Direct characterization of factor VIII in plasma: Detection of a mutation altering a thrombin cleavage site (arginine-372 → histidine)

(hemophilia A/immunoabsorbent/polymerase chain reaction)

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Communicated by K. M. Brinkhous, March 6, 1989

ABSTRACT An immunoabsorbent method has been developed for the direct analysis of normal and variant plasma factor VIII. Using this method, the molecular defect responsible for mild hemophilia A has been identified for a patient whose plasma factor VIII activity is 0.05 unit/ml, even though the factor VIII antigen content is 3.25 units/ml. Although the variant factor VIII has an apparently normal molecular mass and chain composition, the 92-kDa heavy chain accumulates when the variant protein is incubated with thrombin and the 44-kDa heavy chain fragment cannot be detected. In contrast, thrombin cleavage of the 80-kDa light chain to the 72-kDa fragment is normal. As these data indicate a loss of factor VIII cleavage by thrombin at arginine-372, the genetic defect was determined by polymerase-chain-reaction amplification of exon 8 of the factor VIII gene and direct sequencing of the amplified product. A single-base substitution (guanine → adenine) was identified that produces an arginine to histidine substitution at amino acid residue 372. These data identify the molecular basis of an abnormal factor VIII, “factor VIII-Kumamoto,” that lacks procoagulant function because of impaired thrombin activation.

Classic hemophilia, hemophilia A, is an X chromosome-linked disorder of blood coagulation caused by deficient factor VIII activity. Although reduced or absent factor VIII procoagulant activity is consistently identified, hemophilia A is a heterogeneous disorder when characterized immunologically using human antibodies to factor VIII (1-9) and when the specific molecular defects are identified (10-16).

Factor VIII, an essential cofactor for factor X activation by factor IXa, circulates in plasma as a large glycoprotein that is associated with von Willebrand factor in a noncovalent complex. It is modified by several proteolytic cleavages in a process that leads to activation and subsequent inactivation of the molecule (17-20). The mature single-chain protein of 2332 amino acids is proteolytically processed to generate amino-terminal heavy chain polypeptides of 92 to 200 kDa and a 80-kDa carboxyl-terminal light chain. Factor VIII procoagulant activity is generated by thrombin cleavage at arginine-740 to generate a 92-kDa species that is subsequently cleaved at arginine-372 to yield 54-kDa and 44-kDa chains. At the same time, cleavage of the 80-kDa light chain at arginine-1689 yields a 72-kDa fragment.

Almost all plasmas of patients with severe hemophilia A lack factor VIII protein by both procoagulant and immunologic assays (4-9). In some cases, these individuals have been found to have short deletions or point mutations leading to nonsense codons that appear to prevent factor VIII synthesis (10-12). In contrast, nonfunctional antibody-neutralizing factor VIII protein in levels comparable to normal plasma have been identified in 10% of plasmas from patients with mild or moderate hemophilia A (4). These plasmas are termed cross-reacting material positive (CRM∗). The remaining hemophilic plasmas are either CRM-reduced, in which case the reduction in immunologically detectable protein is comparable to the reduction in functional activity, or CRM-negative (CRM-) — i.e., there is no detectable factor VIII by sensitive immunosassays (1-9). Although the nature of the molecular defect has been established in several mild CRM-reduced individuals (14-16) and in one severe CRM∗ patient (13), the properties of their plasma factor VIII protein have not yet been characterized.

We report here a simple and sensitive method for the immunosol钓ation of factor VIII from plasma, the use of this method to identify the functional defect in the plasma of a CRM∗ hemophilia A patient, and the confirmation by gene analysis of the predicted mutation at arginine-372 that is responsible for the abnormal factor VIII.

