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Epitope mapping of human factor VIII inhibitor antibodies by deletion analysis of factor VIII fragments expressed in *Escherichia coli*

(human alloantibodies in hemophilia A/human autoantibodies to factor VIII/neutralization of factor VIII inhibitor antibody)

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ABSTRACT **Epitopes for antibodies that inhibit factor VIII procoagulant protein were analyzed by deletion mapping of factor VIII protein fragments expressed in *Escherichia coli*. A human factor VIII cDNA clone was used to generate *E. coli* expression vectors encoding fragments containing the 80-kDa factor VIII light chain (A3, C1, and C2 domains) and the 44-kDa carboxyl-terminal half of the factor VIII heavy chain (A2 domain). A series of deletions of each fragment was constructed and tested by immunoblotting for the binding of alloantibody and autoantibody inhibitors. Analysis of derivatives of the 80-kDa fragment showed that six inhibitors recognized a major epitope(s) within the carboxyl-terminal 17.3 kDa of factor VIII. These inhibitors also recognized weaker epitopes nearby and one inhibitor recognized epitopes scattered throughout the 80-kDa fragment. Deletions within the heavy chain fragment revealed one epitope-containing region confined to the amino-terminal 18.3 kDa recognized by six inhibitors. Bacterially produced factor VIII fragments containing the major epitopes were capable of neutralizing inhibitors *in vitro* but fragments containing weaker or no epitopes did not. These data suggest a potential therapeutic use of factor VIII fragments for neutralization of inhibitor antibodies.**

Individuals with hemophilia A, an X chromosome-linked, recessive genetic disorder, are deficient or abnormal in factor VIII procoagulant protein. Factor VIII circulates in the plasma in a complex with von Willebrand factor, and it functions in the intrinsic pathway of blood coagulation.

The mature factor VIII protein consists of 2332 amino acids (265 kDa), as deduced from the sequence of a human factor VIII cDNA clone (1, 2). The protein sequence is arranged in a series of homologous repeated domains, A1-A2-B-A3-C1-C2 (3). Factor VIII is activated by thrombin in a series of cleavages that produce an amino-terminal 92-kDa fragment, a carboxyl-terminal 80-kDa fragment, and heterogeneous fragments from the B domain. The 92-kDa fragment is split into 54-kDa and 44-kDa species, and the 80-kDa fragment is reduced to 72 kDa (3, 4). Cleavages of the 92-kDa and 80-kDa fragments have been proposed to yield active factor VIII (5, 6).

Hemophilia A patients are treated for bleeding episodes by the administration of factor VIII from pooled human plasma. A clinical complication in 8–20% of repeatedly transfused hemophiliacs is the formation of alloantibodies that inhibit factor VIII activity (hereafter referred to as inhibitors). Inhibitors can also appear spontaneously as autoantibodies in individuals with immunologic diseases, in post-partum women, or in individuals with no apparent disorders. A number of therapies have been used to neutralize the inhibitor in these patients but none is completely satisfactory (7).

Fulcher *et al.* (8) examined the distribution of inhibitor binding sites (epitopes) along the factor VIII molecule by immunoblotting of factor VIII thrombin cleavage fragments with inhibitors. The majority of inhibitors bound to the 44-kDa and/or 72-kDa thrombin fragments but rarely to the 54-kDa fragment (9).

The purpose of our present study was to localize inhibitor epitopes more precisely within the 44-kDa and 72-kDa factor VIII thrombin fragments by deletion mapping and to determine if bacterially expressed factor VIII fragments containing the epitopes could neutralize inhibitors *in vitro*.

