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Analysis of Factor VIII coagulant antigen in normal, thrombin-treated, and hemophilic plasma*

(NaDodSO$_4$/polyacrylamide gel electrophoresis/Factor VIII coagulant activity)

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ABSTRACT **The relationship between Factor VIII coagulant antigen (VIII:CAg) and Factor VIII-associated von Willebrand factor (VIII:vWF), and the effect of thrombin on VIII:CAg have been determined in plasma by using complexes of VIII:CAg and ^{125}I-labeled human anti-VIII:CAg-Fab. Antibody-treated plasma samples were electrophoresed on NaDodSO$_4$/polyacrylamide agarose gels and analyzed by autoradiography. The major VIII:CAg-^{125}I-labeled Fab complex that persisted in NaDodSO$_4$ had M_r 3.2 × 10^5. This M_r value was confirmed by column chromatography and sucrose density centrifugation and is presumed to reflect a free VIII:CAg of M_r 2.7 × 10^5. Minor bands were also present on autoradiograms of normal plasma corresponding to M_r values of 2.5, 1.85, and 1.7 × 10^5 (free VIII:CAg related proteins with M_r values of 2.0, 1.35, and 1.2 × 10^5, respectively). None of the VIII:CAg bands was present in plasma samples from five patients with severe hemophilia A. No radioactivity was associated with VIII:vWF multimers on NaDodSO$_4$ gels. Thrombin treatment of normal plasma eliminated the radioactive band at 3.2 × 10^5 and increased the intensity of a band of M_r 1.7 × 10^5. Generation of this presumed VIII:CAg fragment of $M_r \approx 1.2 \times 10^5$ coincided with a thrombin-induced increase in Factor VIII coagulant activity. These data demonstrate that the form of VIII:CAg detected in normal plasma is not covalently linked to VIII:vWF multimers and is absent in plasma from five hemophilia A patients. Thrombin-induced proteolysis of VIII:CAg can be detected in microliter quantities of normal plasma.**

Individuals with the X chromosome-linked clotting disorder hemophilia A lack, or have an inactive form of, the protein responsible for Factor VIII coagulant activity (VIII:C). VIII:C is also decreased or absent in patients with an autosomal disorder, von Willebrand disease. In addition, these individuals lack sufficient quantities of functional von Willebrand factor (VIII:vWF), a protein necessary for optimal adhesion of platelets to subendothelial surfaces (1, 2).

The low plasma concentration of VIII:C and VIII:vWF (3, 4), their tendency to copurify, and the susceptibility, particularly of VIII:C, to proteolysis (5–7) have hampered the characterization of these proteins. Whether VIII:C and VIII:vWF are two distinct proteins (8–10) or are covalently linked and become separated only after proteolysis (11, 12) is uncertain. VIII:vWF is a multimeric protein, ranging from $\approx 1 \times 10^6$ to $\approx 20 \times 10^6$ in M_r, and is composed of disulfide-linked monomers of M_r 2.3 × 10^5 (13, 14). When separated from VIII:vWF by high salt concentrations (15, 16) or mild reducing conditions (4), VIII:C has been estimated to have M_r of 2.2 × 10^4 (17) to $\approx 3 \times 10^5$ (4, 7). Trace amounts of thrombin or other plasma proteins result in changes of VIII:C activity and M_r (4, 5, 7).

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Recently, fluid-phase immunoradiometric assays have been developed (18–20) to quantify Factor VIII coagulant antigen (VIII:CAg) in plasma. Human allogeneic antibodies used in these assays were obtained from hemophilia A patients with circulating anti-VIII:C antibodies which developed after transfusions with blood products containing normal VIII:C activity. These antibodies neutralize VIII:C activity but do not usually react with VIII:vWF or other plasma proteins.

We used NaDodSO$_4$ gel electrophoresis to examine the interaction of ^{125}I-labeled anti-(VIII:CAg) Fab with VIII:CAg. The procedure permits determination of variations in the apparent M_r of VIII:CAg forms in small samples of plasma. Because fresh plasma samples are used without further purification, the likelihood of prior *in vitro* proteolysis of VIII:CAg is minimized. The technique was also used to detect a major thrombin product of VIII:CAg in plasma.

