Inhibitors in German Hemophilia A Patients Treated with a Double Virus Inactivated Factor VIII Concentrate Bind to the C2 Domain of FVIII Light Chain


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Summary

To reduce the risk of transmission of hepatitis A virus, an Octapharma produced factor VIII (FVIII) concentrate treated with solvent detergent (FVIII-SD) was further pasteurized after purification. This product, Octavi SDPlus (FVIII-SDP), was marketed in Europe in 1993 to 1995. Inhibitors appeared from September to October, 1995, in 12 of 109 previously treated German hemophilia A patients. A study of similarly treated Belgian patients, who also developed inhibitors, had shown antibodies to the FVIII light chain (domains A3-C1-C2) only. In the present study, the epitope specificity of 8 German inhibitor plasmas was also found to be restricted to the light chain. In radioimmunoprecipitation assays to localize the light chain epitope(s), antibody binding to heavy chain (domains A1-A2-B) was 11-148 fold lower than to the C2 domain, and binding to recombinant A3-C1 was barely detectable. These results were supported by >95% neutralization of a high responder inhibitor titer by the C2 domain.

Introduction

The development of antibodies (inhibitors) that inactivate blood coagulation factor VIII (FVIII) and impair FVIII therapy is a serious complication in the treatment of hemophilia A. In one study, 17 of 71 (23.9%) of previously untreated severe hemophiliacs that received only recombinant FVIII developed an inhibitor. All of the 17 patients who developed inhibitors did so in ≤50 exposure days (1). The association between a particular FVIII concentrate and development of inhibitors was first reported after the introduction of a plasma derived FVIII product purified by controlled-pore silica adsorption and pasteurized (60°C, 10 h) for viral inactivation, FVIII CPS-P in The Netherlands (2) and FVIII P in Belgium (3). This inhibitor outbreak occurred in 19 hemophiliacs of whom 15 had ≥200 exposure days to other FVIII products without developing an inhibitor. Fifteen of 17 inhibitors in the FVIII CPS-P treated patients arose after >50 exposure days (mean 157), which is also unusual. These inhibitors disappeared in all but one case after the patients were switched to other FVIII concentrates, demonstrating the direct role of FVIII CPS-P in the immune response (4).

A possible link between outbreaks of hepatitis A in hemophilia A patients who received solvent detergent treated FVIII in Europe was suggested (5). To address this problem, a new double virus inactivated FVIII concentrate (Bisinact in Belgium and Octavi SDPlus elsewhere in Europe) purified by ion exchange chromatography and treated by solvent-detergent and pasteurization (63°C, 10 h) was introduced in 1995. Both concentrates were produced by the same manufacturing process from cryoprecipitate of German or Belgian plasma. Similarly to FVIII CPS-P, FVIII-SDP induced an inhibitor outbreak in multitransfused hemophiliacs, and the inhibitors disappeared after patients were switched to other FVIII products (6).

The FVIII protein consists of a series of homologous domains arranged in the order A1-A2-B-A3-C1-C2 (7), and it circulates as a heterodimer of a heavy chain (A1-A2-B) and a light chain (A3-C1-C2). The epitope specificity of the FVIII CPS-P induced anti FVIII antibodies was restricted to the C2 domain in radioimmunoprecipitation assays (8). These properties were seen in only 1 of 34 hemophiliacs who developed inhibitors early in their treatment with other FVIII products, but they were common in individuals with autoimmune anti FVIII antibodies (10/21) (9).

A more recent study of Belgian inhibitors that appeared after treatment with FVIII-SDP demonstrated that the anti FVIII antibodies bound mainly to the light chain (6). We have tested a similar group of FVIII-SDP induced German inhibitor plasmas to further determine epitope specificity within the light chain and reactivity with a range of other FVIII concentrates. The predominance of anti C2 antibodies among the FVIII CPS-P induced inhibitors suggested that the FVIII-SDP inhibitors, which are light chain specific (6), may also be primarily C2 specific, and we have tested this hypothesis in our study.

Materials and Methods

Factor VIII concentrates, von Willebrand factor, and antibodies. The source and properties of each FVIII concentrate are summarized in Table 1. Recombinate is the only FVIII purified to homogeneity, and it was provided without albumin by Baxter/Healthcare, Glendale, CA, USA. The AP9 anti FVIII antibodies were raised against A3 domain synthetic peptide 1797-1815 in rabbits, and the IgG was purified by affinity chromatography on Sepharose coupled to the peptide. Anti A3 monoclonal antibody (MoAb) CLB-CaG A was generously provided by Dr. Jan van Mourik, Netherlands Red Cross.