MATERIALS AND METHODS

Construction of Plasmids Containing Factor VIII Fragments. *Escherichia coli* K12 JM101 (10) was used as the host for plasmids pL4 and the pL3 series. *E. coli* K12 LC137 (*supC*^{ts} *htpR165*$_{am}$ *lonR9*), provided by A. Goldberg (Harvard University, Cambridge, MA), was used as a host for expression of fragments from the pH1 and pH2 series. Human factor VIII cDNA clones were isolated from a liver cDNA library (11) and provided by George Ricca (Rorer Biotechnology, King of Prussia, PA). Nucleotide and amino acid residues were numbered as described by Wood *et al.* (1). Modifications of restriction fragment termini were made with oligonucleotide linkers. Factor VIII DNA fragments containing residues 4734–7066 (pL3) or residues 1033–2277 (pH1) were cloned between the *Pvu* II and *Hin*dIII sites of ptac12 (12). pL4 contained residues 5751–7066 cloned into the *Pst* I site of pKK223-3 (Pharmacia). To construct pH2, a *Sph* I site was introduced by changing a cytidine to guanosine (1192) by site-directed mutagenesis (13). The modified fragment, residues 1192–2277, was introduced into pCQV2 (14) at the *Bam*HI site.

Deletions of the factor VIII sequence of pL3, pH1, and pH2 were generated by removing segments between two restriction endonuclease sites and bridging the gap with an oligonucleotide linker. The DNA sequence of all plasmids made with linkers was verified by the dideoxy chain-termination method.

The amino-terminal protein sequence of the 41.2-kDa fragment was determined by automated Edman degradation on an Applied Biosystems model 470A protein sequencer. Phenylthiohydantoin amino acid derivatives were identified by HPLC.

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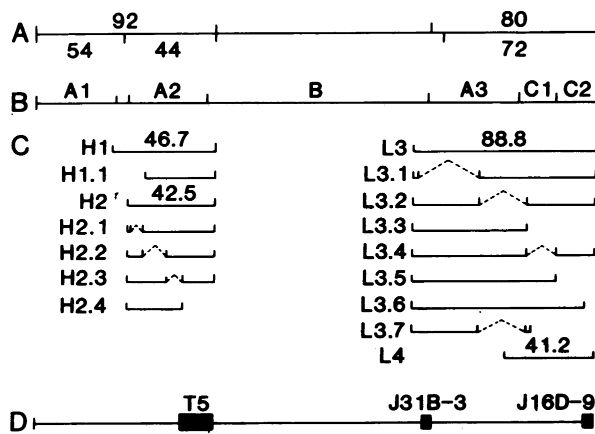

FIG. 1. Map of human factor VIII cDNA and regions cloned for expression in *E. coli*. (*A*) Thrombin cleavage fragments of mature factor VIII, size in kDa. Cleavage to produce the 80-kDa fragments is performed by an unknown enzyme. The B domain cleavage gives fragments of heterogeneous size (data not shown). (*B*) Domain structure of factor VIII, given by amino acid residue number (18): A1, 1–329; A2, 380–711; B, 712–1648; A3, 1649–2019; C1, 2020–2172; C2, 2173–2332. (*C*) Lines represent factor VIII protein fragments expressed in *E. coli*. Sizes (kDa) calculated from amino acid content are listed above the lines. Dotted lines represent internal deletions. Fragments contain the following factor VIII amino acids, given by residue number (1): H1, 326–740; H1.1, 436–740; H2, 373–740; H2.1, 373–379 and 448–740; H2.2, 373–444 and 542–740; H2.3, 373–542 and 606–740; H2.4, 373–606; L3, 1560–2332; L3.1, 1560–1569 and 1822–2332; L3.2, 1560–1829 and 2046–2332; L3.3, 1560–2052; L3.4, 1560–2048 and 2170–2332; L3.5, 1560–2177; L3.6, 1560–2298; L3.7, 1560–1829 and 2046–2056; L4, 1974–2332. (*D*) The solid boxes represent regions containing epitopes for the monoclonal antibodies T5 (19), J31B-3, and J16D-9.

