Anti-Factor VIII Antibodies of Hemophilic Patients Are Frequently Directed Towards Nonfunctional Determinants and Do Not Exhibit Isotypic Restriction

By Jean Guy G. Gilles, Jef Arnout, Jos Vermeylen, and Jean-Marie R. Saint-Remy

A significant proportion of hemophilia A patients receiving transfusions of factor VIII (FVIII) develop a specific antibody response towards FVIII. These antibodies are usually detected by assays in which they inhibit the function of the molecule, such as the Bethesda clotting test. We have prepared anti-FVIII antibodies by specific immunoadsorption from the plasma of four hemophiliaacs with stable inhibitor levels. The isotypic distribution of such antibodies was determined and their capacity to bind to insolubilized FVIII was compared with their inhibitory activity in two functional assays, namely, the Bethesda assay and a chromogenic assay. In addition, the FVIII epitope specificity was determined by competition with monoclonal antibodies for the binding to insolubilized FVIII. We show here that (1) anti-FVIII antibodies are not isotypically restricted; thus, a significant proportion of specific IgG2 was found; (2) antibodies are frequently directed towards epitopes of FVIII that are not directly involved in the function of the molecule and therefore escape detection in the Bethesda method or chromogenic assay; and (3) each patient shows a unique pattern of FVIII epitope recognition. We conclude that evaluation of anti-FVIII antibodies by a functional method does not provide an accurate evaluation of the specific antibody response. These findings have important implications for the comparison of the immunogenicity of FVIII molecules produced by different technologies and for the development of methods to control anti-FVIII antibody production.

The development of more specific therapies is therefore clearly desirable, but requires a more thorough understanding of the mechanisms by which anti-FVIII antibodies are produced as well as an improved assay methodology. Specific antibodies are currently evaluated in vitro by their capacity to inhibit the functional activity of FVIII in coagulation or chromogenic assays. However, antibodies directed towards nonfunctional sites of FVIII that are not detected by current assays could influence in vivo the clearance rate of FVIII and therefore be of pathologic relevance. FVIII epitopes recognized by human antibodies have been mapped using methods in which antibodies react with immunoblots onto which native or thrombin-digested FVIII had been insolubilized or, more recently, by immunoprecipitation with FVIII fragments produced by recombinant DNA technology.

In an attempt to further evaluate the human anti-FVIII antibody response, we have performed a preliminary characterization of affinity-purified antibodies isolated from the plasma of four hemophiliacs with known functional inhibitors. We report here the isotypic distribution, epitope specificity, and FVIII inhibitory activity of these purified antibodies.

MATERIALS AND METHODS

Reagents

Human recombinant FVIII (rFVIII) was obtained from Hyland (Glendale, CA) as material for the laboratory. NHS-LC biotin was purchased from Pierce (Rockford, IL), human serum albumin (HSA) from Institut Merieux (Lyon, France), and phenylmethylsulfonyl fluoride (PMSF) from Sigma Chemicals (St Louis, MO). Monoclonal antibodies (MoAbs) to FVIII (ESH1 to ESH10) were obtained courtesy of Dr Duncan Pepper (Edinburgh, UK), but are now commercialized by American Diagnostica Inc (Greenwich, UK). Buffers used were phosphate-buffered saline at 0.15 mol/L, pH 7.4 (PBS); PBS containing 0.5% bovine serum albumin (PBS-BSA); PBS containing 0.1% Tween 20 (PBS-Tween; Technicon, Tarrytown, NY); glycine-buffered saline at 50 mmol/L, pH 9.2 (GBS); GBS containing 0.5% BSA (GBS-BSA); tri-hydroxymethylaminomethane at 10 mmol/L, pH 7.3 (Tris); Tris containing 0.5% casein (Aldrich Chemicals, Milwaukee, WI) and 0.02% thimerosal (Sigma), pH 7.2 (Tris-casein).

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Patients

The plasma of four adult hemophiliac patients known to have inhibitors was used for the preparation and characterization of specific anti-FVIII antibodies. The patients were 44, 43, 18, and 65 years old and had had an inhibitor to FVIII for 30, 8, 14, and 16 years, respectively. All four patients were considered as high responders, i.e., characterized by a strong anamnestic antibody response after infusion of FVIII. Inhibitor titers at the start of the study were 160, 900, 32, and 28 Bethesda units for patients no. 1, 2, 3, and 4, respectively.

FVIII Immunosorbent

Because most if not all epitopes of FVIII are located in the A and C domains, and because most glycosylated moieties are located on the B domain,11,22 we used an insolubilization procedure involving the sugar residues so as to minimize steric blockade of epitope. FVIII was insolubilized on a hydrazide-coupled solid-phase after mild oxidation. This was performed using Affigel-HZ according to the recommended method (BioRad Laboratories, Richmond, CA). Briefly, 8 mL of a solution of 300 μg/mL human rFVIII containing 20 mmol/L PMFS was dialyzed against the coupling buffer supplemented with 10 mmol/L CaCl₂. HSA was then added to a final concentration of 1.25% (wt/vol) and the volume adjusted to 9 mL with the coupling buffer containing 10 mmol/L CaCl₂. One milliliter of a freshly prepared solution of 100 mmol/L NaIO₄ made in 150 mmol/L NaCl was added and the mixture incubated for 1 hour at room temperature (RT) on a rotating wheel. Glycerol was then added to a final concentration of 20 mmol/L and the solution incubated for a further 10 minutes. Oxidized rFVIII was dialyzed overnight at 4°C against the coupling buffer containing 10 mmol/L CaCl₂ and then mixed with a suspension of Affigel-HZ at a ratio of 1.5 mL (360 μg) per milliliter of packed gel. After a further 24 hours of incubation at RT, the gel was washed extensively with a solution containing 150 mmol/L NaCl and 10 mmol/L CaCl₂ (washing buffer).

