Human Factor VIII Procoagulant Protein

Monoclonal Antibodies Define Precursor-Product Relationships and Functional Epitopes

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Abstract

The human Factor VIII procoagulant protein (VIII:C) purified from commercial Factor VIII concentrate consisted of a polypeptide doublet of 80,000 mol wt, a 92,000-mol wt polypeptide, and additional polypeptides of up to 188,000 mol wt. Thrombin digests contained a doublet of 72,000 mol wt, as well as 54,000- and 44,000-mol wt fragments. Proteolysis studies of purified VIII:C using thrombin and activated protein C have suggested that the 92,000- and 80,000 (or 72,000)-mol wt polypeptides comprise activated VIII:C. We have now used seven monoclonal antibodies raised against purified VIII:C to construct a preliminary epitope map of these VIII:C polypeptides. The specific VIII:C polypeptides with which the monoclonal antibodies reacted were determined by immunoblotting of VIII:C onto nitrocellulose sheets after reduced NaDodSO$_4$-polyacrylamide gel electrophoresis. A minimum of five distinct epitopes were defined by these monoclonal anti-VIII:C antibodies. Identification of polypeptides bearing these epitopes allowed localization of distinct thrombin cleavage sites to the 92,000- and 80,000-mol wt chains, helped define polypeptide chain precursor-product relationships, and suggested that both the 92,000- and 80,000-mol wt polypeptides are necessary for VIII:C function. These data and their interpretation are consistent with the published description of the complete primary structure of VIII:C and its thrombin cleavage products. The 92,000- and 80,000-mol wt chains have been located at the amino- and carboxy-terminal ends of the molecule, respectively.

Introduction

The production of monoclonal antibodies specific for human (1–7) or porcine (8) Factor VIII procoagulant protein (VIII:C)[1] has been reported by numerous investigators. These antibodies have been useful in the identification of VIII:C in plasma and of specific purified VIII:C polypeptides (2, 8). In the studies reported here we have used seven monoclonal antibodies against VIII:C in Western transfers (immunoblotting) (9), as well as immunoadsorption experiments to help define precursor-product relationships of purified VIII:C polypeptides (10) and to localize epitopes on these polypeptides that are important to VIII:C function. The results are consistent with the hypothesis (11, 12) that activated VIII:C (VIII:Ca) consists of an amino-terminal 92,000-mol wt heavy chain and a carboxy-terminal 72,000-mol wt light chain, which are immunologically distinct. Recent publications describing the complete primary structure of VIII:C and its thrombin cleavage products (13, 14) are also in accord with this immunologic evidence for VIII:C polypeptide derivations.

Methods

Purification of VIII:C. VIII:C was purified from commercial Factor VIII concentrate as previously described (10), except that the following protease inhibitors were included as indicated: the VIII:C buffer used to reconstitute the commercial Factor VIII concentrate (0.02 M imidazole, 0.15 M sodium chloride, 0.1 M L-lysine HCl, 0.02% sodium azide, pH 6.8) contained D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone, D-phenylalanyl-L-phenylalanyl-L-arginine chloromethyl ketone, and dansyl-L-glutamyl-L-glycyl-L-arginine chloromethyl ketone (Calbiochem-Behring Corp., La Jolla, CA), all at 10 μM, and leupeptin (Chemicon, El Segundo, CA) at 15 μg/ml (15). The VIII:C buffer at all subsequent steps contained the three chloromethyl ketones at 1 μM and leupeptin at 15 μg/ml, except that in the large volume of VIII:C buffer used to wash the immunoadsorbent column, the chloromethyl ketones were used at 0.1 μM and leupeptin at 1.5 μg/ml.

Thrombin degradation of purified VIII:C. A 194-μg sample of purified VIII:C (which had been stored at −70°C in VIII:C buffer containing 0.3 M calcium chloride and the above inhibitors) was dialyzed overnight at 4°C against 0.05 M Tris-Cl, 0.15 M sodium chloride, and 0.02% sodium azide, pH 7.4, with multiple changes. Purified human alpha thrombin (sp act 2,534 U/mg) was added to the dialyzed VIII:C at a final concentration of 10 U/ml and the mixture was incubated for 2.5 h at 37°C. At this time, the VIII:C had lost >90% of its procoagulant activity and the reaction was stopped by addition of D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (10^{-5} M final concentration), aliquoted, and stored frozen at −70°C.

Assays. Protein concentration was determined using a modification of the Lowry method (16), with bovine IgG as a standard for the monoclonal antibodies and bovine serum albumin (BSA) as a standard for VIII:C. The protein standards were purchased from Bio-Rad Laboratories, Richmond, CA. The VIII:C procoagulant activity assays were done as previously described (17).

Controls. Control monoclonal anti-thyroglobulin or monoclonal anti-Factor IX (IgG-1) antibodies were used in assays, immunoadsorption, and immunoblotting experiments.