Immunoblotting. Bacteria induced for expression of factor VIII fragments were pelleted, suspended in 0.1 vol containing 140 mM Tris·HCl (pH 6.8), 3% (wt/vol) NaDodSO$_4$, 5% (vol/vol) 2-mercaptoethanol, and 10% (vol/vol) glycerol, and boiled 5 min. Samples of bacterial protein were separated by discontinuous NaDodSO$_4$/PAGE (10% polyacrylamide gels) and transferred to nitrocellulose sheets as described (8, 15). When monoclonal antibodies were used, the sheet was preincubated in a solution of 20 mM Tris·HCl (pH 8), 0.5 M NaCl (TBS), and 3% (wt/vol) gelatin. Incubation with antibodies was for 15 hr at 23°C in TBS with 1% gelatin. Blots were washed with TBS containing 0.05% Tween 20, incubated for 30 min with goat anti-mouse antibody conjugated to alkaline phosphatase, washed again, and developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Promega). When inhibitor antibodies were used, the procedure (16) was changed to include preincubation and antibody incubation of blots with 5% (wt/vol) nonfat dry milk in buffer. The secondary antibody was monoclonal anti-human IgG4 (9). After further incubation with ^{125}I-labeled goat anti-mouse antibody, the blots were exposed to x-ray film for 1–2 weeks.

Partial Purification of Factor VIII Fragments. Bacteria induced for factor VIII fragment expression were suspended in a solution of 50 mM Tris·HCl, 1 mM EDTA (pH 8.0), lysozyme (25 μg/ml) and lysed by sonication. The lysates were centrifuged at 12,000 × *g* for 15 min at 5°C, and the pellets were washed twice with buffer containing 0.5% Triton X-100. The pellets were treated with DNase I (20 μg/ml) (Boehringer Mannheim, GmBH) and RNase A (20 μg/ml) (Sigma) for 15 min at 37°C and washed again. Pellets from pH1 and pH1.1 were suspended in buffer with 8 M urea and 0.02% sodium azide for 10 min and dialyzed overnight against barbital-buffered saline (BBS; 0.025 M barbital/0.125 M sodium chloride/0.1% sodium azide, pH 7.5).

Inhibitor Plasma. Frozen citrated plasma samples from factor VIII inhibitor patients were kindly provided by Carol K. Kasper (Orthopaedic Hospital, Los Angeles, CA), Leon W. Hoyer (American Red Cross, Rockville, MD), and Klaus Lechner (First Medical Clinic, University of Vienna, Austria). Plasma samples were heated at 60°C for 30 min before use.

Factor VIII and Inhibitor Assays. Factor VIII coagulant activity was measured by an activated partial thromboplastin time assay with factor VIII-deficient hemophilia A plasma and an MLA Electra model 700 automatic coagulation timer. Factor VIII inhibitor activity was measured in the Bethesda assay (17). BBS buffer was used in all assays.

Factor VIII Inhibitor Neutralization Assays. BBS buffer containing a dilution of inhibitor plasma and 10 μM protease inhibitor [(*p*-amidinophenyl)methanesulfonyl fluoride, Chemicon International, El Segundo, CA] was added to inclusion body pellets of 1 mg of protein, except for L3.3 (1.9 mg). Samples were mixed 15 hr at 23°C on a rotator and centrifuged for 15 min at 12,000 × *g*, and the supernatant was tested for inhibitor activity.

