FVIII Inhibitor IgG Subclass and FVIII Polypeptide Specificity Determined by Immunoblotting

By Carol A. Fulcher, Sytske de Graaf Mahoney, and Theodore S. Zimmerman

We used immunoblotting of purified factor VIII coagulant protein (FVIII) to localize FVIII inhibitor epitopes in 76 inhibitor plasmas to either the 92-kd FVIII polypeptide (and its 54-kd and/or 44-kd thrombin fragments), the 80-kd polypeptide (and its 72-kd thrombin fragment), or both of these polypeptides. We also used immunoblotting to examine the immunoglobulin class and subclass content of 12 inhibitors with monoclonal antibodies specific for human IgG subclasses and IgM. Seven hemophilic (alloantibody) and five spontaneous (autoantibody) inhibitors contained IgG-1 and IgG-4 antibody; one of the spontaneous inhibitors also contained IgG-3. In one hemophilic inhibitor, the IgG-4 component reacted strongly with the 92-kd and 80-kd polypeptides, whereas the IgG-1 component reacted only minimally with the 92-kd polypeptide although its reactivity with the 80-kd polypeptide was strong. Another hemophilic inhibitor was affinity purified and subjected to quantitative radial immunodiffusion, and the presence of IgG-1 and IgG-4 antibody was confirmed. We conclude that the inhibitors examined are not monoclonal but are probably of restricted polyclonal origin and that different IgG subclasses in an inhibitor plasma can have different degrees of FVIII polypeptide reactivity.

MATERIALS AND METHODS

FVIII purification and digestion with thrombin. FVIII was purified from commercial FVIII concentrate by immunoabsorbent chromatography and digested with thrombin as previously described.

Inhibitor assay. Inhibitory activity of FVIII coagulant protein by inhibitor antibodies was measured in Bethesda units (BU) by the method of Kasper and co-workers.

Electrophoresis. Samples of purified FVIII were dialyzed against sample buffer containing 0.01 mol/L of sodium phosphate, 1% NaDodSO4 and 10 mmol/L of disodium EDTA, pH 7.0, overnight at room temperature. Discontinuous NaDodSO4 7.5% polyacrylamide slab gel electrophoresis was performed as previously described. The dialyzed FVIII was applied to the gel at ~4 µg per lane, without prior heating or reduction.

Immunoblotting. Separated FVIII fragments were electrophoretically transferred onto nitrocellulose membranes as previously described, except that 0.1% NaDodSO4 was added to the transfer buffer.

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Fig 1. Line diagram of a single-chain FVIII molecule with enzyme cleavage sites indicated above and below. Top: Purified FVIII is isolated as a series of polypeptides of 80 kd, 92 kd, and multiple higher mol wt polypeptides representing the amino terminal 92-kd portion with various carboxyl terminal cleavages into the midsection of the molecule. Bottom: Thrombin produces the 92-kd polypeptide and further cleaves it to 54 kd and 44 kd. Thrombin also produces the 72-kd fragment from its 80-kd precursor. The enzyme giving rise to the 80-kd polypeptide has not been identified. These data are from published reports of FVIII gene cloning and protein biochemistry.125

Processing of immunoblots. Nitrocellulose membranes containing transferred FVIII fragments were incubated for 6 hours in 100 mL of Blotto buffer [phosphate-buffered saline (PBS) containing 5% nonfat dry milk] prepared according to Johnson and colleagues. All incubations were done on a rotary shaker at room temperature. The membranes were then incubated overnight in 100 mL of Blotto buffer containing plasma samples (up to 2 mL, depending on titer) and protease inhibitors, both prepared as previously described. After incubation with plasma samples, the membranes were washed in 100 mL of PBS containing 0.05% Nonidet P-40 for 30 minutes, with changes of buffer at 10 and 20 minutes. They were subsequently rinsed in Blotto buffer and then incubated for 2 hours in 100 mL of Blotto buffer with 10 µL of ascites fluid containing monoclonal anti-human IgG-1, IgG-2, IgG-3, IgG-4, anti-κ light chains, anti-λ light chains, or anti-IgM antibodies (ICN Immunobiologicals, Lisle, IL). The monospecificity of these monoclonal anti-human immunoglobulin class and subclass antibodies has been determined by the laboratories of Miles-Yeda, Rehovot, Israel, using an enzyme-linked immunosorbent assay (ELISA) assay with freshly prepared antigens. Membranes were then washed for 30 minutes, as above, in Blotto buffer.