Monoclonal anti-VIII:C antibody production. Balb/c mice were immunized by intraperitoneal injection of 1 μg of either purified VIII:C or thrombin-degraded purified VIII:C in complete Freund's adjuvant. 10- and 50-μg boosters in incomplete Freund's adjuvant were given at 1-wk intervals. The final 100-μg booster was given without adjuvant 3 d before fusion. At 4 wk, the mouse spleen cells were fused with P3X63 mouse plasmacytoma cells (18). Cell culture and production of monoclonal antibody in mouse ascites fluid were essentially by Liu

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1. *Abbreviations used in this paper:* PAGE, polyacrylamide gel electrophoresis; VIII:C, Factor VIII procoagulant protein; VIII:Ca, activated VIII:C.

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et al. (19). The antibodies were selected using a solid-phase assay in Linbro-Titertek (Flow Laboratories, Inglewood, CA) plates and an enzyme-linked immunosorbent detection system (20) using a peroxidase-antibody conjugate (Zymed Laboratories, Burlingame, CA). The plates were coated with 100 ng of either purified VIII:C or purified thrombin-degraded VIII:C per well. Clones were also screened against plates coated with 100 ng of human fibrinogen, plates coated with 100 ng of human fibronectin, and plates coated with 100 ng of human von Willebrand factor per well, each protein being a potential contaminant of the immunogen.

Clones which were positive only on the VIII:C-coated plates were also tested for their ability to inhibit plasma VIII:C activity, using a modified Kasper assay (21). Culture supernatants were incubated with an equal volume of normal pooled human plasma, which had been diluted 1:10 or 1:20 into assay buffer (17) to which had been added 2% (vol/vol) of 0.5 M sodium citrate, pH 6.5. Incubation was for 2 h at 37°C, after which samples were assayed for VIII:C procoagulant activity. Culture supernatants from clones C2 and C5 (Table I) inhibited >75% of the residual plasma VIII:C activity, as compared with control (non-anti-VIII:C supernatant) incubations.

Purification and characterization of monoclonal anti-VIII:C antibodies. Culture supernatants were tested for immunoglobulin class and subclass by double diffusion in agarose gel using commercially available antisera (Miles Laboratories, Inc., Elkhard, IN). Protein A-Sepharose chromatography (Pharmacia Fine Chemicals, Piscataway, NJ) of mouse ascites fluid was as described by Ey et al. (22). Purified antibodies were concentrated in a pressure ultrafiltration stirred cell (Amicon Corp., Danvers, MA) using a YM-10 membrane, aliquoted, and stored frozen at −70°C. Protein A-Sepharose purified monoclonal antibodies were assayed for their ability to inhibit plasma VIII:C procoagulant activity using the Kasper assay (21).

Immunoadsorbents were prepared by attaching purified monoclonal antibodies to cyanogen bromide-activated Sepharose 4B (Pharmacia Fine Chemicals), as previously described (10), at a coupling density of 5–10 mg of antibody per milliliter of Sepharose. Minicolumns (Bio-Rad Laboratories) were used for immunoadsorption experiments.

In the experiments of Fig. 4, 50–100 μg of purified VIII:C were diluted 1:10 in VIII:C buffer and recycled 20 times through a minicolumn containing 0.5 ml of immunoadsorbent. Columns were then washed with 40 bed volumes of VIII:C buffer containing either 10 mM calcium chloride (Fig. 4 *B*) or 10 mM disodium EDTA (Fig. 4, *A* and *C*) and eluted with 3 M sodium thiocyanate. Fractions of 0.1 ml were collected and dialyzed against sample buffer for $NaDodSO_4$ polyacrylamide gel electrophoresis (PAGE).

$NaDodSO_4$ 7.5% or 10% PAGE of reduced protein samples and staining with Coomassie Blue R250 was done as previously described (17). Isoelectric focusing in agarose gel in the pH range 3.5–9.5 was done using LKB reagents and equipment (LKB Produkter AB, Bromma, Sweden) according to the manufacturer's instructions. A surface electrode (LKB Produkter) was used to measure the pH gradient. Bovine gamma globulin (Pentex, fraction 11) was from Miles Laboratories.

Immunoblotting procedure. Paired samples of purified VIII:C and purified thrombin-degraded VIII:C were subjected to reduced $NaDodSO_4$ PAGE as previously described (17) except that dithiothreitol was used at 10 mM final concentration, the acrylamide concentration was 7.5%, and the gel thickness was 0.75 mm. The paired samples were run in adjacent lanes in duplicate, one pair on each half of the slab gel.

After electrophoresis, the samples in the gel were immediately electrophoretically transferred onto a 10 × 15-cm sheet of nitrocellulose (Bio-Rad Laboratories) using a Bio-Rad Trans-Blot cell, gel holder with sponge pads, and model 160/1.6 power supply according to the manufacturer's instructions. The transfer buffer contained 25 mM Tris, adjusted to pH 8.3, with glycine, and 20% (vol/vol) methanol. Transfer was for 16 h at 4°C, 0.35 A and 90 V.