METHODS

Materials. Plasma from a hemophiliac with an inhibitor titer of 6500 NIH units/ml was generously contributed by Edward Tuddenham (Hemophilia Centre, London). Hemophilic plasma, VIII:C <1%, was from Tufts New England Medical Center (Boston, MA) and from George King Bio-Medical (Overland Park, KS). Purified thrombin was a gift from John Fenton (New York State Department of Health, Albany, NY).

Plasma Preparation. Venous blood (5 vol) was anticoagulated with 1 vol of 0.085 M trisodium citrate/0.064 M citric acid/2% dextrose (ACD) (21). Platelet-poor plasma was separated by two centrifugations at 5000 × *g* for 3 min (22°C). Some samples also contained diisopropyl fluorophosphate (iPr_2*P*-F; Aldrich) at final concentrations of 2 mM with heparin, Trasylol (Mobay), and hirudin (Sigma) at 2, 100, and 2 units/ml, respectively. Plasma samples were either used immediately or stored at −70°C up to 3 months. A normal plasma pool was composed of plasma from 14 individuals.

VIII:C assays and quantitative electroimmunoassays for VIII:vWf were performed as described (22).

Preparation of Human Anti-VIII:CAg-^{125}I-Labeled Fab. IgG from a patient with an VIII:C inhibitor was isolated by $(NH_4)_2SO_4$ precipitation and DEAE-cellulose chromatography (23) and then was incubated for 2 hr with papain (24). Fab frag-

Abbreviations: VIII:C, Factor VIII coagulant activity; VIII:CAg, Factor VIII coagulant antigen; ^{125}I-Fab, ^{125}I-labeled Fab fragments prepared from human antiVIII:CAg IgG; FVIII:vWF, Factor VIII-related multimer required for normal platelet-endothelial adhesion and ristocetin-induced platelet agglutination, VBS, 0.125 M NaCl/0.028 M sodium barbital, pH 7.3; iPr_2*P*-F, diisopropyl fluorophosphate.

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ments were separated from the mixture on Sephacryl S-200 (Pharmacia), followed by DEAE-cellulose chromatography. The Fab solution, containing 620 NIH units/mg of protein, was iodinated by the IODO-GEN^R (Pierce) method (25). ^{125}I-Fab fragments specific for VIII:CAg were isolated by the method of Lazarchick and Hoyer (18). The ^{125}I-Fab (anti-VIII:CAg) (in a solution containing 0.5 mg of bovine serum albumin/ml), with an initial activity of 2×10^5 cpm/ml, was stored at −20°C and is referred to as "stock ^{125}I-Fab solution."

Fluid-Phase Immunoradiometric Assay of VIII:CAg. This was performed by mixing 10 μl of plasma or plasma diluted with 0.125 M NaCl/0.028 M sodium barbital, pH 7.3 (VBS) with 10 μl of stock ^{125}I-Fab, 0.86 μl of 45% polyethylene glycol 4000 (26), and 1.1 μl of 50 mM iPr_2P-F containing hirudin at 193 units/ml in VBS. After incubation at 37°C for 2 hr, protein was precipitated by addition of 1 mg of human IgG (Miles) in 178 μl of VBS plus 1 ml of 45% saturated $(NH_4)_2SO_4$. After incubation of the mixture at 0°C for 15 min and centrifugation at 12,000 × *g* for 8 min, the radioactivity of the precipitates was determined. Preliminary experiments (not shown) demonstrated that ^{125}I-Fab was in excess of VIII:CAg under conditions used in this assay—i.e., at higher concentrations of plasma, an increased amount of radioactivity could be precipitated.

The VIII:CAg and VIII:C levels were determined for each plasma sample by using standard curves derived from dilutions of normal plasma. In the case of VIII:CAg, the standard curve was derived from a least-squares linear regression analysis of the logarithm of bound radioactivity (cpm) versus logarithm of plasma dilutions. VIII:CAg and VIII:C values determined independently were then plotted against each other to determine the correspondence between these values. Plasma from hemophiliacs was used undiluted in this assay.