The FVIII/inhibitor antibody/monoclonal anti-immunoglobulin antibody complexes were then visualized by incubation of the membranes for at least 30 minutes in 100 mL of Blotto buffer containing 125I-labeled, affinity-purified rabbit anti-mouse immunoglobulin antibody, followed by washing in Blotto buffer as above. After membranes were briefly rinsed in PBS, autoradiography was performed at -70°C as previously described.14 Film exposure times are indicated in Figs 1 through 3.

Negative controls for the immunoblots consisted of electrophoresing 2.5 µg of bovine serum albumin (BSA) in a lane adjacent to FVIII. Positive controls that demonstrated the reactivity of the monoclonal anti-human immunoglobulin antibodies with human immunoglobulins under these conditions consisted of substituting affinity-purified goat anti-human IgG, IgA, and IgM antibody for purified FVIII, as well as substituting normal human plasma for inhibitor plasma. Thus, immobilized goat anti-human immunoglobulin antibody was used to trap normal human immunoglobulins. This procedure was necessary because some of the monoclonal anti-human immunoglobulin antibodies did not react well with human immunoglobulins that were immobilized directly on nitrocellulose.

Affinity purification of inhibitor antibody. Inhibitor antibody was purified directly from plasma by passage over a column of FVIII-Sepharose. The column was prepared by coupling 1 mg (2,000 U) of partially purified FVIII to ~1 mL of cyanogen bromide-activated Sepharose 4B (Pharmacia, Piscataway, NJ) according to the manufacturer’s instructions. A 0.5-mL sample of inhibitor plasma was diluted to 4 mL in 0.02 mol/L of imidazole, 0.15 mol/L of sodium chloride, pH 7.0, containing protease inhibi-
**RESULTS**

**FVIII polypeptide specificity of FVIII inhibitors as determined by immunoblotting.** In a previous report, we used immunoblotting to localize FVIII inhibitor epitopes in 25 FVIII inhibitor patients to either the 72-kd thrombin fragment of FVIII or the 44-kd thrombin fragment or both of these fragments. These two fragments are derived from the 80-kd and 92-kd precursor polypeptides, respectively (Fig 1). We extended our study to include 76 hemophilic and spontaneous FVIII inhibitor patients, and the results are presented in Table 1. In all but two patients, the FVIII inhibitor epitopes were localized to either or both the 72-kd and 44-kd thrombin fragments. The two exceptions were hemophilic inhibitors with epitopes on both the 54-kd and 44-kd thrombin fragments. These results extend the preliminary FVIII inhibitor epitope map to include all fragments believed to be important to FVIII procoagulant activity. Most FVIII inhibitor epitopes, however, were restricted to the 72-kd and 44-kd thrombin fragments.

Due to the smaller number of spontaneous FVIII inhibitor samples tested, we cannot yet draw conclusions about possible differences in the distribution of FVIII fragment specificities between spontaneous and hemophilic FVIII inhibitors. Also, as indicated in Table 1, some immunoblots gave inconclusive data due to very weakly reactive inhibitors. This

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Table 1. Immunoblotting Analysis of FVIII Polypeptide Specificity of 54 Hemophilic and 22 Spontaneous FVIII Inhibitors

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>72 kd</th>
<th>44 kd</th>
<th>72 kd and 44 kd</th>
<th>54 kd and 44 kd</th>
<th>Inconclusive</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemophilic</td>
<td>24</td>
<td>5</td>
<td>16</td>
<td>2</td>
<td>7</td>
<td>54</td>
</tr>
<tr>
<td>Spontaneous</td>
<td>9</td>
<td>6</td>
<td>6</td>
<td>3</td>
<td>2</td>
<td>22</td>
</tr>
</tbody>
</table>
weak reactivity could result from loss of conformationally dependent epitopes during the immunoblotting process, low antibody titer, low-affinity antibodies, or technical reasons. In some cases, repeating the immunoblots was sufficient to resolve the uncertainty. In addition, 14 of the inhibitor plasmas we analyzed were identified by Hoyer and co-workers as being either type I or type II, based on their kinetics of FVIII inactivation. Type I inhibitors completely inactivate FVIII activity, whereas type II inhibitors do not. Among the ten type I and four type II inhibitors we studied, there was no clear correlation between type I or type II behavior and FVIII fragment specificity.

