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Research Article

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Immunoradiometric Measurement of the Factor VIII Procoagulant Antigen

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ABSTRACT A fluid-phase immunoradiometric assay has been developed which identifies an antigen on the Factor VIII (antihemophilic factor) procoagulant protein. This sensitive and quantitative assay is not influenced by levels of Favor VIII-related antigen (von Willebrand factor) or other plasma proteins. There is a close correlation of procoagulant activity and immunologically detectable protein in normal and von Willebrand's disease plasmas. In contrast, several different patterns have been identified in hemophilic plasmas. Neither procoagulant activity nor procoagulant antigen is detectable in plasmas from patients with severe classic hemophilia. Patients with mild and moderate hemophilia have either comparable plasma concentrations of procoagulant activity and procoagulant antigen or relatively greater levels of immunologically detectable protein.

INTRODUCTION

Human antibodies to Factor VIII (antihemophilic factor) occur in multiply-transfused hemophilic patients and infrequently, as auto-antibodies in nonhemophilic individuals. Although these antibodies inactivate Factor VIII procoagulant activity, their use as an immunologic probe of Factor VIII structure has been limited because they do not form immunoprecipitates nor do they fix complement (1). The obvious potential of these antibodies as diagnostic reagents has been used to some extent in antibody neutralization assays that detect antigenic determinants related to Factor VIII (antihemophilic factor) procoagulant activity [VIII:C (Ag)].[1] Nonfunctional antibody-neutralizing protein has been identified in ≅10% of hemophilic plasmas by this method (2–4). Patients whose plasmas neutralize these antibodies have been designated cross-reacting material positive (CRM$^+$); the other 90% of hemophilic plasmas have been considered CRM negative (CRM$^-$). Unfortunately, antibody neutralization assays are relatively insensitive and are qualitative rather than quantitative. We report here the development of a fluid-phase immunoradiometric assay that identifies antigenic determinants related to Factor VIII procoagulant activity. The sensitivity and reproducibility of this method have permitted a more precise analysis of the immunologic properties of hemophilic plasmas.

METHODS

Factor VIII measurements: The methods used to measure VIII:C, Factor VIII-related antigen (VIII R):Ag, and Factor VIII inhibitor levels have been published as has the description of the pooled normal human plasma that served as the standard (1 U/ml) for measurement of VIII:C, VIII R-Ag, and VIII:C (Ag) (5). Plasmas from patients with classic hemophilia and von Willebrand's disease were obtained at least 9 days after any transfusion therapy. All samples were either assayed on the day of collection or after storage at −70°C. Although repeated freezing and thawing did not alter VIII:C (Ag) values, assays were done on previously unthawed samples. Measurements of VIII:C (Ag) in plasmas stored at −70°C for as long as 7 yr did not have any apparent reduction when compared to fresh samples obtained from the same individuals.

Preparation of radiolabeled human anti-Factor VIII. The human anti-Factor VIII used in these studies was purified from the plasma of a patient with a high titer (3,800 National Institutes of Health (NIH) U/ml) spontaneous inhibitor (5). Immunoglobulin (Ig)G was separated from the other plasma proteins by addition of caprylic acid and Fab' fragments were prepared by sequential pepsin digestion, reduction with beta-mercaptoethanol, and Sephadex G-100 (Pharmacia Fine Chemicals, Div. of Pharmacia Inc.) gel filtration (6, 7). The anti-Factor VIII titer of this Fab' preparation was 500 NIH U/mg and <10% of this activity was lost when

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[1] *Abbreviations used in this paper*: CRM$^+$, cross-reacting material positive; CRM$^-$, cross-reacting material negative; VIII:C (Ag), the antigenic determinant identified by human anti-Factor VIII; VIII:C, Factor VIII (antihemophilic factor) procoagulant activity; VIII R:Ag, the Factor VIII-related antigen that is identified by heterologous anti-human Factor VIII.