After transfer, the nitrocellulose was divided into halves and one half was stained with Coomassie Blue R250 as described by Burnette (23) and preserved in distilled water for photography. The other half was reacted with monoclonal antibody using the Burnette (23) method, with the following modifications: all steps were done at room temperature in plastic boxes on a slowly rotating shaker; 0.5% gelatin (Eastman Kodak Co., Rochester, NY) was substituted for 5% BSA; purified monoclonal antibody was used at 10 μg/ml; and commercially available affinity-purified rabbit anti-mouse IgG labeled with ^{125}I was substituted for ^{125}I-labeled protein A. Labeling was done using the chloramine-T method (24) and 50 μCi of the labeled antibody was used per transfer. Processed nitrocellulose sheets were put into plastic freezer bags and exposed to Kodak XRP-1 film (Eastman Kodak Co.). Exposure was either at room temperature at least overnight (Figs. 5–10), or for several hours at −70°C using a Cronex Quanta III (E. I. duPont de Nemours & Co., Inc., Wilmington, DE) intensifying screen (Fig. 3).

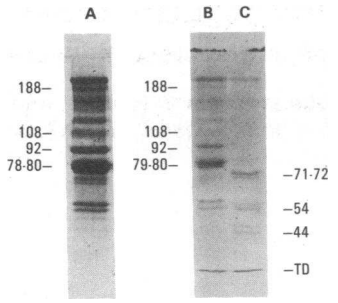

Figure 1. Transfer of VIII:C to nitrocellulose. (*A*) $NaDodSO_4$ PAGE of purified VIII:C stained with Coomassie Blue and dried onto filter paper. (*B* and *C*) Coomassie Blue-stained nitrocellulose sheet containing an electrophoretic transfer of purified VIII:C (*B*) and purified thrombin-degraded VIII:C (*C*). Transfer was from $NaDodSO_4$ 7.5% PAGE of reduced 20-μg samples. Apparent molecular weights of VIII:C polypeptides (188,000 to 79,000–80,000) and thrombin-degraded VIII:C polypeptide molecular weights (71,000–72,000, 54,000, and 44,000) are shown (× 10^{-3}). The heavily staining polypeptide above 188,000 and those below 79,000–80,000 in *A* and *B* are non-VIII:C contaminants, as are those above the 54,000 and 44,000 polypeptides in *C*. These contaminants can be largely removed by extensive absorption of the purified VIII:C with monoclonal anti-von Willebrand factor, anti-fibrinogen, and anti-fibronectin antibodies bound to Sepharose. TD represents the position of the tracking dye.

Results

Characterization of monoclonal anti-VIII:C antibodies. Monoclonal antibodies designated C1, C2, C4, and C5 were produced using purified VIII:C (Fig. 1, *A* and *B*) as immunogen. Those designated T2, T5, and T7 were produced using purified thrombin-degraded VIII:C (Fig. 1 *C*). Table I gives the IgG heavy chain subclass of each antibody as determined by double diffusion analysis and/or protein A-Sepharose chromatography.

The antibodies were purified from ascites fluid using protein

Table I. Characterization of Monoclonal Anti-VIII:C Antibodies

Antibody	Subclass	Titer
		*BU/mg**
C1	IgG-1	0
C2	IgG-2a	1,731
C4	IgG-1	0
C5	IgG-1	1,488
T2	IgG-1	0
T5	IgG-1	13
T7	IgG-1	12

*BU/mg, Bethesda units per milligram of purified IgG. One BU represents 50% inhibition of plasma VIII:C activity as compared with a control incubation (21).

A-Sepharose and purity was assessed by NaDodSO$_4$ PAGE. Isoelectric focusing showed restricted heterogeneity of the monoclonal antibodies as compared with a bovine gamma globulin fraction. Isoelectric points were between pH 6.2 and 7.0, with each antibody having a different pattern of microheterogeneity (Fig. 2). The ability of the purified antibodies to inhibit plasma VIII:C activity was reflected by the anti-VIII:C titers given in Table I. When attached to Sepharose and used as immunoadsorbent minicolumns, the purified C1, C2, C4, C5, T2, and T5 antibodies specifically removed purified VIII:C activity and antigen from solution, as compared with a control monoclonal anti-thyroglobulin immunoadsorbent. The T7 antibody did not remove purified VIII:C activity from solution. Because the T7 antibody was produced using thrombin-degraded VIII:C as immunogen, it is possible that the T7 epitope was not exposed. None of these anti-VIII:C antibodies convincingly removed VIII:C activity from plasma or commercial Factor VIII concentrate (see Discussion).

VIII:C polypeptide specificity of monoclonal anti-VIII:C antibodies. Immunoblot analysis using the purified monoclonal anti-VIII:C antibodies and purified VIII:C revealed precursor-product relationships of the VIII:C polypeptides and defined epitopes involved in VIII:C function. Fig. 1 shows VIII:C stained with Coomassie Blue before and after transfer to nitrocellulose, demonstrating transfer of all high molecular weight polypeptides. Figs. 3 and 5–10 show immunoblot analysis of the polypeptides using the C1, C2, C4, C5, and T7 antibodies.

