmas before (well no. 1) and after depletion (well no. 2) of vW_{Ag} by immobilized antibody and following repletion with vW_{Ag} isolated from normal plasma (well no. 3). The height of the rockets are proportional to vW_{Ag} content.

concentrations of antibody greater than 2.0 U/ml (3). At concentrations of antibody less than this $VIII_c$ antigens were detected only in a minority of plasmas. In the studies reported here, however, antibody concentrations of 0.75 U/ml were effective in this regard.

The reason for the differences between the previously reported work and our study may reflect modifications we have introduced into the assay procedure. In these experiments, $VIII_c$ activity carry over was eliminated by introducing $VIII_c$ activity into the assay only in the second, i.e., indicator, step and then in small amounts (1:15 dilution of a normal plasma pool). Only small amounts of anti- $VIII_c$ antibody were required to inactivate 75–80% of the $VIII_c$ activity in this diluted normal plasma pool. Thus the ratio of the blocking antigen (hemophilic plasma, etc.) to antibody could be kept relatively high without concentrating the plasmas to be tested. However, this modification of the assay prevents direct comparison of the

blocking capacity of normal plasma with hemophilic plasma because the blocking antigen cannot have detectable $VIII_c$ activity.

Blocking of anti- $VIII_c$ cannot be attributed to non-specific effects resulting from the relatively high ratio of blocking plasma to antibody. This was shown by removing the blocking capacity of the plasma with immobilized rabbit antibody directed against vW_{Ag} and $VIII_c$. Plasmas similarly treated with nonimmune immobilized rabbit globulin were not depleted of the capacity to block in the assay. In addition, the high concentrations of blocking plasma used were shown to have no significant influence on the $VIII_c$ assay.

We have previously shown that our rabbit antibody to vW_{Ag} removes both this antigen and $VIII_c$ activity from normal human plasma though it does not do so in a stoichiometric 1:1 ratio (11). vW_{Ag} isolated from normal plasma and rendered free of $VIII_c$ procoagulant activity by gel filtration in 0.8 M NaCl and by repeated freeze thawing appeared to retain $VIII_c$ antigens since it blocked human antibodies in a fashion similar to hemophilic plasmas containing comparable amounts of vW_{Ag} . Previous studies have also shown blocking of human anti- $VIII_c$ antibody with vW_{Ag} freed of $VIII_c$ activity (19). Whether both $VIII_c$ antigen and vW_{Ag} reside on the same molecule or on molecules which co-purify is a source of continuing debate.

These findings have implications for the molecular nature of the abnormality giving rise to hemophilia A as well as to the development of anti- $VIII_c$ antibodies in individuals with this disease. They suggest that antigenic differences between normal $VIII_c$ and the form of $VIII_c$ present in hemophilic plasma may be less marked than previously supposed. The presence of these $VIII_c$ antigens in all severe hemophiliacs tested may explain the relatively low incidence (8–14%) (20) of anti- $VIII_c$ antibodies developing in these individuals in spite of repeated transfusion therapy with active Factor VIII preparations.

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