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the protein was labeled with ^{125}I by the lactoperoxidase method (8). Immune complexes were prepared by adding partially purified Factor VIII (150 mg of lyophilized Factor VIII concentrate; Abbott Diagnostics, Diagnostic Instruments, South Pasadena, (Calif.) to 0.14 mg radiolabeled (0.24–0.42 mCi) Fab'. After a 2-hour incubation at 37°C, the immune complexes were separated from nonreactive Fab' by gel filtration on a 2.5 × 53-cm column of 6% agarose (Bio-Gel A1.5m, Bio-Rad Laboratories, Richmond, Calif.). Fractions corresponding to proteins >200,000 daltons were pooled; the 25–30-ml contained 0.8–1.5% of the added radioactivity. After the protein concentration of the pooled fractions was increased by the addition of bovine serum albumin (final concentration, 1 mg/ml), the immune complexes were dissociated by incubation at pH 3.5 for 30 min (37°C). The solution was then returned to pH 7.4, concentrated with Aquacide (Calbiochem, San Diego, Calif.), and chromatographed using 1.6 × 90-column of Sephadex G-200. The purified antibody (late eluting fractions corresponding to free Fab') contained 50–88% of the added radioactivity.

Radioimmunoassay of VIII:C (Ag). Preliminary experiments established that reproducible separation of bound and free antibody could be best obtained in 38% saturated ammonium sulfate. The assays were carried out using 12 × 75-mm polystyrene tubes to which were added sequentially: 0.1 ml of test material or a dilution of test material in borate-buffered saline, pH 7.5 (9); 0.1 ml of human IgG (20 mg/ml in borate-buffered saline) (fraction II, Miles Laboratories, Inc., Kankakee, Ill.) included as a carrier protein so that the precipitate was large enough for easy separation; and 0.1 ml of purified radiolabeled human anti-Factor VIII Fab'. The mixture was incubated at 37°C for 4 h to obtain maximal complex formation, and the bound antibody was separated by addition of 0.2 ml of a solution of 95% saturated ammonium sulfate. After a 30-min incubation at room temperature, the precipitated proteins were separated by centrifugation (2,800 *g* for 30 min) and were washed twice with 1 ml of 38% saturated ammonium sulfate. The radioactivity in the washed precipitates was then determined using a crystal scintillation detector (Searle Analytic, Inc., Des Plaines, Ill). Duplicate determinations were done for at least two dilutions of test material and the assay values were determined by reference to a standard curve obtained with pooled normal plasma (5). The dose-response relationship was most easily made linear by plotting the net percentage of antibody bound (the measured value minus the buffer-blank value) vs. antigen concentration using a logit plot.

RESULTS

Radiolabeled Fab' prepared from the serum of a patient with a high titer anti-Factor VIII was purified by forming immune complexes with Factor VIII, separating these complexes from unreactive Fab', and dissociating the anti-VIII (Ag). This purified antibody represented 0.5% of the Fab' and it had 450 NIH U anti-VIII:C activity/mg and ^{125}I incorporated at 1.7–3 mCi/mg. The detailed procedure for antibody preparation is given in Methods.

Preliminary experiments established that free and complexed antibody (^{125}I-Fab') could be separated best by their differential solubilities in 34–40% saturated ammonium sulfate solutions; a final concentration of 38% saturation was chosen for the immunoassay. The mixtures of test material, labeled anti-VIII, and carrier protein were incubated for at least 4 h at 37°C to assure maximal complex formation before the addition of ammonium sulfate. The percentage of antibody bound was slightly lower when the initial incubations were only 30 min.

A satisfactory dose-response relationship was obtained in this immunoradiometric assay for dilutions of pooled normal plasma between 1:3 and 1:100 (Fig. 1). Values for more dilute samples could not be distinguished from the control tubes that did not contain antigen. Thus, Factor VIII coagulant protein [VIII:C (Ag)] levels as low as 0.01 U/ml could be detected by this method. Because plasma samples were assayed at the dilutions of 1:3 or greater, the assay sensitivity for plasmas was considered to be 0.03 U/ml. As much as 89% of the radiolabel was precipitated when excess Factor VIII-containing material was tested. The values for the buffer-blank tubes were 12–16% of the added radioactivity. The reproducibility of the assay was established by three separate series of measurements carried out with samples of normal plasmas. The value for each determination was obtained from the standard curve for that day's assay. The number of measurements, the mean±SD, and the range of values were as follows: 9 assays of a plasma that had 1.13±0.08 U/ml (0.96–1.24); 18 assays of a plasma that had 1.04±0.13 U/ml (0.80–1.24); and 10 assays of a plasma with 0.94±0.09 U/ml (0.79–1.08). These values indicate a coefficient of variation of 7, 12, and 9%, respectively.