The monoclonal anti-VIII:C antibodies could be divided into three types on the basis of their VIII:C polypeptide specificity as follows. The first type of antibody (which included C5, T2, T5, and T7) reacted with VIII:C polypeptides from 92,000 to 188,000 mol wt, but not with the 80,000-mol wt doublet (see Fig. 1 for molecular weight designations). The second type (which included C2 and C4) reacted with the 80,000-mol wt doublet and some or most of the higher molecular weight polypeptides, but not with the 92,000-mol wt chain. The third type, exemplified by the C1 antibody, reacted with chains from 108,000 to 188,000 mol wt, but not with either the 92,000-mol wt chain or the 80,000-mol wt doublet. The 92,000- and 80,000-mol wt polypeptides and probably the higher molecular weight forms are analogous to those described by Vehar et al. (13), although the numbers vary slightly. The immunoblotting data for five of the seven monoclonal anti-VIII:C antibodies, C2, C4, C5, T7, and C1, are presented below. Immunoblotting with control monoclonal anti-thyroglobulin or anti-Factor IX antibodies showed no reaction with any protein on the nitrocellulose transfers.

C2

Fig. 3 shows the reactivity of the C2 antibody against VIII:C and thrombin-degraded VIII:C. The antibody reacted strongly with the 80,000-mol wt doublet (Fig. 3 *A*) and other exposures of the autoradiograph revealed that both members of the doublet had reacted. There was also a weak but distinct reaction with VIII:C chains in the 188,000-mol wt region and with a chain between 108,000 and 188,000 mol wt. The presence of the C2 epitope on both the 80,000-mol wt doublet and high molecular weight chains suggests that the doublet was derived from them by proteolysis. There was no reaction of C2 with either the 92,000- or the 108,000-mol wt chains, indicating the absence of this epitope on these polypeptides. This antibody also did not react with any of the three major thrombin fragments of VIII:C (Fig. 3 *B*). Apparently, thrombin cleavage of VIII:C destroyed this epitope, and it has, therefore, been designated as thrombin-sensitive. Primary structure data indicate a thrombin cleavage site at arg 1689-ser 1690, which generates the carboxy-terminal 72,000-mol wt polypeptide (13). It is likely that the C2 epitope is located near this bond. In addition, this antibody was a strong inhibitor of plasma VIII:C activity (Table I), which suggests that this epitope on the carboxy-terminal 80,000-mol wt chain is located in a region important to VIII:C procoagulant function.

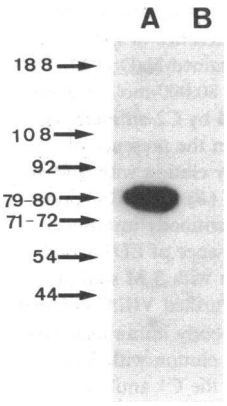

Figure 3. Autoradiograph of a nitrocellulose sheet containing an electrophoretic transfer of purified VIII:C (*A*) and purified thrombin-degraded VIII:C (*B*) reacted with the C2 monoclonal anti-VIII:C antibody. The transfer was identical to that shown in Fig. 1 (*B* and *C*), except that here ~3 g of protein was transferred, and in place of Coomassie Blue staining, the VIII:C was first reacted with the C2 monoclonal anti-VIII:C antibody and then with ^{125}I-labeled rabbit anti-mouse IgG to visualize polypeptides containing the C2 epitope. As in Fig. 1, VIII:C polypeptide molecular weights (188,000 to 79,000–80,000) and thrombin-degraded VIII:C polypeptide molecular weights (71,000–72,000, 54,000, and 44,000) are shown. × 10^{-3}.

When the C2 antibody was attached to Sepharose and used as an immunoadsorbent minicolumn, it adsorbed all VIII:C polypeptides (80,000–188,000 mol wt), demonstrating that they form an aggregate in solution. All VIII:C polypeptides could be recovered after elution of the column with 3 M sodium thiocyanate (Fig. 4 *B*). However, if EDTA was included in the column buffer, C2 removed primarily the 80,000-mol wt doublet from solution, which could also be subsequently eluted with 3 M sodium thiocyanate (Fig. 4 *A*). In addition, the polypeptides of 92,000–188,000 mol wt could be recovered in the column pass-through. This preferential reaction of C2 with the doublet showed that C2 had a high affinity for this chain and that a divalent cation(s) is involved in the association between the 80,000-mol wt doublet and the 92,000-mol wt chain, as well as some of the larger chains. Fass et al. (8) have previously reported a similar finding for analogous chains of porcine VIII:C. When thrombin-degraded VIII:C was passed through the C2 immunoadsorbent in experiments similar to those of Fig. 4, none of the fragments were bound, and they

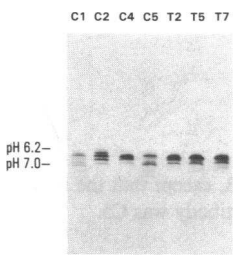

Figure 2. Isoelectric focusing of the monoclonal anti-VIII:C antibodies (Table I) in agarose gel. The pH range of the major isoelectric forms of the monoclonal antibodies is shown at the left. (*Top*) anode. (*Bottom*) cathode.