MATERIALS AND METHODS

Materials. Tween 20 and Tween 80 were purchased from Sigma; bovine serum albumin was from J. T. Baker; and Sepharose CL-2B was from Pharmacia. Purified human thrombin (specific activity, 2377 units/mg of protein) was a gift from J. Fenton II (New York State Department of Health, Albany). Anti-factor VIII monoclonal antibody MAB038 was obtained from Chemicon and monoclonal antibody C8 was from J. R. Scientific. A monoclonal antibody (J16D-9) against a factor VIII synthetic peptide (amino acid residues 2318-2332) was a gift from C. A. Fulcher ( Scripps Clinic and Research Foundation, La Jolla, CA). 125I-labeled affinity-purified rabbit anti-mouse immunoglobulin was obtained from Amersham.

Patient Samples. CRM∗ hemophilia A was identified in patients ARC-1, -2, and -3 by assays carried out in the Department of Clinical Pathology of Tokyo Medical College (21), and extensive studies were carried out for patient ARC-1 (also designated JH-35 in the series of patients studied by the Pediatric Genetics Unit of the Johns Hopkins University School of Medicine). This 62-year-old man has moderately severe hemophilia A, first symptomatic at age 2, that requires factor VIII therapy for bleeding episodes two or three times each year. No family data are available. Studies were also carried out with plasma from patient DE in whom

Abbreviations: CRM, cross-reacting material; PCR, polymerase chain reaction.

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CRM⁺ hemophilia was first identified in 1968 (1) (Table 1, ARC-4). None of the patients had received any factor VIII concentrates within the 10 days prior to the collection of citrate-anticoagulated plasma (1). The procedure for isolation of genomic DNA from peripheral blood leukocytes has been described (10).

**Immunopurification of Factor VIII.** IgG was prepared from the plasma of a patient with a high-titer (3600 Bethesda units) factor VIII autoantibody by the caprylic acid method (22) and was coupled to cyanogen bromide-activated Sepharose CL-2B at a concentration of 4 mg/ml of settled gel (23). After washing, the immunobeads were suspended in 0.15 M NaCl/4 mM CaCl₂/20 mM Tris-HCl, pH 7.4 (TBS). Pooled normal plasma (4) or variant hemophilia A patient plasma were brought to 0.8 M NaCl and Tween 80 at 0.5 ml/dl, and 0.6–2.5 ml was incubated with 20 µl of immunobeads overnight at room temperature with gentle agitation. After centrifugation, the residual factor VIII antigen in the supernatant was determined (4). The beads were washed into a polypropylene column (0.8 × 4 cm; Bio-Rad) using 20 ml of 50 mM imidazole/40 mM CaCl₂/ethylene glycol (5 ml/dl)/Tween 80 (0.5 ml/dl), pH 6.4, and then washed with 5 ml of TBS. The beads were then transferred into 1.0-ml conical polystyrene tube, the wash buffer was removed by centrifugation, and factor VIII was eluted by adding 40 µl of 0.125 M Tris-HCl (pH 6.8) containing NaDodSO₄ (2 g/dl), glycerol (10 ml/dl), and bromophenol blue (5 mg/ml). After a 1-hr incubation at 37°C with gentle agitation, the tube was centrifuged and the eluted factor VIII was recovered in the supernatant.

**Factor VIII Measurements.** Factor VIII procoagulant activity was measured by a one-stage method using factor VIII-deficient plasma as substrate (24). Factor VIII antigen was measured by immunoradiometric assay using 125I-labeled Fab' prepared from the plasma of a patient with an alloantibody to factor VIII (4). von Willebrand factor antigen was measured by immunoradiometric assay using a rabbit antibody (25). Citrate-anticoagulated normal plasma, pooled from 10 donors, served as a standard (1 unit/ml) for factor VIII procoagulant activity, factor VIII antigen, and von Willebrand factor antigen measurements and was used as the control for immunopurification studies.

**Thrombin Treatment of Factor VIII.** Some aliquots of immunopurified factor VIII were treated with thrombin before the NaDodSO₄ elution step. The amount of factor VIII antigen immunoadsorbed on the beads was estimated by measuring the difference between the plasma and postadsorption supernatant fluid. Thrombin, diluted to 10 units/ml with TBS, was added to the beads in sufficient quantity to achieve the desired concentration (0.2–2 units of thrombin per unit of factor VIII antigen), and the mixture was incubated at room temperature for 0.5–60 min with gentle agitation before adding the NaDodSO₄ elution buffer.