FVIII and von Willebrand factor assays. FVIII activity (IU/ml) was measured by the chromogenic assay Coatest FVIII (Chromogenix, Mölndal, Sweden) using the 3rd international FVIII standard (1993), von Willebrand factor (vWF) concentration (IU/ml) was determined by enzyme linked immunosorbent assay (ELISA) (10) and by ristocetin cofactor activity (11) (European Pharmacopeia). Inhibitor titers were measured by the Bethesda assay (12).

Activation of factor VIII by α-thrombin. FVIII-SD, Bisinact, Octavi SDPlus, or Recombinate were reconstituted in water to a concentration of 200 IU FVIII/ml. Purified bovine α-thrombin (108 U/mg; Sigma, St. Louis, MO, USA) was added to a final ratio of 0.008 IU/1 IU FVIII for 30 min at 25°C. The reaction was stopped by adding 1/4 volume reducing buffer (250 mM Tris-HCl, pH 6.8,
8% SDS, 200 mM dithioerythritol, 40% glycerol) and heating at 95 °C for 4 min.

**Immunoblotting.** Methods for these procedures were previously described (6). Samples of FVIII concentrates containing 2.7 IU FVIII per lane, with or without thrombin activation as above, were analyzed by SDS 8% polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. After electrophoretic transfer of the proteins to nitrocellulose, the membrane was incubated overnight at room temperature with 5 μg/ml AP9 rabbit anti FVIII antibodies or a 1/5 dilution of inhibitor plasma. Antibody binding was detected with 0.6 μg/ml biotinylated MoAb to rabbit IgG (Sigma) or 0.4 μg/ml biotinylated goat anti human IgG (Sigma) followed by 0.4 mg/ml streptavidin-peroxidase conjugate (Sigma). Bound peroxidase was measured by absorbance at 410 nm. Protein purification and radiolabeling. These methods were described in detail (17). Molar concentrations of polypeptides were calculated using the molecular weight derived from the translated cDNA sequence (18). Purity of all proteins was >95% by SDS-PAGE and Coomassie blue or silver staining. A3-C1 domain (0.75 nM final concentration). Immune complexes were bound to Protein G Sepharose (Pharmacia Biotech, Uppsala, Sweden), followed by centrifugation and 3 washes. The total and Protein G bound radioactivity were measured in a gamma counter. The mean of 4 controls without antibody was subtracted from values for test samples. Immunoprecipitation units/ml (IPU/ml) were calculated as follows: (average bound-total 125I-FVIII polypeptide minus background × plasma dilution). Undiluted inhibitor plasma (20 μl) was added to 100 μl 35S-A3-C1 in growth medium, immunoprecipitation was performed, and detection of bound protein was by SDS-PAGE and autoradiography (16).

**Expression of FVIII fragments.** Construction of expression vectors for the A2 (13) and C2 (14) domain cDNAs and recombinant polypeptide production in Sf9 insect cells (9) were described (14, 15). A construct encoding the A3-C1 domain (0.75 nM final concentration). Immune complexes were bound to Protein G Sepharose (Pharmacia Biotech, Uppsala, Sweden), followed by centrifugation and 3 washes. The total and Protein G bound radioactivity were measured in a gamma counter. The mean of 4 controls without antibody was subtracted from values for test samples. Immunoprecipitation units/ml (IPU/ml) were calculated as follows: (average bound-total 125I-FVIII polypeptide minus background × plasma dilution). Undiluted inhibitor plasma (20 μl) was added to 100 μl 35S-A3-C1 in growth medium, immunoprecipitation was performed, and detection of bound protein was by SDS-PAGE and autoradiography (16).