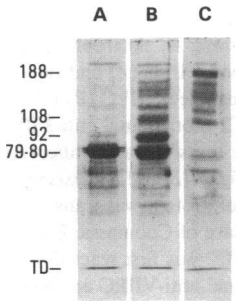

Figure 4. Immunoadsorption of VIII:C in the presence and absence of EDTA. (*A*) Coomassie Blue-stained $NaDodSO_4$ 7.5% PAGE of purified 80,000-mol wt doublet of VIII:C prepared by C2 antibody immunoadsorption in the presence of EDTA followed by elution with 3 M sodium thiocyanate. (*B*) All VIII:C polypeptides after C2 antibody immunoadsorption in the absence of EDTA and subsequent elution with 3 M sodium thiocyanate. (*C*) Purified VIII:C polypeptides of 108,000–188,000 mol wt after C1 antibody immunoadsorption in the presence of EDTA and subsequent elution with 3 M sodium thiocyanate. In the absence of EDTA, the C1 antibody results were similar to those in *B*. Molecular weights at the left are shown ($\times 10^{-3}$). To increase contrast, the gel was dried onto filter paper before photography. TD, tracking dye.

could be recovered in the minicolumn pass-through. Thus, the C2 epitope was also thrombin-sensitive under nondenaturing conditions, and is probably at or near the thrombin cleavage site. Such thrombin cleavage sites are potentially important in the activation and inactivation of VIII:C.

C4

Like C2, the C4 antibody was of the second type. It reacted with the 80,000-mol wt doublet, but not with the 92,000-mol wt chain (Fig. 5 *A*). Thus, two of the seven antibodies reacted only with the doublet and not with the 92,000-mol wt chain.

However, C4 differed from C2 by its reaction with all chains of 108,000–188,000 mol wt (the 188,000-mol wt chain is poorly visible in Fig. 5 due to multiple photographic reproductions), whereas C2 reacted with only some of them. This again indicates the derivation of the doublet from high molecular weight material. The presence of the C4 epitope on chains as low as 108,000 mol wt suggested the occurrence of overlapping cleavages in the high molecular weight material, although our previous studies (10, 11) suggested that the 92,000-mol wt chain was contained in all high molecular weight material. Another possible explanation for the C4 reactivity is presented in the Discussion section. Although the C4 epitope appeared to be thrombin-sensitive, there was a slight reaction with the 72,000-mol wt doublet of thrombin-degraded VIII:C (Fig. 5 *B*). However, when used as an immunoadsorbent, C4 failed to remove thrombin-degraded VIII:C from solution. It is possible that the C4 epitope was incompletely destroyed by thrombin, resulting in a low affinity for VIII:C in solution, as compared with the C2 epitope that was completely destroyed. In addition, C4 showed no inhibition of plasma VIII:C activity (Table I), while C2 was a strong inhibitor, demonstrating a difference in the epitopes recognized by these two antibodies.

The C4 antibody was unique among the seven monoclonal anti-VIII:C antibodies in its apparently low affinity for VIII:C. When used as an immunoadsorbent for intact VIII:C, it was possible to elute VIII:C protein from C4 in an active state using 0.5 M calcium chloride as eluant. Thus, it could be used as a purification step for VIII:C.

C5

This antibody is an example of the first type, which reacted with VIII:C chains of 92,000–188,000 mol wt, but not with the 80,000-mol wt doublet. It also reacted with a smaller VIII:C fragment of ~50,000 mol wt present in the VIII:C preparation (Fig. 6 *A*). This C5 epitope was not thrombin-sensitive, as evidenced by its presence on the 54,000-mol wt fragment of thrombin-degraded VIII:C (Fig. 6 *B*). This is consistent with the hypothesis that the 54,000-mol wt fragment was derived from the 92,000-mol wt chain by thrombin cleavage (10), which has been confirmed by primary structure analysis (13, 14). The 54,000-mol wt fragment represents the amino-terminus of the molecule (13, 14). As seen in Table I, C5 was a strong inhibitor of plasma VIII:C activity, thus demonstrating an epitope on the amino-terminal 92,000-mol wt chain involved in VIII:C procoagulant activity.

When used as an immunoadsorbent in experiments similar to those shown in Fig. 4, this antibody removed all VIII:C chains from solution, but in the presence of EDTA, only the 92,000–188,000-mol wt chains were bound. Like the C2 antibody, which could be used to purify the 80,000-mol wt doublet in the presence of EDTA, this result suggested divalent cation involvement in the association of the doublet and 92,000-mol wt chains (8).