**Immunoblotting of Factor VIII.** Eluted factor VIII (40 µl) was analyzed by discontinuous NaDodSO₄/PAGE using 1.5-mm-thick 5–12% polyacrylamide slab gels (26) and transferred to nitrocellulose sheets (Schleicher & Schuell) using a Bio-Rad Trans-Blot cell.

<table>
<thead>
<tr>
<th>Patient</th>
<th>VIII:C, units/ml</th>
<th>VIII:Ag, units/ml</th>
<th>vWF:Ag, units/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARC-1</td>
<td>0.05</td>
<td>3.25</td>
<td>2.58</td>
</tr>
<tr>
<td>ARC-2</td>
<td>0.05</td>
<td>1.38</td>
<td>1.15</td>
</tr>
<tr>
<td>ARC-3</td>
<td>0.37</td>
<td>1.06</td>
<td>2.22</td>
</tr>
<tr>
<td>ARC-4</td>
<td>0.05</td>
<td>0.57</td>
<td>0.71</td>
</tr>
<tr>
<td>Normal plasma</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
</tbody>
</table>

Table 1. Characterization of CRM⁺ hemophilic plasmas

After transfer, the nitrocellulose sheet was blocked by incubation at 37°C for 1 hr with bovine-serum albumin (3 g/dl) and Tween 20 (0.05 ml/dl). The sheet was then incubated with anti-factor VIII monoclonal antibodies (1 µg of IgG per ml) in PBS containing bovine serum albumin (1 g/dl) and Tween 20 (0.05 ml/dl) (dilution buffer). After overnight incubation at room temperature, the sheet was washed three times with PBS containing Tween 20 (0.05 ml/dl) (washing buffer) and then incubated with 125I-labeled affinity-purified sheep antihuman immunoglobulin in dilution buffer (80,000 cmp/ml). After a 4-hr incubation at room temperature, the sheet was again washed three times with washing buffer and dried, and radioactive bands were visualized by autoradiography at −70°C for 48–72 hr using Kodak X-1 film.

**Amplification of Genomic DNA and Sequencing.** Exon 8 and flanking regions of the factor VIII gene from genomic DNA of patient ARC-1 were amplified by the polymerase chain reaction (PCR) (27) using Thermus aquaticus (Tag) DNA polymerase (28) and two oligonucleotide PCR primers. Genomic DNA (500 ng) was added to 100 µl of buffer [10 mM Tris-HCl, pH 8.3/50 mM KCl/1.5 mM MgCl₂/gelatin (10 mg/ml)] that contained each PCR primer at 400 nM and each dNTP at 200 µM. Thirty cycles of PCR were carried out, each cycle consisting of a 30-sec denaturation at 94°C, a 30-sec reannealing at 55°C, and a 90-sec extension at 72°C, using a DNA thermal cycler (Perkin–Elmer/Cetus). Amplified DNA was desalted and excess dNTPs were removed by spin-dialysis on a Centricon 30 (Amicon). The purified DNA was sequenced directly by the method of Wong et al. (34). sp2 sequencing primer (10 ng), end-labeled with [γ-32P]ATP using T4 DNA kinase, was annealed with 80 ng of PCR product on ice after heat denaturing at 95°C for 5 min. The reaction mixture was divided into four tubes containing unlabeled dNTPs at 62 µM, deoxynucleotide triphosphates at 6.2 µM, and 2 units of T7 DNA polymerase (Sequenase, United States Biochemical) in the sequencing reaction buffer (25 mM Tris-HCl, pH 7.5/10 mM MgCl₂/70 mM NaCl/7 mM dithiothreitol). After incubation at 37°C for 15 min, the reaction was stopped with 95% (vol/vol) formamide/20 mM EDTA/bromophenol blue (50 mg/ml)/xylene cyanol FF (0.05 mg/dl), and boiled samples were electrophoresed in an 8% polyacrylamide/8 M urea gel at 58 W for ∼2.5 hr. Gels were fixed in 10% (vol/vol) methanol/90% acetic acid, dried, and exposed to Kodak XAR-5 film for 16 hr.