**Immunoprecipitation assays.** Details were described previously (17). Duplicate dilutions of inhibitor plasmas were mixed with 125I-heavy chain or 125I-C2 domain (0.75 nM final concentration). Immune complexes were bound to Protein G Sepharose (Pharmacia Biotech, Uppsala, Sweden), followed by centrifugation and 3 washes. The total and Protein G bound radioactivity were measured in a gamma counter. The mean of 4 controls without antibody was subtracted from values for test samples. Immunoprecipitation units/ml (IPU/ml) were calculated as follows: (average bound-total 125I-FVIII polypeptide minus background × plasma dilution). Undiluted inhibitor plasma (20 μl) was added to 100 μl 35S-A3-C1 in growth medium, immunoprecipitation was performed, and detection of bound protein was by SDS-PAGE and autoradiography (16).
Laub et al.: C2 Domain Restricted Anti Factor VIII Antibodies
could not be distinguished in efficacy of treatment or in inductor induc-
tion, the term FVIII-SDP (solvent-detergent pasteurized) will be used for
simplicity, and the earlier unpasteurized product will be referred to
as FVIII-SD. FVIII-SDP was treated with solvent-detergent followed
by ion exchange chromatography and heat pasteurization at 63 °C for
10 h in the presence of a high concentration of stabilizers. Inhibitors to
FVIII-SDP appeared after 84-300 exposure days, but they disappeared
and FVIII recovery was normalized when FVIII-SDP was replaced by
other FVIII concentrates.

Patient B1, a severe hemophilia A patient, was treated exclusively
with FVIII-THP in a Brussels Hemophilia Center (Hôpital Brugmann),
and he developed a high titer inhibitor in 144 exposure days. Patient B1
plasma was used as a control, as the inhibitor developed under condi-
tions usually seen for previously untreated patients (PUPs).

Epitope specificity of patient anti FVIII antibodies by immunoblot-
ning. Plasma from the patients with FVIII-SDP induced inhibitors was
tested for epitope specificity after SDS-PAGE of a single batch of
FVIII-SD and immunoblotting. In Figs. 1A and 1B, specific immuno-
reactive bands are also FVIII specific. As it would be difficult to distinguish
that the binding was specific for the light chain as well. As Recombi-

The epitope specificity of the inhibitors induced by FVIII-SDP was
compared for FVIII concentrates Recombinate, FVIII-SD, FVIII-THP,
Haemoctin, and FVIII-SDP. In Fig. 3, lanes 1-4, the 80 kDa light chain
was recognized in all the concentrates by immunobLOTS with a plasma
pool from three of the German patients. No additional immunospecific-
ity was observed when two batches of FVIII-SDP associated with in-
hibitor induction were used (not shown). Inhibitor reactivity toward
Recombinant 80 kDa and 72 kDa light chain fragments as well as the 44 kDa A2 domain heavy chain fragment. This specificity of the B1 antibodies is more similar to
that seen in severe hemophilic PUPs treated with various other FVIII
concentrates (9), and it is different from that of the antibodies elicited
by FVIII-SDP.

A sample from a plasma pool of 3200 normal donors was used as a
specificity control, as protein bands other than FVIII were apparent
in the immunoblots. No binding of the pool to the FVIII-SDP 80 or
72 kDa fragments was detectable (Fig. 2, lane 1), which confirmed the
specificity of the inhibitor plasmas for these fragments (Fig. 1). Four
polypeptides of 220, 170, 130, and 100 kDa and several of 50 to 55 kDa
were also detected. Binding of control anti vWF MoAb QED 10103
demonstrated that ≥3 polypeptides ≥100 kDa comigrated with vWF spe-
cific polypeptides present in FVIII-SD with (+) or without (-) thrombin
cleavage (Fig. 2, lanes 2, 3). This is consistent with the fact that vWF
makes up 40% of the total protein of FVIII-SD. Several bands in FVIII-
SD stained with Coomassie blue (lane 4) and some bands in the inhibi-
tor immunobLOTS comigrated with the vWF polypeptides. These results
suggest that both normal individuals and the hemophiliacs in this study
have low levels of anti vWF antibodies. The variability among the pa-
tients in their detection of the proposed vWF bands (Figs. 1A and 1B)
may indicate a corresponding variability in the specificity of their anti-
bodies for the different vWF fragments. We did not detect any modifi-
cation in vWF activity, vWF antigen, or multimer composition (results
not shown).

Inhibitor immunoreactivity towards different FVIII concentrates.
The epitope specificity of the inhibitors induced by FVIII-SDP was
expected 72 kDa fragment lacking the amino-terminal light chain poly-
peptide, amino acids 1649-1689. The 80 kDa and 72 kDa fragments
were identified by binding of AP9 (Figs. 1A, 1B, lane AP9), a rabbit
antibody specific for peptide 1797-1815 of the A3 domain. Identical re-
results were obtained when FVIII-SDP was used (data not shown).