Preparation of Samples for NaDodSO$_4$ Gel Electrophoresis. In a typical experiment, 22 μl of pooled normal plasma containing 200 units of Trasylol per ml were mixed with 22 μl of stock ^{125}I-Fab, 1.9 μl of 45% polyethylene glycol 4000, and 2.5 μl of iPr_2P-F/hirudin, and then incubated for 2 hr at 37°C.

Samples were also prepared from thrombin-activated plasma. Purified thrombin (20 units/ml in VBS) was added at 22°C to pooled normal plasma, containing 200 units of Trayslol per ml, to give a final thrombin concentration of 0.25 unit/ml of plasma. Aliquots (22 μl) were withdrawn at intervals and added to 26.4 μl of the ^{125}I-Fab/iPr_2P-F/hirudin/polyethylene glycol solution. A final concentration of 2.5 units of thrombin per ml was also used to achieve more extensive proteolysis. The latter samples were incubated at 37°C for 10 min before addition to the ^{125}I-Fab/iPr_2P-F/hirudin/polyethylene glycol solution and then incubated for an additional 2 hr at 37°C. Aliquots (10 μl) were also withdrawn from thrombin-activated plasma at 3-min intervals, diluted in 0.8 ml of VBS, and analyzed immediately by the one-stage VIII:C assay.

The capacity of hirudin plus iPr_2P-F to block thrombin activity was measured by a colorimetric assay (27). Thrombin activity was undetectable within 30 sec after the addition of iPr_2P-F/hirudin.

NaDodSO$_4$ Gel Electrophoresis and Autoradiography. After 2-hr incubation at 37°C, the plasma plus ^{125}I-Fab samples were mixed with an equal volume of 2% NaDodSO$_4$/8 M urea/2 mM EDTA/0.05 M Tris·HCl, pH 8.0. The solution was incubated at 37°C for 20 min, and 30-μl aliquots were applied to 3-mm-thick 3% polyacrylamide/0.5% agarose slab gels, 13 × 10.5 cm (28) cast on Gel-Bond (Marine Colloids) prepared the previous day. Electrophoresis was performed at 75 V for 0.5 hr and 120 V for 1.5 hr.

The gels were fixed and stained overnight in 0.0035% Coomassie blue R-250/10% isopropanol/10% acetic acid, destained for 7 hr in 7.5% acetic acid/5% methanol, soaked for 1 hr in 2% glycerol/10% acetic acid, and kept in a drying oven at 50°C for 18 hr.

For autoradiography, the dried gels were kept for 24 hr at −70°C with Kodak XR-5 x-ray film in a cassette containing two Cronex Lightning-Plus intensifying screens.

The apparent M_r of antigen–antibody complexes was determined by interpolation on a straight line derived by plotting migration distance versus logarithm of M_r of the Coomassie-stained proteins fibronectin, α_2-macroglobulin, fibrinogen, IgG, albumin, thyroglobulin, ferritin, and ^{14}C-labeled phosphorylase *b* (New England Nuclear).

Column Chromatography and Sucrose Density Centrifugation. A 0.5-ml aliquot of normal citrated platelet-poor plasma with proteolytic inhibitors (see above) was incubated within 15 min of venipuncture with an equal volume of stock ^{125}I-Fab and polyethylene glycol/iPr_2P-F/hirudin in the same proportion used for NaDodSO$_4$ gel electrophoresis. After incubation at 37°C for 2 hr, the sample was chromatographed at 37°C on Sephacryl S-300 (1.6 × 88 cm) in VBS at pH 7.5 and containing 38 mM ε-aminocaproic acid, 2 mM iPr_2P-F, Trasylol at 2000 units/liters, hirudin at 200 units/liter, bovine serum albumin at 0.1 g per liter, and 0.02% Na azide. Plasma samples collected in citrate alone and stored frozen at −70°C had the same elution pattern as citrated plasma containing proteolytic inhibitors. The partition coefficient, K_{av} (29) was used (30) to determine the Stoke's radius. Fibrinogen, apoferritin, aldolase, catalase, IgG, and albumin were standards.