T7

T7 was another antibody of the first type that reacted with VIII:C polypeptides of 92,000–188,000 mol wt, but showed no distinct reaction with the 80,000-mol wt doublet (Fig. 7 *A*). Like C5, it was not thrombin-sensitive, because it reacted with the 44,000-mol wt fragment of thrombin-

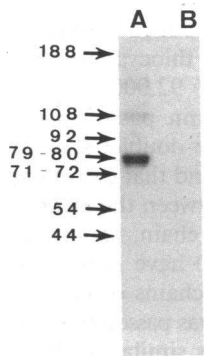

Figure 5. As in Fig. 3, except that the monoclonal anti-VIII:C antibody was C4.

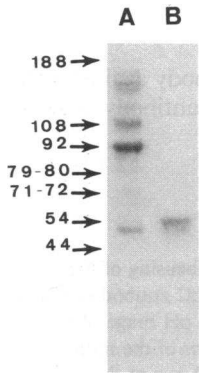

Figure 6. As in Fig. 3, except that the monoclonal anti-VIII:C antibody was C5.

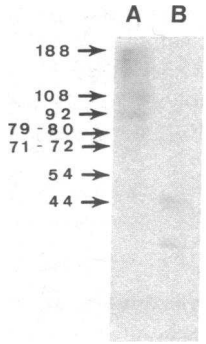

Figure 7. As in Fig. 3, except that the monoclonal anti-VIII:C antibody was T7.

degraded VIII:C, as well as with some VIII:C polypeptide material at the tracking dye (Fig. 7 *B*). This is consistent with the hypothesis that the 44,000-mol wt fragment was also a thrombin cleavage product of the 92,000-mol wt chain (10), which has been confirmed by primary structure analysis (13, 14). As seen in Table I, this antibody inhibited plasma VIII:C activity, but not as strongly as C2 or C5. This demonstrated that a third epitope was involved in the procoagulant function of VIII:C.

C1

An example of the third type of antibody chain specificity was illustrated by C1. As seen in Fig. 8 *A*, C1 reacted only with VIII:C polypeptides of 108,000–188,000 mol wt. It showed no reaction with either the 80,000-mol wt doublet or the 92,000-mol wt chain. The C1 epitope is probably not thrombin-sensitive, as evidenced by the diffuse polypeptide material, heterogeneous in molecular weight, present in Fig. 8 *B*. This may represent portions of the middle section of the VIII:C molecule (13, 14, and see Fig. 11). As shown in Table I, C1 did not inhibit plasma VIII:C activity. When C1 was used as an immunoadsorbent in the presence of EDTA, only the VIII:C polypeptides containing the C1 epitope were bound and subsequently eluted (Fig. 4 *C*).

Mixing experiments

Immunoblotting was also done using mixtures of the monoclonal anti-VIII:C antibodies in order to confirm the assignments of VIII:C polypeptide molecular weight specificity. Fig. 9 shows that a mixture of C4 and C5 recognized all VIII:C polypeptides, as well as the low molecular weight

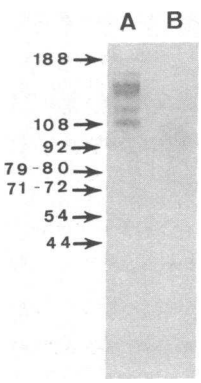

Figure 8. As in Fig. 3, except that the monoclonal anti-VIII:C antibody was C1.

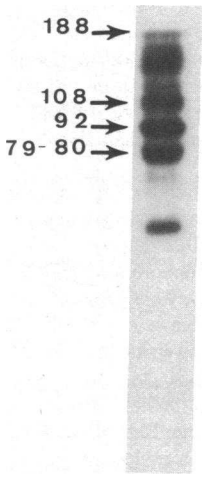

Figure 9. VIII:C as in Fig. 3 (*A*), except that the monoclonal anti-VIII:C antibody used was a mixture of C4 (Fig. 5) and C5 (Fig. 6).

fragment reactive with C5. Fig. 10 shows that a mixture of T7 and C5 reacted with both the 54,000- and 44,000-mol wt fragments of thrombin-degraded VIII:C, as well as the VIII:C polypeptide material at the tracking dye reactive with T7. None of the seven monoclonal anti-VIII:C antibodies in Table I reacted with the thrombin-degraded 72,000-mol wt doublet, with the possible exception of C4, which showed a very weak reaction (Fig. 5 *B*).

Epitope map

Using the preceding data, it was possible to construct a preliminary epitope map of the monoclonal anti-VIII:C antibodies. Fig. 11 (top) shows a diagrammatic representation of the structure of single chain VIII:C (13, 14) containing the epitopes for C5, T7, C1, C2, and C4. The second line depicts a partially proteolyzed VIII:C molecule representing at least some of the polypeptides of 108,000–188,000 mol wt present in our VIII:C preparations. Below it are the 92,000-mol wt chain containing the C5 and T7 epitopes and the 80,000-mol wt doublet containing the C2 and C4 epitopes, indicating their derivation from the higher molecular weight material. To the extent that four of the antibodies in Table I reacted with the 92,000-mol wt chain,

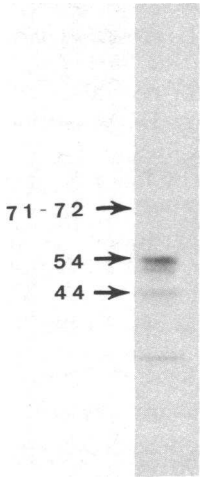

Figure 10. Thrombin-degraded VIII:C as in Fig. 3 (*B*), except that the monoclonal anti-VIII:C antibody used was a mixture of C5 (Fig. 6) and T7 (Fig. 7).