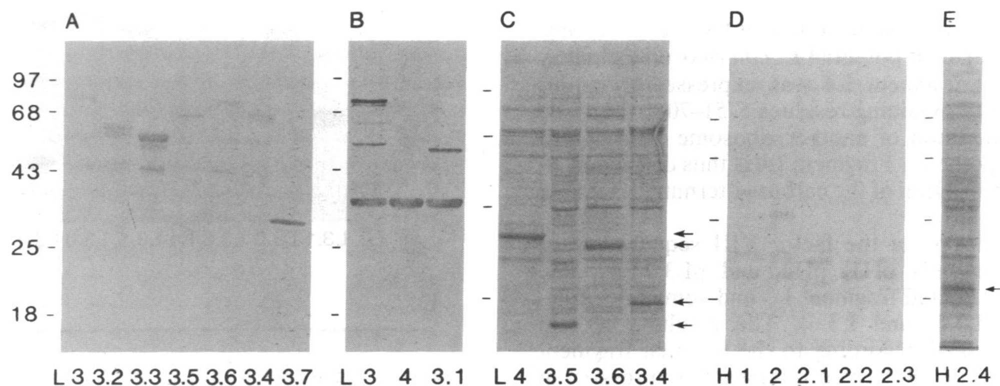

FIG. 2. Analysis of expression of factor VIII fragments in *E. coli*. Plasmid containing strains were grown in L broth with ampicillin (50 μg/ml) to an absorbance of 0.4 at 595 nm. Plasmid pL4 and pL3, or its derivatives, were grown at 37°C and induced with 0.5 mM isopropyl β-D-thiogalactoside for 1 hr; plasmids pH1 and pH1.1 were grown at 30°C and induced at 40°C with 3 mM isopropyl β-D-thiogalactoside for 2 hr. Aliquots of total bacterial protein were separated by NaDodSO$_4$/PAGE and analyzed by immunoblotting with monoclonal antibodies (Fig. 1*D*). Control bacterial extracts with no factor VIII fragments were negative for all antibodies tested. (*A*) Immunoblot with antibody J31B-3. Each lane contained 30 μg of protein. (*B*) As in *A* with antibody J16D-9. (*C*) Coomassie blue stained gel of 30-μg protein samples, as indicated. The arrows indicate the positions of the factor VIII fragments. (*D*) Immunoblot with antibody T5. Each lane contained 40 μg of protein for all extracts except lane H2, which contained 10 μg. (*E*) Same as *C*.

Table 1. Immunoblot reaction of inhibitors with deletions of the H2 and L3 fragments

Inhibitor			Factor VIII fragment												
Name	Type	Specificity	L3	L3.1	L3.2	L3.3	L4	L3.4	L3.5	L3.6	H2	H2.1	H2.2	H2.3	H2.4
NF	Hemophilia A	72	+	+	+	−	+	+	w	w	−†				
1824	Hemophilia A	72	+	+	+	−	+	+	w	w	−†				
F	Spontaneous	72	+	+	+	−	+	+	−	w	−†				
TB	Hemophilia A	44,72	+	+	+	+	+	+	−/+*	+/w*	+†				
CC	Hemophilia A	44,72	+	+	+	−	+	+	w	w	+	−	−	+	w
HA	Spontaneous	44,72	+	+	+	−	+	+	−	w	+	−	−	+	+
PB	Hemophilia A	44	−								+	−	−	w	w
GK	Spontaneous	44	−								+	−	−	w	+
CHA	Hemophilia A	44	−								+	−	−	+	+

Experimental conditions are as described in Figs. 2 and 3. Reactions were rated as positive (+), negative (−), or weak (w). In weak reactions the intensity of the band in the inhibitor immunoblot was much less than that of the same band in an immunoblot with monoclonal antibody or in a stained gel (Fig. 2). Inhibitors had been tested by immunoblotting (8) for binding to the 44-kDa (44) and 72-kDa (72) factor VIII thrombin fragments (specificity) and were from hemophilia A patients or patients with spontaneous inhibitors.

*The reactions with derivatives of the L3 fragment (upper values) and the L4 fragment (lower values) are given separately since both types of fragments appeared on the autoradiographs. The other inhibitors detected bands only of the L4 derivatives.

†For these inhibitors H1 was used for immunoblotting.

RESULTS

Expression of Factor VIII Fragments in *E. coli*. Factor VIII cDNA segments encoding the major factor VIII inhibitor epitopes or deletions thereof were subcloned into plasmids with expression signals for *E. coli*. The position of the segments in relation to the factor VIII coding sequence and the domains and thrombin cleavage sites of the factor VIII protein are shown in Fig. 1. Protein fragments were named according to their location in the 92-kDa heavy chain (H) or the 80-kDa light chain (L). Plasmids (p) carry the name of the fragment they encode.