The sedimentation coefficient of the ^{125}I-Fab–VIII:CAg complex was determined in a 15–45% linear sucrose density gradient in 0.14 M NaCl/0.02 M imidazole, pH 7.2. Samples were centrifuged for 24 hr at 20°C and 260,000× *g*. Albumin, IgG, and catalase served as marker proteins.

RESULTS

Correlation Between VIII:CAg and VIII:C. Fluid-phase immunoradiometric assays demonstrated that there was good correlation between VIII:C activities and quantities of VIII:CAg in normal individuals and in hemophiliacs with VIII:C activities <1% (Fig. 1). The linear regression for the normals was $y = -0.0475 + 1.14x$ with a correlation coefficient (r) of 0.94. Hemophiliacs had VIII:CAg values ≤3% and VIII:vWF antigen

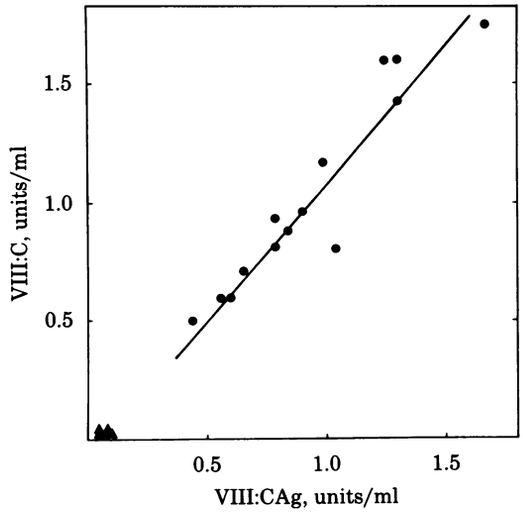

FIG. 1. Relationship between VIII:C and VIIICAg determined by quantitative radioimmunoassay. Samples included plasma from 14 normal individuals (●) and 5 hemophiliacs (▲).

levels determined by electroimmunoassay ranging from 100% to 240%.

Reaction of ^{125}I-Fab with Normal and Hemophilic Plasma Examined by NaDodSO$_4$/Polyacrylamide Gel Electrophoresis. To determine the M_r of VIII:CAg–^{125}I-Fab complexes in normal and hemophilic individuals, we used NaDodSO$_4$/polyacrylamide agarose gel electrophoresis (Fig. 2). Normal plasma samples were prepared under various conditions to test the possibility that M_r heterogeneity of VIII:CAg might be caused by *in vitro* proteolysis during collection or storage. Blood was drawn directly into solutions containing ACD, heparin, Trasylol, and hirudin or ACD alone. In addition, iPr_2P-F was added to the plasma containing the proteolytic inhibitor mixture within 15 min of venipuncture (after centrifugation of blood samples). Plasma from one donor and pooled normal plasma in ACD were stored at −70°C for 2 months before examination. All of these plasma samples were incubated for 2 hr at 37°C with a solution of ^{125}I-Fab/iPr_2P-F/hirudin and then subjected to NaDodSO$_4$ gel electrophoresis.

The patterns of Coomassie blue-stained proteins from normal and hemophilic plasma samples were indistinguishable except for variation in intensity of individual bands (Fig. 2*A*).

On autoradiographs of 3% polyacrylamide/0.5% agarose gels, the ^{125}I-Fab–VIII:CAg complex that formed in normal plasma was a major band with apparent M_r 3.2×10^5 (Fig. 2*B*). In addition, minor bands were present in all normal plasma samples. The most prominent of these had M_r 1.85×10^5. Less distinct bands corresponded to M_r values of 2.5×10^5 and 1.7×10^5. These minor bands were present in all normal plasma samples regardless of storage time or presence of inhibitors. These observations suggest that ^{125}I-Fab detects several types of VIII:CAg which occur *in vivo*, perhaps related to proteolysis. However, the presence of some *in vitro* proteolysis of the major VIII:CAg, despite the presence of proteolytic inhibitors, cannot be excluded absolutely. No additional bands were associated with VIII:vWF when plasma samples were examined on 1.5% polyacrylamide/0.5% agarose gels which resolve VIII:vWF multimers into many oligomeric components (22).