The specificity of the assay was verified most directly by experiments which demonstrated that the VIII:C (Ag) assay was not affected by the concentration of VIII R:Ag or other plasma proteins in the test material (Table I). These VIII:C (Ag) assays were carried out using mixtures of partially purified Factor VIII procoagulant protein (10) and plasmas from patients with severe hemophilia or von Willebrand's disease. Because the measured VIII:C (Ag) was not changed by

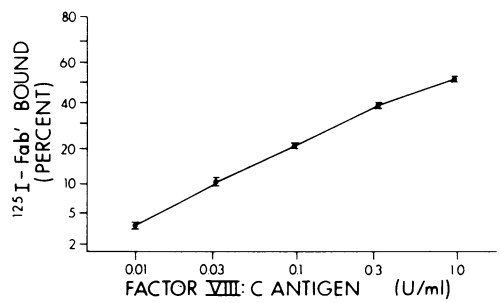

FIGURE 1 Immunoradiometric assay of VIII:C (Ag). The net percentage of labeled antibody (anti-Factor VIII Fab') in the precipitates is indicated on the vertical axis. The mean and 1 SEM are indicated for data obtained in 11 separate assays.

TABLE I
Immunoassay of Partially Purified Factor VIII Procoagulant

Assay mixture*	VIII:C	VIII R:Ag	VIII:C (Ag)
		U/ml	
Factor VIII procoagulant + borate-buffered saline	0.49	<0.003	1.29, 1.32, 1.41
+ hemophilic plasma	0.49	0.60	1.35, 1.36, 1.40
+ von Willebrand's disease	0.49	<0.005	1.32, 1.50, 1.53

* Immunoadsorbent-purified Factor VIII procoagulant used in this experiment had VIII:C of 0.97 U/ml and VIII R:Ag <0.005 U/ml (10). Equal volumes of the Factor VIII coagulant and diluent were assayed for VIII:C (Ag) content at 1:3, 1:10, and 1:30 dilutions, and the three results are indicated. The values for VIII:C and VIII R:Ag in these mixtures were calculated from the values for the two components.

the addition of either plasma, the assay values appear to be independent of other plasma proteins.

We have used this immunoradiometric assay to study the relative values of VIII:C and VIII:C (Ag) in plasma samples from normal individuals, patients with classic hemophilia, and patients with von Willebrand's disease (Fig. 2). Good correspondence for the two measurements was obtained for normal plasma samples: the linear regression analysis for the 17 samples was $y = 0.81x + 0.14$ and the correlation coefficient (r) was 0.90. Von Willebrand's disease plasmas also had similar concentrations of VIII:C and VIII:C (Ag): the linear regression pattern for the 17 samples was $y = 0.56x + 0.04$ with an r value of 0.85. Readily detectable levels of VIII:C (Ag) (>0.05 U/ml) were found in even the four severe von Willebrand's disease plasmas that had <0.01 U/ml VIII:R Ag.