**RESULTS**

**Immunopurification of Factor VIII Protein.** Factor VIII was directly separated from plasma by immunoadsorption using a human antibody to factor VIII that had been coupled to agarose beads. An antibody that reacts with both heavy and light chain determinants was chosen for this purpose so that any partial or complete factor VIII protein with heavy or light chain determinants would be purified (29). The adsorbent removed 65–95% of factor VIII antigen in normal or CRM⁺ plasmas; <7% of the von Willebrand factor antigen was removed (30). Because the interaction of human factor VIII with these antibodies is very avid, usual elution techniques such as low pH and chaotrophic agents are only partially successful in separating the two components of the immune complex (31). For this reason, and because pH extremes have been shown to destroy factor VIII immunogenicity, NaDodSO₄ was used to separate the bound factor VIII from the immunoadsorbent. Characterization of the protein was then accomplished by NaDodSO₄/PAGE followed by immunoblotting using a monoclonal anti-factor VIII antibody. Preliminary experiments in which the nitrocellulose membranes were probed with the same or different human anti-factor VIII were unsatisfactory because of multiple additional im...
munologic reactions that were not related to factor VIII. It was not surprising that these were detected, since factor VIII inhibitor patients are multiply transfused with therapeutic products that have the potential for generating other antibodies to human plasma proteins. Three other high-titer (570-3600 Bethesda units) human anti-factor VIII and a murine monoclonal anti-factor VIII heavy chain have been successfully used as the immunoadsorbent when coupled to agarose beads.

Normal factor VIII eluted from the immunoadsorbent was characterized using three monoclonal antibodies (Figs. 1 and 2). MAB038 bound to the 80-kDa light chain fragment but did not detect the thrombin-cleaved 72-kDa light chain fragment (Fig. 2A); J16D-9 detected both 80-kDa and 72-kDa light chain fragments (Fig. 2B); and C8 detected (weakly) factor VIII heavy chain fragments as large as 200 kDa for native protein isolated from plasma and the 92-kDa and 44-kDa thrombin-cleaved heavy chain fragments (Fig. 2C) as well as a 17-kDa fragment that was only detected after longer incubation or when higher concentrations of thrombin were used. Variable strength nonspecific bands were seen at 150-170 kDa in each strip. They were reduced in intensity when normal human IgG was included with the 125I-labeled anti-mouse immunoglobulin and are presumed to be due to small amounts of the inhibitor IgG that eluted from the immunoadsorbent. When the immunoadsorbent beads were not incubated with plasma or when CRM+ hemophilic plasma was used, only weak nonspecific reactivity was observed.

The sensitivity of the method was established by incubating 20 µl of immunobeads with mixtures of normal plasma and CRM+ hemophilic plasma. Both heavy and light chain factor VIII determinants were detected when factor VIII was immunoadsorbed from 2-ml volumes that contained at least 0.5 ml of normal plasma. As factor VIII antigen removal by the immunobeads is the same (>65%) for volumes of 2-20 ml, it is likely that this method can characterize the factor VIII protein in 20 ml of CRM+ or CRM-reduced hemophilic plasmas that contain as little as 0.05 unit of factor VIII antigen per ml. Factor VIII adsorption was reduced by 40% when the total volume was increased to 40 ml.