Plasmids pH1, pH2, and pL3 were constructed in such a way as to have independent translation initiation sites for factor VIII fragments. Expression of the fragments was verified by immunoblotting (Fig. 2 *A*, *B*, and *D*) with monoclonal antibodies to purified human factor VIII (T5) or to factor VIII peptide residues 1660–1674 (J31B-3) or residues 2318–2332 (J16D-9) (Fig. 1*D*). The expected full-length fragments from each plasmid, as well as shorter fragments that may have arisen by proteolysis or incomplete translation, were seen.

Extracts of plasmid pL3 contained fragment L3 and an abundant fragment of 41.2 kDa, detected only by antibody J16D-9 (Fig. 2*B*). Sequencing of the amino terminus of this fragment, which was designated L4, showed that it began at Met-1973. This suggested that it was generated from translation initiating at a methionine codon within the L3-coding sequence and an adjacent potential *E. coli* ribosome binding site. The fact that fragment L4 was expressed from the nucleotide segment containing residues 5751–7066 (Fig. 2*B*, L4) without the addition of another ribosome binding site supported this hypothesis. Fragment L4 is thus derived from an independent translation of the carboxyl-terminal 41.2 kDa of fragment L3.

A series of deletions of the factor VIII sequence were constructed in plasmids pH1, pH2, and pL3 (Fig. 1*C*). Deletions in pL3 affected fragment L3 and sometimes fragment L4 (L3.4, L3.5, and L3.6). The resulting protein fragments were named according to the parental fragment with the exception of L4-derived fragments. Expression of the fragments was confirmed as shown in Fig. 2. A few fragments were detectable only on stained polyacrylamide gels (Fig. 2 *C* and *E*) because they did not contain the epitopes for the monoclonal antibodies used.

Deletion Mapping of Inhibitor Epitopes. Expression of factor VIII fragments in *E. coli* allowed us to localize factor VIII inhibitor epitopes by deletion mapping and to compare the epitope specificity of several inhibitor antibodies.

Nine inhibitors (NF, 1824, F, TB, CC, HA, PB, GK, and CH) were used to analyze deletions of fragments L3 and H2 by immunoblotting. Their reactivities are summarized in Table 1. All inhibitors bound to fragments L3, H2, or to both.

Fragment L3 was divided into three subfragments by the deletions that generated fragments L3.1, L3.2, and L3.3 (Fig. 1*C*). Inhibitors NF, 1824, F, CC, and HA, with specificity for the 72-kDa thrombin fragment, bound to fragments L3.1, L3.2, and L4 but not to L3.3 (Table 1). An immunoblot with inhibitor CC is shown in Fig. 3*C*. The region deleted from L3.3 was subdivided by the construction of deletion frag-