Three distinct patterns were detected for hemophilic plasmas (Table II and Fig. 2C). Six patients with very severe disease (>0.01 U/ml VIII:C) had undetectable levels of VIII:C (Ag) (Table II, group A). These results correspond to a CRM^- state; neither immunoradiometric assay nor antibody neutralization is able to detect evidence of a protein that reacts with human anti-Factor VIII. A second group of patients had reduced content of both VIII:C and VIII:C (Ag) (Table II, group B). These 17 plasmas had similar values for the two assays; an arbitrary assignment to group B was made if the ratio of VIII:C (Ag) to VIII:C was <1.6:1 or if the absolute difference between the two assays was <0.05 U/ml. The VIII:C (Ag) content of the other 18 hemophilic plasmas was >1.6 times that of the procoagulant activity (Table II, group C). This difference indicates that the immunoassay is mesuring a different property than the coagulation assay in these plasmas. The most striking differences were noted for five plasmas that had normal VIII:C (Ag) levels and very low VIII:C activity (Table II, patients 37–41). These five plasmas were all CRM^+ by antibody neutralization assay (2). The complex heterogeneity observed for the hemophilic plasmas prevented the calculation of

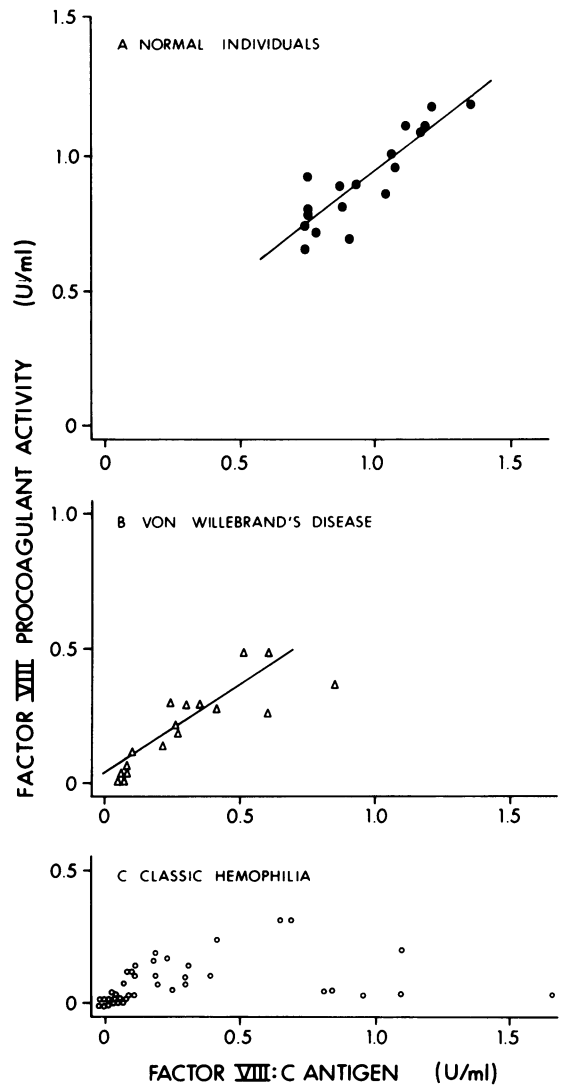

FIGURE 2 The relationship of Factor VIII procoagulant activity and VIII:C (Ag). (A) 18 normal plasmas. (B) Plasmas from 17 patients with von Willebrand's disease. (C) Plasmas from 41 patients with classic hemophilia.

TABLE II
Factor VIII Measurements for Plasmas from Patients with Classic Hemophilia

Group	Patient	VIII:C	VIII:C (Ag)	VIII R:Ag
			U/ml	
A	1	<0.01	<0.03	0.74
	2	<0.01	<0.03	1.60
	3	<0.01	<0.03	0.78
	4	<0.01	<0.03	0.84
	5	<0.01	<0.03	0.72
	6	<0.01	<0.03	2.03
B	7	<0.01	0.04	0.85
	8	<0.01	0.06	1.24
	9	<0.01	0.06	0.90
	10	0.01	0.04	1.12
	11	0.02	0.05	1.06
	12	0.02	0.07	1.71
	13	0.03	0.04	0.89
	14	0.04	0.03	0.85
	15	0.07	0.07	1.20
	16	0.10	0.11	0.71
	17	0.10	0.11	0.90
	18	0.12	0.09	0.23
	19	0.12	0.10	0.83
	20	0.14	0.11	1.66
	21	0.16	0.18	2.40
	22	0.17	0.23	0.70
	23	0.19	0.19	2.08
C	24	0.03	0.09	0.69
	25	0.03	0.10	2.80
	26	0.05	0.25	0.88
	27	0.07	0.20	0.67
	28	0.07	0.30	1.16
	29	0.10	0.19	1.54
	30	0.10	0.30	1.06
	31	0.10	0.39	1.60
	32	0.14	0.31	1.68
	33	0.20	1.10	1.21
	34	0.24	0.42	1.84
	35	0.31	0.65	1.32
	36	0.31	0.69	1.16
	37	0.03	0.96	1.35
	38	0.03	1.10	0.74
	39	0.03	1.66	1.78
	40	0.04	0.81	0.91
	41	0.05	0.84	0.97