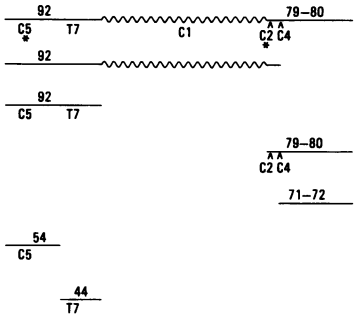

Figure 11. Preliminary epitope map of the monoclonal anti-VIII:C antibodies described in this report. (*Top* line) Model of high molecular weight single chain VIII:C (13, 14), showing theoretical epitope locations. Asterisks indicate antibodies that were strong inhibitors of plasma VIII:C activity. Carats indicate thrombin-sensitive epitopes. Straight lines labeled 92 or 79–80 represent the lower molecular weight VIII:C polypeptides of 92,000 and 79,000–80,000, which reacted uniquely with the indicated monoclonal antibodies. Wavy lines represent other polypeptide sequences. (Second line) Representation of the multiple chains of partially proteolyzed high molecular weight VIII:C as seen in Fig. 1 (*A* and *B*), which retained all of the above epitopes with the exception that some lacked the C2 epitope. (Third and fourth lines) Amino-terminal 92,000-mol wt VIII:C polypeptide and carboxy-terminal 79,000–80,000-mol wt VIII:C doublet (13, 14) seen in Fig. 1 (*A* and *B*), that were reactive with the indicated monoclonal antibodies as shown in Figs. 3 and 5–8 (*A*). Lines 5–7 represent thrombin-degraded fragments of VIII:C seen in Fig. 1 (*C*). The 54,000-mol wt fragment is at the amino-terminus of VIII:C and the 72,000-mol wt chain is carboxy-terminal (13, 14). Loss of the thrombin-sensitive C2 and C4 epitopes is shown in Figs. 3 and 5 (*B*), while the T7 and C5 epitopes were retained (Figs. 6 and 7, *B*), consistent with their derivation from the 92,000-mol wt chain. In previous studies (10, 11), we have provided evidence that the 92,000-mol wt chain is associated with VIII:C activity. In addition, by analogy with activated Factor V, this 92,000-mol wt chain, the 80,000-mol wt doublet and/or the 72,000-mol wt doublet, may constitute a thrombin-activated form of VIII:C (12).

but not with the 80,000-mol wt doublet, and two reacted with the doublet, but not with the 92,000-mol wt chain, the data suggest that these two chains are immunologically distinct and are cleaved from different segments of a precursor VIII:C molecule. This has been confirmed by primary structure data (13, 14), which have shown that the 92,000-mol wt chain is at the amino-terminus and the 80,000-mol wt chain is at the carboxy-terminus of the VIII:C molecule.

The three major thrombin cleavage products of VIII:C, the 72,000-mol wt doublet and the 54,000- and 44,000-mol wt chains, are also shown in Fig. 11. The thrombin sensitivity of the C2 and C4 epitopes on the 80,000-mol wt doublet is reflected by their absence (or near absence) on any of the thrombin cleavage products. Derivation of the 54,000- and 44,000-mol wt thrombin fragments from the 92,000-mol wt chain is demonstrated by following the C5 and T7 epitopes, respectively.

At least two distinct thrombin cleavage sites on VIII:C were shown by these antibodies. A site on the 92,000-mol wt chain giving rise to the amino-terminal 54,000- and 44,000-mol wt fragments was demonstrated by C5 and T7, and one or two sites on the 80,000-mol wt doublet were shown by the thrombin-sensitive C2 and C4 epitopes. According to primary structure data, these thrombin cleavage sites would correspond to arg 372-ser 373 on the 92,000-mol wt chain, and arg 1689-ser 1690 on the 80,000-mol wt chain (13, 14). In addition, these epitopes reflected areas important to VIII:C procoagulant activity, because C5 was a strong inhibitor and T7 a weak inhibitor of plasma VIII:C activity on the 92,000-mol wt chain, and C2 was a strong inhibitor on the 80,000-mol wt doublet (Table I).