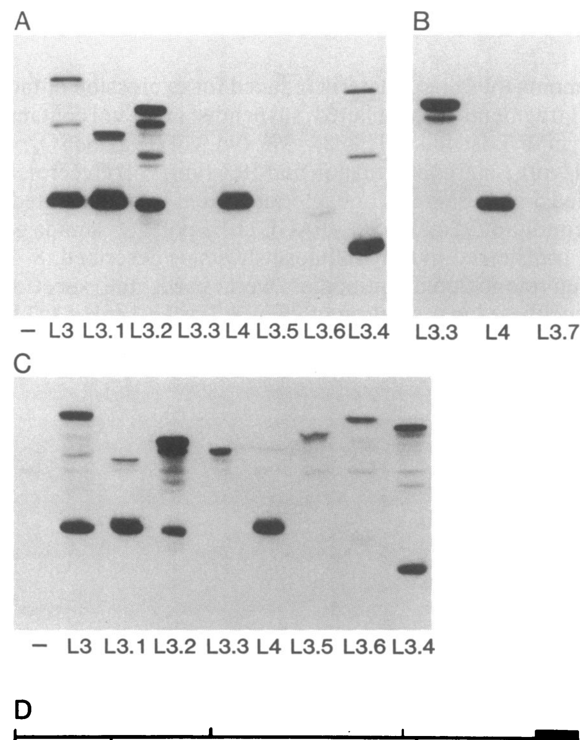

FIG. 3. Immunoblot analysis of the reactivity of factor VIII inhibitors with the L3 factor VIII fragment and its derivatives. Lanes —, extract from a similar strain containing no plasmid; other lane labels refer to protein fragments. (*A*) Inhibitor TB (Table 1) was tested. Eight micrograms of L3.3 and 40 μg of the other extracts were used. (*B*) Separate gel with lanes as in *A* with the addition of a lane containing L3.7 extract. (*C*) Inhibitor CC was tested; 40 μg of each extract was used. The L4-derived fragment from pL3.5 (Table 1) was too weak to detect by photography. (*D*) Map of factor VIII as in Fig. 1*A*. Solid box, inhibitor epitope(s).

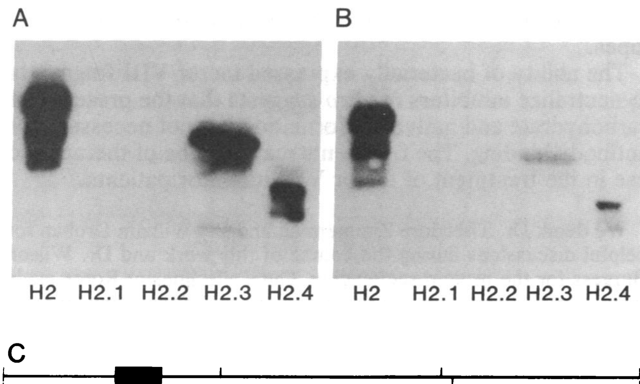

FIG. 4. Immunoblot analysis of the reactivity of factor VIII inhibitors with the H2 factor VIII fragment and its derivatives. Experimental conditions were as in Fig. 2. The amounts of extract used were adjusted to keep the amount of factor VIII fragment constant: H2, 40 μg; H2.1, 200 μg; H2.2, 300 μg; H2.3, 80 μg; H2.4, 40 μg. Lane labels refer to fragments. Inhibitors HA and PB (Table 2) were used in *A* and *B*, respectively. (*C*) Map of factor VIII as in Fig. 1*A*. Solid box, inhibitor epitope(s).

ments L3.4, L3.5, and L3.6 (Fig. 1*C*). Fragment L3.4 reacted strongly with all five inhibitors but there was a marked reduction in binding when they were tested with fragment L3.6 and a further reduction with fragment L3.5 (Fig. 3*C*).

In extracts containing fragments L3.5 and L3.6, the weakly reactive fragments migrated in the gel as expected for fragment L4, rather than L3, deletion derivatives. The L4 derivatives were present at high levels in total protein extracts (Fig. 2*C*); thus the faint bands in Fig. 3*C* arose from weak binding of the inhibitor to the fragments and not from low fragment concentration. In the same extracts (Fig. 3*C*, L3.5 and L3.6) the deletion derivatives of fragment L3 were not detectable in the immunoblots because their concentration in the extract was lower than that of the L4 derivatives (Fig. 2*B*, L3). The variability in response to fragment L3.5 may be due to differences in epitope specificity among inhibitors or to differences in inhibitor concentration in the immunoblotting. Reactions with L3.5 were weak at best.