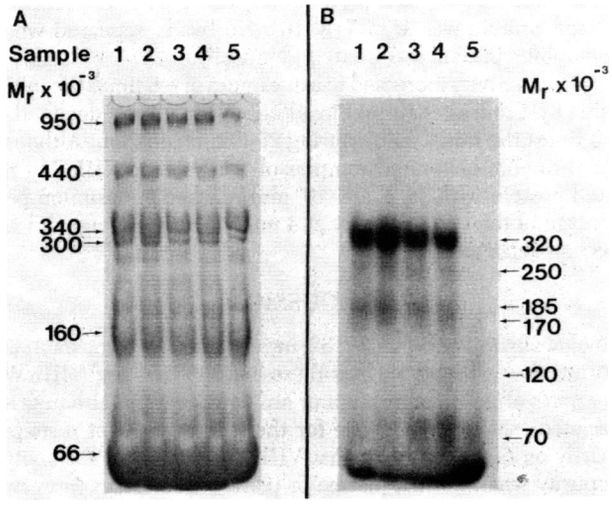

FIG. 2. NaDodSO$_4$/3% polyacrylamide/0.5% agarose gel electrophoretic analysis of plasma incubated previously with stock ^{125}I-Fab. Gels were stained with Coomassie blue (*A*) and then autoradiograms were prepared (*B*). Lanes: 1, fresh plasma from one normal individual anticoagulated with ACD, heparin, Trasylol, iPr_2P-F, and hirudin; 2, fresh plasma from same normal individual anticoagulated with ACD alone; 3, plasma from same normal individual collected on ACD and stored at −70°C for 2 months; 4, pooled normal plasma collected on ACD and stored at −70°C for 2 months; 5, hemophilic plasma.

In contrast, all five hemophilic plasma samples examined in this study had no radioactive band at M_r 3.2×10^5 and a barely detectable band at M_r 1.7×10^5. Faint bands which may reflect non-antigen-specific interaction of Fab with other plasma proteins were visible in both normal and hemophilic samples at M_r 1.2×10^5 and 0.7×10^5. Free ^{125}I-Fab has apparent M_r 0.55×10^5 in this gel system.

Number of ^{125}I-Fab Molecules Bound per VIII:CAg. If more than one ^{125}I-Fab were to be bound per VIII:CAg then, under conditions of antigen excess, additional bands should be seen on the gels. These additional radioactive bands would represent VIII:CAg forms with less than the maximal number of bound ^{125}I-Fab fragments and should be separated by migration distances equivalent to one ^{125}I-Fab fragment.

To evaluate the stoichiometry of binding, equal volumes of undiluted plasma were incubated with 100% stock ^{125}I-Fab and with 1:1 and 1:3 dilutions of stock ^{125}I-Fab. Previous experiments with the fluid phase assay demonstrated that antibody was in excess of antigen at the 1:1 (vol/vol) ratio of plasma to 100% stock ^{125}I-Fab, whereas antigen was in excess of antibody with 1:1 and 1:3 dilutions of stock solution. All ratios of ^{125}I-Fab–VIII:CAg produced the same radioactive banding pattern as in Fig. 2, suggesting that only a single Fab fragment combines with each VIII:CAg molecule. The major VIII:CAg, free of the ^{125}I-Fab fragment, has a calculated M_r of 2.7×10^5, and the M_rs of other VIII:CAg forms are 2.0, 1.35, and 1.2×10^5.