No antibody binding to the 92 kDa heavy chain or its thrombin
clavage fragments of 44 kDa (A2 domain) or 54 kDa fragment (A1 do-
main) was detected. The bands migrating above the 92 kDa marker are
not likely to be FVIII related as their position did not change after
thrombin cleavage. This was verified by the AP9 control of rabbit anti
human FVIII light chain in which no bands larger than 80 kDa or small-
er than 72 kDa were observed. G8 plasma drawn five months after the
inhibitor episode did not bind to 80 kDa light chain, demonstrating the
disappearance of these antibodies (Fig. 1B, lane G8*). The immuno-
blotting results demonstrate that the anti FVIII antibodies of patients
G1-G8 bind only to the uncleaved or thrombin cleaved light chains of
both FVIII-SD and FVIII-SDP. A new antibody epitope was thus prob-
ably not induced by the heat pasteurization.

The anti FVIII antibodies of PUP B1 (Fig. 1B, lanes 8, 9) appeared
after treatment by FVIII-THP, and they recognized the 80 kDa and
72 kDa light chain fragments as well as the 44 kDa A2 domain heavy chain fragment. This specificity of the B1 antibodies is more similar to
that seen in severe hemophilic PUPs treated with various other FVIII
concentrates (9), and it is different from that of the antibodies elicited
by FVIII-SDP.

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A control without inhibitor plasma is shown in lane 7. The visible bands in this lane are probably due to the presence of the IgG (<0.1 mg) in the concentrate and its detection by the goat anti human IgG used for detection. The patient bands in this region were probably stronger due to the additional binding to vWf fragments of this size (Fig. 2).

**Determination of C2 domain specificity of the anti-fVIII antibodies by immunoprecipitation and inhibitor neutralization assays.** We performed immunoprecipitation assays with highly purified, radiolaabeled fragments of plasma derived fVIII to further define the domain specificity of the anti fVIII antibodies. Representative dose response binding curves to heavy chain and C2 domain by G2 and G8 plasmas are shown in Fig. 4, and IP units/ml were calculated (Materials and Methods) from the linear portion of such curves for plasmas G1-G4 and G8 (Table 3). Insufficient plasma was available to test the others. The IP units/ml for heavy chain were 11 to 148-fold lower than those for C2. As the immunoblots (Figs. 1-3) contained a number of bands >80 kDa that were stained, the IP results more clearly confirm that anti heavy chain antibody titers are very low or negative. Nonquantitative IP assays of antibody binding to the \(^{35}\text{S}-\text{A3-C1}\) portion of the light chain (Fig. 5) indicated the barely detectable binding by the inhibitor plasmas (1:5 dilution) compared to anti A3 MoAb CLB-CAg A (19), which is present at saturating concentrations (lane 1), confirming that the antibodies bound primarily to the C2 domain of the light chain.

Inhibitor neutralization by purified light chain and the A2 and C2 domains was performed. Increasing concentrations of C2 and light chain both led to complete neutralization of the inhibitor titer (Fig. 6). As equimolar concentrations of each were required for neutralization, the antibodies appear to bind to each with similar affinity, and binding is restricted to the C2 domain as also demonstrated by their minimal binding to A3-C1 (Fig. 5). The A2 domain did not neutralize the inhibitors at concentrations up to 50 nM.

**Discussion**

The development of anti fVIII antibodies is a serious complication in the treatment of hemophilia A patients with fVIII concentrates. Patients with severe hemophilia are most likely to develop inhibitors as they are not tolerant to fVIII, but only about 25% of them have such antibodies during the course of their treatment (20). The absence of fVIII and some gene mutations such as the intron 22 inversion (21) are important factors for the pathogenesis of inhibitor formation. A weaker association is the HLA class II genotype (22). Our results emphasize in addition the potential effect of changes in the biochemical and structural integrity of the fVIII molecule in inducing inhibitors in hemophiliacs (PTP) that were previously tolerant to fVIII infusion.

From December 1994 to September 1995, 12 multitransfused patients in Germany and 8 patients in Belgium developed inhibitors after treatment with new fVIII concentrates, Octavi SDPlus in Germany and Bisinact in Belgium, which we refer to as FVIII-SDP. The production process of FVIII-SDP included two viral inactivation steps: solvent-detergent treatment and pasteurization at 63° C for 10 h. A similar outbreak of product related inhibitors was detected in 1990 to 1991 in pre-

![Fig. 4](image)

Anti-fVIII antibody binding to heavy chain and C2 domain in radioimmunoprecipitation assays. Details are described in Materials and Methods. Serial two-fold dilutions of patient G2 and G8 plasmas were tested for binding to the C2 domain (■) and the heavy chain (○). The plasma volume per total volume of 60 µl is shown on the x axis.