The deletion analysis suggests the existence of multiple epitopes within the 72-kDa factor VIII thrombin fragment because elimination of all binding of the five inhibitors required deletion of two or three separate segments. The major epitope(s) (Fig. 3*D*) was still present in fragment L3.4 but it was deleted from fragments L3.6 and L3.5 (17.3 kDa). Removal of the major epitope(s) revealed weaker epitopes encoded by the factor VIII sequence between the deletion endpoints of plasmids pL3.6 and pL3.5 or between the endpoints of pL3.5 and pL3.3.

Inhibitor TB (Table 1 and Fig. 3 *A* and *B*) was distinct because it showed binding to fragment L3.3 and to the L3-derived fragments in L3.5 and L3.6 extracts. It resembled the other inhibitors in its weak binding to the L4-derived fragments present in the same extracts. Inhibitor TB also reacted weakly with deletion fragment L3.7 (Fig. 1*C*). Thus inhibitor TB recognized the major carboxyl-terminal epitope(s) identified above, weaker epitopes nearby and one or more additional epitopes on fragments L3.3 and L3.7.

Six inhibitors specific for the 44-kDa factor VIII thrombin fragment were tested for their reactivity with deletions of fragment H2 (Table 1). These inhibitors reacted with fragments H2.3 and H2.4 but not H2.1 and H2.2. Immunoblots of inhibitors HA and PB are shown in Fig. 4 *A* and *B*. For each inhibitor, the reaction with fragment H2 was greater than with the deletion fragments H2.3 and H2.4. However, the weaker binding of inhibitors to these deletion proteins did not indicate that epitopes were scattered throughout fragment H2. If there were other epitopes located nearer to the amino terminus, they should have been detected in immunoblots of fragments H2.1 and H2.2 but this was not the case. The epitope(s) on fragment H2 thus appeared to be confined to the amino-terminal 18.3 kDa (Fig. 4*C*).

Neutralization of Inhibitors by Factor VIII Fragments. We tested the ability of partially purified factor VIII fragments to neutralize inhibitors *in vitro* as a way of independently confirming the epitope mapping results by immunoblotting.

Factor VIII fragments in *E. coli* were produced as insoluble inclusion bodies that could be separated from 70 to 80% of the bacterial protein by differential centrifugation (16) (data not shown). Fragment L4 was chosen for neutralization of the 72-kDa-specific inhibitors since it carried the major carboxyl-terminal epitope(s) and some weaker epitopes. Fragment L3.3 (Fig. 1*B*) was used as a control because only inhibitor TB bound to it strongly. For similar reasons, fragment H1 was chosen for neutralization of the 44-kDa-specific inhibitors, and fragment H1.1 was used as the control. All inhibitors tested by immunoblotting and one additional inhibitor were assayed for neutralization by the factor VIII fragments. The results are summarized in Table 2.

Inhibitors WS, PB, CHA, and GK (specific for the 44-kDa factor VIII thrombin fragment) were neutralized by fragment H1 but not by H1.1 whereas the control inhibitor NF was not neutralized by either. Neutralization of 72-kDa-specific inhibitors NF and 1824 was achieved by fragment L4 but not by L3.3, whereas the control inhibitor WS was not neutralized

Table 2. Neutralization of inhibitors by factor VIII fragments

Inhibitor			Preincubation of inhibitor with factor VIII fragment								
Name	Type	Specificity	–	H1.1	H1	–	L3.3	L4	–	H1.1 + L3.3	H1 + L4
NF	Hemophilia A	72	<1	<1	<1	<1	<1	100			
1824	Hemophilia A	72				53	59	98			
F	Spontaneous	72				<1	<1	<1			
TB	Hemophilia A	44,72	<1	<1	<1	<1	<1	76	<1	<1	90
CC	Hemophilia A	44,72	<1	<1	49	<1	<1	18	<1	<1	90
HA	Spontaneous	44,72	<1	<1	<1	<1	<1	20	<1	ND	70
WS	Hemophilia A	44	9	9	91	<1	<1	8			
PB	Hemophilia A	44	24	17	100						
GK	Spontaneous	44	<1	<1	83						
CHA	Hemophilia A	44	32	44	100						