**Determination of inhibitor class and subclass by immunoblotting.** An example of the use of immunoblotting to analyze the immunoglobulin class and subclass composition of FVIII inhibitors is shown in Fig 2. This inhibitor (A in Table 2) was a high-titer inhibitor with epitopes on the 80-kd FVIII polypeptide (and its 72-kd thrombin fragment). It was positive for IgG-1, IgG-4, and light chains. Although the inhibitor was also weakly positive for IgG-2, IgG-3, and light chains, this was not apparent after multiple photographic reproductions. Due to the very weak nature of the reactions, these latter immunoblots were considered inconclusive. The inhibitor was negative for IgM. Also shown in Fig 2 are the positive controls used in these experiments, indicating that all monoclonal anti-immunoglobulin antibodies were reactive with human immunoglobulins in this system. The monoclonal anti-IgG-2 antibody showed a weaker than expected positive control, however (based on the relative amount of IgG-2 in plasma), and, in most subsequent immunoblots, the IgG-2 positive control was too weak to yield interpretable results. In addition, several inhibitors tested negative for both light chains, resulting in a lack of reliable data for light chain typing. It is possible that light chain variants exist that lack the epitopes for these monoclonal antibodies. Therefore, immunoglobulin class and subclass determination using this technique was largely limited to the heavy chains of IgG-1, IgG-3, IgG-4, and IgM.

**Table 2. IgG Subclass Content of 12 FVIII Inhibitor Plasmas as Determined by Immunoblotting.**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>FVIII Type</th>
<th>Titer (BU/mL)</th>
<th>IgG-1</th>
<th>IgG-2</th>
<th>IgG-3</th>
<th>IgG-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>A HA 80</td>
<td>7,800</td>
<td>Pos</td>
<td>—</td>
<td>—</td>
<td>Pos</td>
<td>Neg</td>
</tr>
<tr>
<td>B HA 80</td>
<td>167</td>
<td>Neg Pos</td>
<td>Neg</td>
<td>Pos</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>C HA 92</td>
<td>5.3</td>
<td>Pos Neg</td>
<td>Neg</td>
<td>Pos</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>D HA 92</td>
<td>135</td>
<td>Neg Pos</td>
<td>Pos</td>
<td>Neg</td>
<td>Pos</td>
<td>Neg</td>
</tr>
<tr>
<td>E HA 92,80</td>
<td>150</td>
<td>Neg Pos</td>
<td>Neg</td>
<td>Pos</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>F HA 92,80</td>
<td>375</td>
<td>Neg Pos</td>
<td>Pos</td>
<td>Neg</td>
<td>Pos</td>
<td>Neg</td>
</tr>
<tr>
<td>G HA 92,80</td>
<td>3,000</td>
<td>Pos Neg</td>
<td>Pos</td>
<td>Neg</td>
<td>Pos</td>
<td>ND</td>
</tr>
<tr>
<td>H SP 92</td>
<td>3,600</td>
<td>Neg Pos</td>
<td>Neg</td>
<td>Pos</td>
<td>Neg</td>
<td>ND</td>
</tr>
<tr>
<td>I SP 92</td>
<td>500</td>
<td>Neg Pos</td>
<td>Pos</td>
<td>Neg</td>
<td>Pos</td>
<td>ND</td>
</tr>
<tr>
<td>J SP 92</td>
<td>58</td>
<td>Neg Pos</td>
<td>Pos</td>
<td>Neg</td>
<td>Pos</td>
<td>ND</td>
</tr>
<tr>
<td>K SP 80</td>
<td>1,700</td>
<td>Pos Neg</td>
<td>Neg</td>
<td>Pos</td>
<td>ND</td>
<td>Neg</td>
</tr>
<tr>
<td>L SP 80</td>
<td>960</td>
<td>Pos Neg</td>
<td>Neg</td>
<td>Pos</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

HA, hemophilia A; SP, spontaneous; Pos, positive; Neg, negative; ND, not determined; BU, Bethesda units.

**IgG subclass composition and FVIII polypeptide specificity of seven hemophilic and five spontaneous FVIII inhibitors as determined by immunoblotting.** Table 2 and Fig 3 summarize data on the IgG subclass composition of 12 FVIII inhibitors using this technique. Seven inhibitors were from severe, multitransfused hemophilia A patients and five were of the spontaneous type. The data in Table 2 show that all 12 inhibitors were positive for both IgG-1 and IgG-4 antibodies. Of seven tested, none was positive for IgM. All except one of the inhibitors were negative for IgG-3. The exception was a spontaneously arising inhibitor (H in Table 2), that showed a weak, but positive reaction with the monoclonal anti-IgG-3 antibody (Fig 3). As noted above, most data for IgG-2 were inconclusive.