Column Chromatography of Normal and Hemophilic Plasma Treated with ^{125}I-Fab. To confirm the M_r determination of the major antibody–antigen complex estimated by the modified NaDodSO$_4$ gel procedure, column chromatography and sucrose density gradient centrifugation were performed. In the elution profile of hemophilic plasma (Fig. 3*A*) most of the radioactivity was free ^{125}I-Fab with much smaller amounts of radioactivity in a region extending from the void volume to the ^{125}I-Fab peak. In contrast, major quantities of radioactivity were in the void volume (peak 1) and near the position of fibrinogen (peak 2) in

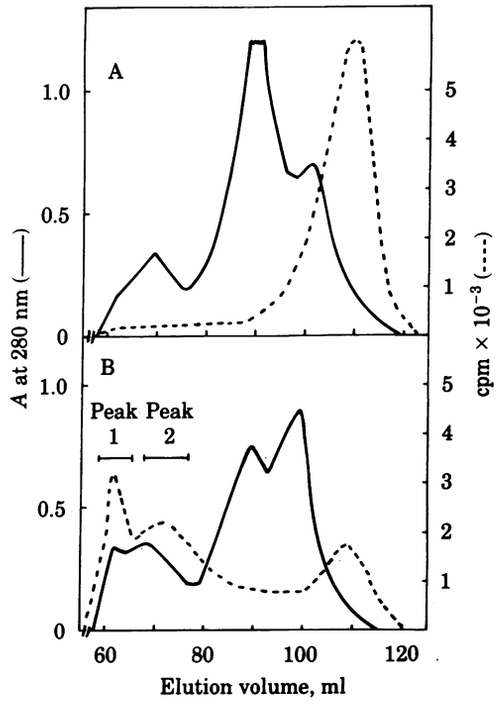

FIG. 3. Elution profile of hemophilic (*A*) and normal (*B*) plasma incubated with ^{125}I-Fab and chromatographed at 37°C on Sephacryl S-300.

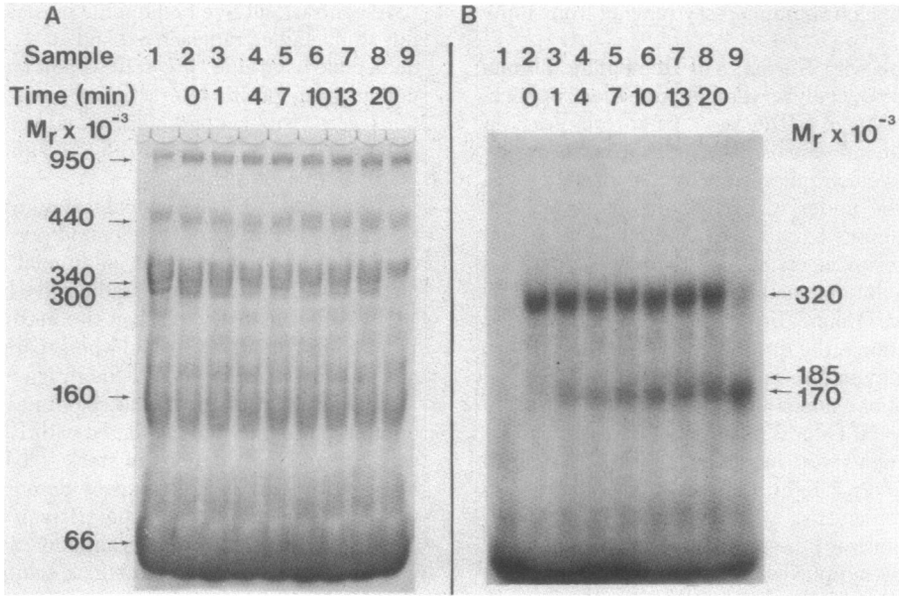

FIG. 4. Thrombin-induced proteolysis of VIII:CAg in plasma. Samples were analyzed by incubation with ^{125}I-Fab followed by NaDodSO$_4$/3% polyacrylamide/0.5% agarose gel electrophoresis. Gels were stained with Coomassie blue (*A*) and autoradiograms were prepared (*B*). Lanes: 1, hemophilic plasma; 2–8, samples withdrawn from normal plasma into iPr_2P-F/hirudin at the indicated times after addition of thrombin/ml (final concentration, 0.25 unit/ml); 9, one normal plasma sample incubated for 10 min at 37°C with thrombin at 2.5 unit/ml (final concentration). Fibrin was removed before addition of thrombin-treated samples to the gels.

the elution profile obtained from normal plasma (Fig. 3*B*). The Stoke's radius of the ^{125}I-Fab–VIII:CAg complex in peak 2 was calculated (29) to be 90 Å. This value, coupled with a sedimentation coefficient of 8.8 S, yields a M_r of 3.3×10^5 for the ^{125}I-Fab–VIII:CAg complex, in excellent agreement with the value 3.2×10^5 determined by NaDodSO$_4$ gel electrophoresis.