![Fig. 5](image)

Inhibitor antibody binding to A3-C1 by immunoprecipitation. Details are described in Materials and Methods. Analysis was by SDS-10% PAGE and autoradiography. Lanes 1-8 are anti A3 MoAb CLB-CAg A, G1, G3, G4, G5, G6, G7, and G8, respectively.

![Fig. 6](image)

Inhibitor neutralization assay of G8 plasma by fVIII polypeptides. Increasing concentrations of light chain (■), C2 domain (○), and A2 domain (○) were added to the same dilution of G8 plasma, and the inhibitor titers and % neutralization were determined as described in Methods. The A2 domain at 50 nM also had no effect (not shown).

**Table 3** Summary of inhibitor plasma characterization

<table>
<thead>
<tr>
<th>Patient Number</th>
<th>Bethesda units/ml</th>
<th>Immunoprecipitation units/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Heavy chain</td>
<td>C2</td>
</tr>
<tr>
<td>G1</td>
<td>2.1</td>
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</tr>
<tr>
<td>G2</td>
<td>4.0</td>
<td>3.4</td>
</tr>
<tr>
<td>G3</td>
<td>3.4</td>
<td>36.0</td>
</tr>
<tr>
<td>G4</td>
<td>2.9</td>
<td>5.0</td>
</tr>
<tr>
<td>G8</td>
<td>211</td>
<td>33.0</td>
</tr>
</tbody>
</table>

IP units/ml were calculated as described in Materials and Methods.

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viously treated hemophiliacs who received fVIII CPS-P, another concentration that was heat pasteurized (60°C for 10 h). The exact reason why these concentrates became immunogenic is unknown and still under investigation.

In the present study we demonstrated that the inhibitors induced by fVIII-SDP in German hemophilic patients had an epitope specificity restricted to the 72 kDa thrombin cleaved light chain in immunoblotting assays, as did the inhibitors from the previously tested Belgian patients (6). In the only German patient with a high inhibitor titer, inhibitor neutralization assays demonstrated that these antibodies were directed solely against the C2 domain of the fVIII light chain. The other 4 patients could not be similarly analyzed due to their lower inhibitor titers, but it was evident from radioimmunoprecipitation assays of all 5 patients with the fVIII heavy chain, the C2 domain, and the A3-C1 domains that ≥90% of all anti-fVIII antibodies bound to C2. In contrast, patient B1, a PUP who developed inhibitors against fVIII-THP which had only a solvent-detergent viral inactivation step, had both anti heavy chain and anti light chain antibodies, which is common for hemophilic inhibitors (9).

As the C2 domain restricted epitope specificity was identical to those of the fVIII CPS-P induced inhibitors (8), it was possible that heat pasteurization was the sole cause of the inhibitor outbreak in both cases. However, as only some lots of fVIII-SDP were associated with inhibitor formation, this is unlikely. In addition, no other pasteurized fVIII concentrates used for treatment of hemophilia were associated with inhibitor formation in PTP. The inhibitors induced by fVIII-SDP recognized several different fVIII concentrates with similar specificity, suggesting that fVIII-SDP immunogenicity, similarly to that of fVIII CPS-P, was not due to the appearance of novel antibody binding sites (8).

Recent unpublished results obtained in our laboratory suggested an alteration of biological properties of the fVIII-SDP light chain such that thrombin activation occurred more slowly but factor Xa generation and phospholipid binding were enhanced (23). Additional data in one study suggested the association of inhibitor development with the presence of a proteolytic 40 kDa fVIII-SDP fragment generated prior to pasteurization (24). In another study this fragment was present in a batch of fVIII-SDP strongly associated with the appearance of inhibitors (25). As the 40 kDa fragment was also found in other batches, a definitive conclusion for the role of this fragment in the novel immunogenicity of fVIII-SDP was not possible.

Although a neonatal mouse tolerance induction model was previously used to determine if B domain deleted fVIII was immunologically different from intact fVIII, this could not be demonstrated (26). It is also not clear whether this model is capable of detecting the increased immune responsiveness of partially altered molecules such as fVIII-SDP. The more recently developed hemophilic mice with a knockout of the fVIII gene (27) may prove to be a more useful immunogenicity model. For the present, no in vitro or animal model can be used to assure the immunological safety of a new fVIII concentrate. The inhibitor induction by fVIII-SDP and fVIII CPS-P stresses the need for future investigations in this direction and clinical trials in hemophilic PTP without inhibitors when a new fVIII concentrate is to be evaluated.

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