meaningful values for linear regression analysis or correlation.

DISCUSSION

This paper describes a specific sensitive, and reproducible assay for VIII:C (Ag), the antigen determinant associated with Factor VIII procoagulant activity. The assay, and a similar method described by Peake and Bloom (11), has obvious potential value for further study of the nature of the protein that has VIII:C activity under conditions that may interfere with standard coagulation assays. A limiting factor in the establishment of such assays is the availability of very potent human anti-Factor VIII. In the case of the inhibitor plasma which we have used, the VIII:C neutralization titer of 3,800 NIH U/ml represented the effect of ≅0.1 mg/ml of antibody.

We have applied this VIII:C (Ag) assay to obtain more quantitative immunologic evaluation of Factor VIII in normal plasmas and in Factor VIII deficiency diseases. The correspondence of VIII:C and VIII:C (Ag) values was very close in normal plasmas (Fig. 2). Significant correlation (an r value that indicates $P < 0.01$) was also noted for von Willebrand's disease plasmas. The slope of the linear regression equation was reduced, however, indicating there was more immunologically detectable VIII:C (Ag) than VIII:C. Although the interpretation of this observation is not certain, it suggests that either the specific activity of Factor VIII coagulation protein is reduced in this disease or that in vivo loss of VIII:C activity is more rapid than the metabolism of the protein identified by the VIII:C (Ag) assays.

These studies have also identified VIII:C (Ag) in plasmas of all patients with mild and moderate classic hemophilia. This is a different conclusion from that which we and others derived from less sensitive antibody neutralization assays (2–4). Thus, there appears to be a more complex molecular heterogeneity than had been recognized previously and these hemophilic plasmas contain similar, (Table II, group B) or greater (group C) quantities of VIII:C (Ag) than VIII:C activity. The pattern of procoagulant-immunologic relationship is like that observed in Christmas disease (12), and the same three groups (A, B, and C; Table II) are detected. We recognize that the distribution of patients in the three groups may be biased in the population of hemophiliacs that we have studied; most severe hemophiliacs could not be tested because of recent transfusions, many mild and moderate hemophiliacs are seen to confirm the diagnosis, and four of the five CRM^+ hemophiliacs (Table II, patients 37–41) are members of one family (2).

This immunoassay for VIII:C (Ag) did not identify any antigenic material in six of nine hemophilic plasmas that had <0.01 U/ml VIII:C. We had expected to find some evidence of Factor VIII coagulant antigen in these plasmas because others have reported that all hemophilic plasmas have immunoreactive protein when studied by sensitive, qualitative methods (13, 14). Our data indicate that the concentration of normal antigen determinants in these hemophilic plasmas must be <3% of that present in normal human plasma. The identification of rabbit antibody neutralizing material,

in three hemophilic plasmas that were CRM-when tested with human antibody, has also suggested that a nonfunctional protein is synthesized by all hemophilic patients (15). Inasmuch as human anti-Factor VIII may be reacting with very different sites on the protein responsible for procoagulant activity, our data are not inconsistent with that observation.

As is apparent in these studies of Factor VIII deficient plasmas, the immunoradiometric assay for VIII:C (Ag) has many advantages when compared to antibody neutralization methods. This quantitative immunoassay should permit a more flexible approach to studies of Factor VIII structure-function relationships, and it may lead to a better understanding of the properties of the protein responsible for Factor VIII coagulant activity.

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