Proteolysis of VIII:CAg by Thrombin. To determine the effect of thrombin on the apparent M_r of VIII:CAg, thrombin-treated plasma samples were mixed with ^{125}I-Fab and analyzed by NaDodSO$_4$ gel electrophoresis (Fig. 4).

An increase in the ^{125}I-Fab–VIII:CAg complex, M_r 1.7×10^5, was detected in plasma within 1 min after the addition of thrombin. Subtraction of the M_r of free ^{125}I-Fab, 0.5×10^5, yields 1.2×10^5 for the M_r of this thrombin-generated VIII:CAg-related protein. The complex formed between ^{125}I-Fab and thrombin-generated VIII:CAg-related protein was not well separated from the minor radioactive band with M_r 1.85×10^5 seen both in the absence and presence of thrombin. By densitometry of the autoradiograph it was determined that the percentage of the ^{125}I-Fab–Ag complex with M_r 1.7×10^5 attained a maximum between 4 and 7 min after thrombin addition and remained constant thereafter.

A plasma sample was also treated with thrombin (2.5 units/ml, final concentration) to determine the effect of increased thrombin proteolytic activity on VIII:CAg. Densitometric analysis showed that ≈80% of the total complex of ^{125}I-Fab–VIII:CAg-related protein was M_r 1.7×10^5. No bands appeared when hemophilic plasma was treated identically.

VIII:C activity increased to a maximum of ≈8 times the initial value by 4 min after thrombin addition (Fig. 5) and declined to ≈3 times the initial value during 20-min incubation. Although the thrombin-generated complex of ^{125}I-Fab and VIII:CAg-related protein with M_r 1.7×10^5 also reached a maximum percentage of total radioactivity at 4 min, this percentage did not decline subsequently.

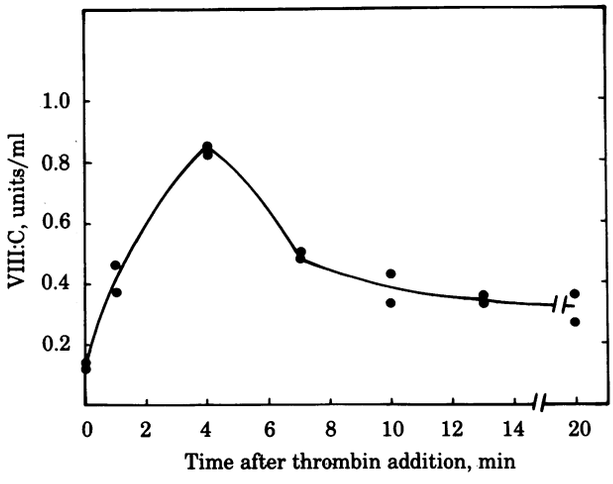

FIG. 5. Thrombin activation of VIII:C in plasma. At the times indicated after addition of purified thrombin (0.25 unit/ml, final concentration) to pooled normal plasma, 10-μl aliquots were withdrawn and diluted 1:80, and the VIII:C activity of the sample was determined by one-stage VIII:C assay using hemophilic plasma devoid of VIII:C activity.

DISCUSSION

Several current reviews (31, 32) have summarized the data supporting the two opposing hypotheses—that VIII:C and VIII:vWF are parts of the same protein or are two separate, although associated, entities. Evidence for the former concept rests primarily on the observation that VIII:C and VIII:vWF activities copurify when normal plasma or plasma derivatives (prepared in the presence of high concentrations of proteolytic inhibitors) are analyzed by gel filtration chromatography at physiological ionic strength (6, 33). One series of studies (11, 12) suggested that VIII:C exists in a precursor, inactive form covalently bound to VIII:vWF. Only after activation by thrombin or a thrombin-like enzyme was dissociation of the two activities in the presence of high salt concentration reported to occur.