Characterization of CRM+ Hemophilic Plasmas. To screen the factor VIII-like proteins in CRM+ plasmas, the three monoclonal antibodies were used together to characterize the immunoadsorbent eluates (Fig. 3). The factor VIII fragments (before and after thrombin treatment) of plasmas ARC-2, ARC-3, and ARC-4 could not be distinguished from those of normal plasma. In contrast, while the variant factor VIII immunoisolated from ARC-1 plasma had apparently normal fragments (Fig. 3, lane 3), the pattern after thrombin treatment was different. The 92-kDa band was more prominent than that for normal factor VIII and the 44-kDa fragment was not present (lane 4). Light chain cleavage was normal, however, with loss of the 80-kDa band and the appearance of the 72-kDa fragment. To characterize thrombin proteolysis of ARC-1 in more detail, samples were incubated with thrombin.
for 0.5–60 min and the immunoblots were compared to those for normal plasma (Fig. 4). Although formation of the 92-kDa heavy chain fragment was noted for ARC-1 after 0.5 min, the 44-kDa fragment was barely detectable, even at 20–60 min. In contrast, the 44-kDa heavy chain fragment could be detected after 0.5 min for normal factor VIII and the 92-kDa fragment did not accumulate. When the time-course study was carried out with a higher ratio of thrombin to factor VIII (2 units of thrombin per unit of immunoassayed factor VIII antigen), the rate of cleavage was increased, but the results for ARC-1 were otherwise unchanged. The 17-kDa fragment variably detected for ARC-1 after the 20-min incubation was prominent after a 5-min incubation for both ARC-1 and normal plasma when the higher ratio of thrombin was used.

The Molecular Defect in ARC-1. These data suggested that the abnormality of ARC-1 factor VIII is due to a change in its structure near arginine-372, the site of thrombin cleavage that produces 44-kDa and 54-kDa heavy chain fragments from the 92-kDa heavy chain (20, 31). Exon 8 of the factor VIII gene contains the codon for arginine-372, and it was amplified from ARC-1 genomic DNA using the PCR (27, 28) (Fig. 5). The DNA sequence showed a single nucleotide change, guanine → adenine, at the codon for arginine-372 (Fig. 6). This missense mutation causes a histidine substitution for arginine and thereby prevents thrombin cleavage at this site. These data identify the molecular basis of an abnormal factor VIII designated “factor VIII-Kumamoto” according to the residence of the propositus.

DISCUSSION

The cloning of the factor VIII gene has made molecular analysis of hemophilia A possible. The large number of deletions and point mutations characterized to date have provided important information about the variety and nature of mutations responsible for this coagulation disorder. However, the molecular defect has been established in only a fraction of hemophilic samples studied using standard probes and restriction enzymes (10–12). For this reason, Gitschier et al. (13) sought evidence of mutations in specific factor VIII regions by amplifying specific genomic DNA regions using the PCR. They examined regions that included the CpG sequence within codons for arginines at thrombin and activated protein C cleavage sites. The CpG dinucleotide sequences are candidates for cytosine to thymine transitions; they have been present in many of the hemophilic mutations that have been detected. Two mutations were identified when 215 DNAs were characterized. One, a nonsense mutation, was found at the activated protein C cleavage site (amino acid 336) in a patient with severe CRM- hemophilia. The other, a patient with CRM+ severe hemophilia, had a missense mutation at a thrombin activation site, arginine-1689 → cysteine.

While these and similar DNA-based approaches are the only way to characterize the molecular defects in CRM- hemophiliacs, we describe in this paper an alternative method for screening CRM-reduced and CRM+ plasmas. It can detect qualitative factor VIII changes in chain length as well as differences in susceptibility to proteolytic enzymes. This plasma immunopurification technique uses human anti-factor VIII that binds both heavy and light chain factor VIII determinants and thereby concentrates the protein from plasma. Subsequent immunoblotting with a mixture of monoclonal antibodies identifies factor VIII chains and proteolytic fragments. As it does not depend in any way on factor VIII procoagulant function and can characterize factor VIII protein when present in hemophilic plasmas at concentrations as low as 5% of those in normal plasma, it is applicable to all

![Fig. 4](image-url) Time course of thrombin proteolysis of factor VIII. Normal (NPP) and ARC-1 factor VIII fragments were detected using a mixture of the three monoclonal antibodies as described in Fig. 2, except that the thrombin incubation was terminated by adding NaDodSO4 elution buffer as noted. Immunoadsorbed normal factor VIII (1.74 units of factor VIII antigen per lane) was analyzed in lanes 1–5 and immunoadsorbed ARC-1 factor VIII (1.78 units of factor VIII antigen per lane) was analyzed in lanes 6–10.