Percent residual factor VIII activity in the Bethesda assay (performed in duplicate) was converted to relative percent compared to a control (100%) without inhibitor or factor VIII fragment. Factor VIII activity without fragment preincubation (–) reflects how well that particular dilution of inhibitor inactivates factor VIII; therefore, this value varies slightly for each inhibitor. An increase in factor VIII activity indicates that neutralization of inhibitor by factor VIII fragments has occurred. Due to the insolubility of the fragments, results for fragments H1 and L4 in replicate experiments were variable. In some cases, neutralizations were repeated up to four times, and the best results are given. ND, not done; 44, 44-kDa fragment; 72, 72-kDa fragment.

by either. Inhibitor F was not neutralized in four independent experiments. Perhaps the epitope recognized by this inhibitor during immunoblotting (Table 1) is not involved in its neutralization. The important epitope may not be recognizable by immunoblotting or in the bacterially expressed fragments.

Results from inhibitors with specificity for both the 44-kDa and the 72-kDa fragments were variable. Inhibitor CC was partially neutralized by fragments H1 and L4 and more completely by a mixture of the two. Inhibitor HA was partially neutralized by fragment L4 but only weakly neutralized (data not shown) by fragment H1 in one of three attempts. Inhibitor TB was neutralized by fragment L4 but it could not be neutralized by fragments H1 or L3.3 to which it bound on immunoblots (Table 1). Since TB and HA are inhibitors specific for different regions of factor VIII in the same plasma sample, the results may reflect the presence of one strong inhibitor that masks the effects of the weaker one.

DISCUSSION

Human factor VIII inhibitors of alloantibody (hemophilia A) or autoantibody (spontaneous) origin bound to analogues of the 44-kDa and/or 72-kDa factor VIII thrombin cleavage fragments produced in *E. coli*. Deletion mapping of the fragments by immunoblotting with factor VIII inhibitor antibodies revealed two epitope-containing regions to which inhibitors bound strongly. These regions were located at the amino terminus of the 44-kDa thrombin fragment (18.3 kDa, amino acids 379–538) and at the carboxyl-terminus of the 72-kDa thrombin fragment (17.3 kDa, amino acids 2178–2332). Epitopes for the nine inhibitors tested were located in one or both of the two regions. Alloantibody and autoantibody inhibitors were similar in their epitope specificity. The epitope(s) on the 44-kDa fragment was in the A2 factor VIII domain and the major epitope(s) on the 72-kDa fragment was in the C2 domain. Since these domains are not homologous, the epitopes we have described are not likely to be similar in amino acid sequence.

Inhibitor reactivity with deletions of the 72-kDa fragment analogue revealed the presence of additional epitope(s). In six inhibitors, one or more weak epitopes were located upstream of the major epitope(s) within the carboxyl-terminal 32.2 kDa of factor VIII. One inhibitor with 72-kDa and 44-kDa specificity reacted with an epitope(s) as far away as the amino-terminal one-third of fragment L3. The existence of multiple inhibitor epitopes has already been suggested from competitive immunoradiometric assays (19–21).

Since the deletion maps were generated from denatured proteins, an inhibitor epitope may be a linear sequence of amino acids. However, the contribution of conformationally dependent determinants that may still exist under the conditions used cannot be ruled out by our data. In addition the epitope may not be a single binding site within the relatively large regions (17–18 kDa) defined.

In vitro neutralization of 10 inhibitors by factor VIII fragments showed that inhibitors could be neutralized only by those fragments to which they bound in immunoblotting assays. The epitope localization by immunoblotting was thus supported by the neutralization results. The two methods appear to have detected the major common inhibitor epitopes.

The ability of bacterially expressed factor VIII fragments to neutralize inhibitors *in vitro* suggests that the presence of carbohydrate and native conformation are not necessary for antibody binding. The fragments may thus be of therapeutic use in the treatment of factor VIII inhibitor patients.

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