In contrast, many investigators have collected plasma in the presence of proteolytic inhibitors and have separated VIII:C

and VIII:vWF by high salt concentrations (8, 10), mild reducing conditions (4), or rapid quantitative ultrafiltration of fresh plasma samples (34). With some exceptions (17), most investigators have found that the M_r of human Factor VIII is in the range $\approx 2 \times 10^5$ to $\approx 3 \times 10^5$ (7, 34, 35).

We determined the M_r of ^{125}I-Fab–VIII:CAg complexes by $NaDodSO_4$ gel electrophoresis and concluded that a property of VIII:CAg was measured. This conclusion is based on: (*i*) the ability of Fab to neutralize VIII:C activity; (*ii*) correlation between VIII:C and VIII:CAg in fluid-phase radioimmunoassays; and (*iii*) only slight reactivity (by Sephacryl column chromatography) of ^{125}I-Fab with plasma of severe hemophiliacs, even though these patients had levels of VIII:vWF that were normal or above.

^{125}I-Fab–VIII:CAg complexes were formed prior to electrophoresis in order to avoid possible loss of VIII:CAg during sample preparation or electrophoretic runs. To preserve ^{125}I-Fab–VIII:CAg complexes, we used less-severe denaturing conditions than usual in preparing samples for $NaDodSO_4$ gel electrophoresis. Preliminary experiments demonstrated that ^{125}I-Fab–VIII:CAg complexes were destroyed by heating the sample to 70°C for 10 min, giving rise to non-VIII:CAg interaction of ^{125}I-Fab with other plasma proteins. At the same time, we observed no change in the electrophoretic migration of Coomassie blue-stained proteins when the sample was incubated at 37°C rather than 70°C. Thus, the migration distance of these stained proteins prepared under mild conditions were used to estimate the apparent M_r of ^{125}I-Fab–VIII:CAg complexes.

These mild denaturing conditions limit structural characterization of VIII:CAg, however. For example, VIII:CAg may be composed of several noncovalently linked, but tightly bound, chains held together by forces similar to those joining the ^{125}I-Fab–VIII:CAg complexes. VIII:CAg might also be composed of several disulfide-linked chains analogous to bovine VIII:C (4). Also, it is possible that only certain forms of the ^{125}I-Fab–VIII:CAg complex survive $NaDodSO_4$ treatment. This latter possibility is supported by observations that ^{125}I-Fab interacts with VIII:CAg present in large complexes eluted in the void volume of Sephacryl columns.

The M_r 2.7×10^5 of the major form of free VIII:CAg determined by the $NaDodSO_4$ gel method is in excellent agreement with that observed by other investigators using salt dissociation techniques to separate VIII:C from VIII:vWF (7, 34, 35). Our data indicate that several forms of VIII:CAg can be separated from VIII:vWF, whether or not proteolytic inhibitors are added to blood as it is collected.

Besides the major VIII:CAg species of M_r 2.7×10^5, there are three other VIII:CAg-related proteins with free antigen M_r values of 2, 1.35, and 1.2×10^5. It is not yet known which one (or more) of these proteins with VIII:CAg activity has VIII:C activity. With thrombin-activation of VIII:C, the antigen–antibody band of M_r 3.2×10^5 decreased whereas the band of M_r 1.7×10^5 increased in intensity and then remained constant as VIII:C activity declined over time. Possibly, this latter antigenic species represents an "activated" form of VIII:CAg which is altered in solution to an inactive structural form shortly after generation. The fate of the remaining nonantigenic material with an estimated free M_r of 1.5×10^5 is unknown.

The $NaDodSO_4$/polyacrylamide gel procedure described here should prove useful for detecting VIII:CAg forms present during the purification of Factor VIII as well as in the plasma of patients with abnormalities of VIII:C.

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