![Fig. 6](image-url) Sequencing gel identifying the ARC-1 mutation. The normal factor VIII exon 8 sequence coding for amino acid residues 368–376 is shown on the left and that from ARC-1 is on the right. The guanine → adenine mutation is indicated by an asterisk.

![Fig. 5](image-url) Strategy of PCR amplification of exon 8 of the factor VIII gene and flanking regions. The 365-base-pair region was amplified using HEM55 (5' primer: 5'TCT TGG TAT AGA ACA GCC TA-3') and HEM54 (3' primer: 5'-AG AGA GTA CCA ATA GTC AAA-3'). The end-labeled primer sp2 (5'-GAA ATG GAT GTG GTC AGG-3') was used for nucleotide sequencing.
CRM$^+$ and most CRM-reduced plasmas. In concept, it is similar to the approach developed by Yoshioka et al. (32) for the screening and analysis of patients with hemophilia B variant proteins. In their procedure, the absorption from plasmas is carried out with barium chloride instead of an immunoadsorbent, but the subsequent steps follow the same pattern: protease treatment, NaDodSO$_4$/PAGE, and immunoblotting using a monoclonal antibody.

One of the first four CRM$^+$ samples studied by our technique, ARC-1, had an abnormal immunoblot pattern after incubation with thrombin even though the dysfunctional factor VIII in plasma had a normal pattern (Fig. 3). The 92-kDa heavy chain fragment was generated, but it was not cleaved to produce procoagulant activity (20, 33). This finding suggested that a mutation at or near the relevant thrombin cleavage site (arginine-372) is responsible for the molecular defect. This hypothesis was verified by PCR amplification of exon 8 and sequencing the amplified segment. The guanine → adenine mutation in the codon for arginine-372 leads to replacement of this amino acid with histidine so that this thrombin cleavage is prevented in ARC-1 factor VIII. The markedly reduced procoagulant activity is consistent with data of Pittman and Kaufman (33), for a factor VIII variant in which arginine-372 was converted to isoleucine by site-directed mutagenesis. Pittman and Kaufman (33) also characterized other altered forms of factor VIII, and they determined that conversions of arginine residues to isoleucine residues at positions 740, 1648, and 1721 resulted in cleavage resistance at the modified sites, but they did not diminish factor VIII procoagulant activity. Similar modifications at positions 372 or 1689 resulted in inactive factor VIII molecules that were not cleaved to thrombin (33).

The molecular defect that we have identified for patient ARC-1, and data for one other CRM$^+$ and two CRM-reduced hemophiliacs (13–15) are, to the best of our knowledge at the present time, the only instances in which the genetic defect has been correlated with a structural change that has reduced or abolished procoagulant function. The immunopurification method can be used to screen additional CRM-reduced and CRM$^+$ plasmas to identify additional instances in which mutations have caused functional defects. These are likely to include losses of cleavage sites, as in ARC-1 and one other hemophilic patient (13). They may also be due to changes in factor VIII interaction with factor IXa, factor X, phospholipid, or von Willebrand factor. Such data should further clarify the structure-function relationships that are critical for factor VIII procoagulant activity.

We thank Carol Fulcher for providing monoclonal antibody J16D-9, John Fenton for providing purified human thrombin, physicians in the Department of Clinical Pathology (Tokyo Medical College) for obtaining patient samples used in this study, and Debbie Wilder for manuscript preparation. This work was supported in part by U.S. Public Health Service Grants HL36099 and RR05737.