The IgG-1 and IgG-4 components of inhibitor F showed differences in the degree of reactivity with some of the FVIII polypeptides when immunoblotted and autoradiographed simultaneously (Fig 3). Although the IgG-1 component was strongly reactive with the 80-kd FVIII polypeptide, it reacted only minimally with the 92-kd polypeptide (and its higher mol wt precursors). In contrast, the IgG-4 component was strongly reactive with all polypeptides. This is the first evidence for differences between FVIII polypeptide reactivity and IgG subclass.

**Affinity purification of a hemophilic FVIII inhibitor and quantitation of the IgG subclass content by radial immunodiffusion.** Inhibitor G in Table 2 was affinity purified from plasma by a one-step adsorption onto FVIII-Sepharose as described in the Materials and Methods section. Of 1,622 BU applied to the column, 1,440 BU were bound, and 240 BU (17% of bound) were recovered in the final concentrated pool. The IgG-1, IgG-2, IgG-3, and IgG-4 contents of the affinity-purified inhibitor were quantitated by RID against monospecific polyclonal anti-human IgG subclass antisera, as described in the Materials and Methods section. The affinity-purified inhibitor sample contained 8.8 µg of IgG-1 and 11.6 µg of IgG-4. No IgG-3 was found and, as with the immunoblotting system, the results for IgG-2 were inconclusive due to weak positive controls. The quantitative RID results therefore confirmed the qualitative immunoblotting results, which showed that the inhibitor contained both IgG-1 and IgG-4 components.

**DISCUSSION**

Hoyer and colleagues noted the predominance of IgG-4 in FVIII inhibitors, even though IgG-4 constitutes only ~4% of total serum IgG. Although the reason for this is not known, Hoyer and colleagues note that a predominantly IgG-4 response has been hypothesized by Aalberse and co-workers to be associated with chronic antigenic stimulation. Aalberse and co-workers showed that in novice beekeepers, the antibody response to the phospholipase A2 of bee venom shifts over time from an IgG-1 dominated response to an IgG-4 dominated response following repeated bee stings. These data provide an attractive hypothesis that FVIII inhibitors, particularly in multitransfused hemophiliacs, would be characterized by an IgG-4-dominated response. Quantitation of IgG-1 and IgG-4 in patient G by
RID showed them to be present in nearly equal amounts. However, the low (17%) yield of purified inhibitor IgG raises the possibility that only a selected population of the total inhibitor content may have been isolated, and it may not represent the actual relative amounts of IgG subclasses present.

Previous FVIII inhibitor subtyping data based on immunoneutralization assays compiled from many laboratories suggested that approximately half of FVIII inhibitors were IgG-4 only and half were IgG-4 plus at least one other heavy chain subclass.15 In only one report12 did six of seven inhibitors tested by immunoneutralization assay contain both IgG-1 and IgG-4. The immunoblotting data presented here clearly show that in each of the 12 inhibitor plasmas tested, both IgG-1 and IgG-4 antibody were present, suggesting that the occurrence of IgG-1 antibody in FVIII inhibitors may be a more general phenomenon than previously thought.

The immunoblotting data also show that in each of the 12 inhibitor plasmas tested, the restriction of FVIII epitopes to the 80-kd FVIII polypeptide, the 92-kd polypeptide, or both of these polypeptides was maintained by each IgG subclass analyzed. Analysis of inhibitor F, however, showed that differences in FVIII polypeptide reactivity between IgG subclasses in the same individual are possible. In this inhibitor both the IgG-1 and IgG-4 components reacted equally well with the 80-kd polypeptide, whereas the IgG-4 component reacted much more strongly with the 92-kd polypeptide than did the IgG-1 component.

The spontaneous, autoantibody inhibitors showed an IgG subclass composition similar to that of hemophilic alloantibodies, although the only inhibitor found to contain IgG-3 in addition to IgG-1 and IgG-4 was of the spontaneous type. It seems likely that, as previously suggested,13 FVIII inhibitors are of a restricted polyclonal, or oligoclonal origin. The present data, however, cannot rule out the possibility that some of these inhibitors might be biclonal (composed of two monoclonal antibodies) in origin. Further investigation of the number of FVIII epitopes for these inhibitors should help to answer this question.

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