Discussion

The monoclonal anti-VIII:C antibodies described in this report all reacted with purified VIII:C both in solution and after denaturation, reduction, and transfer to nitrocellulose. Although some degree of polypeptide refolding could have occurred during the processing of the nitrocellulose sheets, it is likely that these epitopes do not require native conformation for their expression. When used as immunoadsorbents, the antibodies bound both purified VIII:C and partially purified VIII:C from solution. The partially purified VIII:C had been freed of von Willebrand factor by calcium chloride dissociation after immunoadsorption of commercial Factor VIII concentrate onto a column of monoclonal anti-von Willebrand factor-Sepharose (14). However, these antibodies did not significantly remove VIII:C activity from plasma or commercial Factor VIII concentrate. Similar findings have been reported by others (1, 8) for their monoclonal anti-VIII:C antibodies. Possibly, the binding between von Willebrand factor and VIII:C in plasma or commercial Factor VIII concentrate either decreases the affinity of the antibodies for VIII:C, or blocks their interaction (8).

The usefulness of these antibodies both in immunoadsorption and immunoblotting experiments has provided supporting data for our working hypothesis of structure-function relationships of human VIII:C. We previously proposed (10, 11) that VIII:C and its activated form, VIII:Ca, are structurally analogous to Factor V and its activated form, Va. Thrombin cleavage of high molecular weight precursor VIII:C (13, 14) gives rise to an amino-terminal 92,000-mol wt heavy chain and a carboxy-terminal 80,000- or 72,000-mol wt light chain doublet. These chains could constitute a two-chain VIII:Ca similar to the thrombin activation of human Factor V to a two-chain activated form (Va) consisting of an amino-terminal 105,000-mol wt heavy chain and a carboxy-terminal 71,000–74,000-mol wt light chain doublet (25). The monoclonal antibody data presented here support the derivation of a 92,000-mol wt heavy chain and an 80,000-mol wt light chain from high molecular weight material. The data also suggest that cleavage of the 92,000-mol wt chain, which has been shown to be associated with loss of VIII:C activity (10, 11), gave rise to the 54,000- and 44,000-mol wt fragments. The finding (Table I) that the C5 antibody, with an epitope on the 92,000-mol wt chain, and the C2 antibody, with an epitope on the 80,000-mol wt doublet, are both relatively strong inhibitors of plasma VIII:C activity, also suggests a major functional role for both chains.

Data from the immunoadsorption experiments presented here (Fig. 4) implicate divalent cation involvement in the association between VIII:C polypeptides. The analogy with human factor Va (26) and the work of Fass et al. (8) with porcine VIII:C suggest that calcium ions are involved in the association between the polypeptides of human VIII:Ca. Recent data on the homology between ceruloplasmin and VIII:C have

also demonstrated potential copper binding sites in VIII:C (13, 14, 26).

Other epitopes of possible functional significance are those designated as thrombin-sensitive. Destruction of these epitopes by thrombin cleavage suggests that they are located at or near thrombin cleavage sites. These sites could be important in VIII:C activation or inactivation by thrombin. Although the destruction of an epitope due to conformational changes resulting from thrombin cleavage at a distant site cannot be ruled out, the immunoblotting data suggest that these antibodies recognize proteolytic fragments of at least partially denatured VIII:C. Thus, the epitopes are probably not highly dependent upon polypeptide conformation and are more likely to be disrupted only when proteolytic cleavages are nearby.

Because the thrombin-cleaved 72,000-mol wt doublet reacted not at all with the C2 antibody and only weakly with the C4 antibody, its derivation from the 80,000-mol wt doublet was not proven. Nevertheless, this derivation seemed likely, especially in view of the weak reactivity of the 72,000-mol wt doublet with C4. Vehar et al. (13) have reported that the 72,000-mol wt chain is, indeed, derived from the 80,000-mol wt chain. This would localize the C2 and C4 epitopes on or near an amino-terminal 41 residue peptide (13, 14) on the 80,000-mol wt doublet.

As noted in the Results, the C5, T7, T2, and T5 antibodies reacted uniquely with the 92,000-mol wt chain and all larger chains, while C4 reacted uniquely with the 80,000-mol wt chain and all larger chains except for that of 92,000 mol wt. To be in accord with the model presented in Fig. 11, this would require even the 108,000-mol wt polypeptide to contain segments from both the 92,000- and 80,000-mol wt chains. However, these two chains are located at the amino- and carboxy-terminal ends of the 264,763-mol wt (13) VIII:C molecule, making it unlikely that the 108,000-mol wt chain could contain both epitopes. A possible explanation for the C4 antibody reactivity with all chains of 108,000–188,000 mol wt, but not the 92,000-mol wt chain, is suggested by the regions of internal homology (domains) in VIII:C (13, 14, 26). Location of the C4 epitope in one of the repetitive sequences could account for these results. The C4 epitope could be located in the second and third A domains (13), but not in the first A domain. A thrombin cleavage site which probably produces the 92,000-mol wt chain is located in the second A domain (13), and cleavage at this site could destroy the C4 epitope on the 92,000-mol wt polypeptide.

In summary, these studies provide evidence that human VIII:Ca, like Factor Va (27), is comprised of immunologically distinct heavy and light chains, both of which participate in function. Additional investigations with these antibodies should further illuminate structure function relationships of this biologically important molecule.

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