Preparation of Human Anti-FVIII Antibodies

Human anti-FVIII antibodies were prepared from plasma by salt precipitation, gel filtration chromatography, and specific immunoadsorption on FVIII. Ten milliliters of plasma was obtained from each of the four patients. Salt precipitation was performed at 21°C by dropwise addition of phosphate buffer at 200 mmol/L, pH 8.2, containing 360 g/L Na₂SO₄. The precipitate was allowed to form overnight at RT and was recovered by centrifugation at 8,000g for 20 minutes. After resuspension of the pellet in PBS containing 1 mol/L NaCl, the sample was applied to a 90 x 5 cm Fractogel (Merck, Darmstadt, Germany) column equilibrated in the same buffer. Fractions containing IgG were pooled, concentrated to 20 mL by ultrafiltration on YM10 membrane (Amicon, Danvers, MA), and dialyzed against PBS. Five-milliliter aliquots of such IgG preparations were passed over 6-mL columns of FVIII-Affigel at a flow rate of 10 mL per hour. The gel was then washed successively with 100 mL of washing buffer, 20 mL of 400 mmol/L NaCl containing 40 mmol/L CaCl₂ and 25 mmol/L imidazole, and finally 100 mL of washing buffer.

Anti-FVIII antibodies were recovered by sequential elution with acid and alkaline buffers. Twenty milliliters of acid buffer (50 mmol/L imidazole, pH 4.5, containing 60% ethylene glycol, 0.5% HSA, and 40 mmol/L CaCl₂) was passed through the column, followed by 100 mL of washing buffer and 20 mL of alkaline buffer (50 mmol/L diethylamine, pH 10, containing 60% ethylene glycol and 40 mmol/L CaCl₂). A final wash was performed with 100 mL of washing buffer. The fractions eluted from the immunosorbent were adjusted to pH 7 and pooled. Both the preparations of specific antibodies and the bulk of nonadsorbed antibodies (flow-through fraction) were dialyzed against the washing buffer and concentrated to 4 mL by ultrafiltration.

The percentage of antibody recovery was calculated by comparing the capacity of antibodies to neutralize the activity of FVIII before and after immunoadsorption, and in the fraction eluted from the column. This was performed using a chromogenic assay (see below) in which the concentration of antibodies required to reduce the activity of FVIII by 50% was evaluated; the number of inhibitory units contained in each sample was obtained by calculating the dilution factor necessary to measure such a reduction, starting from a solution containing 10 mg/mL IgG.

Table 1. Yield and Subclass Distribution of Specific Anti-FVIII Antibodies

<table>
<thead>
<tr>
<th>Patient Antibodies</th>
<th>IgG Subclass Distribution (%)</th>
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</thead>
<tbody>
<tr>
<td>No.</td>
<td>IgG1</td>
</tr>
<tr>
<td>1</td>
<td>58</td>
</tr>
<tr>
<td>2</td>
<td>70</td>
</tr>
<tr>
<td>3</td>
<td>66</td>
</tr>
<tr>
<td>4</td>
<td>49</td>
</tr>
<tr>
<td>Normal</td>
<td>72</td>
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* Obtained by adsorption of 10 mg total IgG on FVIII-Affigel HZ.

Fig 1. Correlation between the inhibitory capacity of anti-FVIII antibodies in plasma and after purification by immunoaffinity. The capacity of anti-FVIII antibodies to inhibit the function of FVIII was measured in the plasma of the four patients studied here using a conventional coagulation method12 and is expressed in Bethesda units. The capacity of affinity-purified antibodies to neutralize the activity of FVIII was measured with a chromogenic assay14 and expressed as the concentration of Ab required to inhibit FVIII activity by 50%. The figure shows the close correlation between these two assays (r = .86). Each data point represents a single patient as identified by the corresponding number.
Preparation of Mouse MoAbs to FVIII

The initial immunization was performed by injecting human rFVIII in complete Freund's adjuvant in the foot pads of 6-week-old Balb/c mice. Three injections were made at 2-week intervals, using 50 μg rFVIII for the first injection and 20 μg for the two others. Four days after the last injection, splenocytes were fused with the SP207 mouse myeloma cells.23 Hybridomas secreting anti-FVIII antibodies were identified in an enzyme-linked immunosorbent assay (ELISA) system using FVIII-coated polystyrene plates. This was performed as follows. Polystyrene microtiteration plates (Nunc, Roskilde, Denmark) were coated with 50 μL of 50 mmol/L glycine buffer, pH 9.2, containing 2 μg/mL rFVIII and were incubated for 2 hours at 21°C. The plates were then washed three times with PBS-Tween and 25 μL of hybridoma supernatant was added. After a further incubation for 2 hours at RT followed by washing, the binding of mouse IgG was detected by sequential addition of 50 μL biotin-labeled antimouse Fc-γ goat IgG (Tago Inc, Burlingame, CA) for 2 hours at 21°C, 50 μL avidin-peroxidase (Sigma) diluted to 1 μg/mL in PBS-BSA for 30 minutes, and 50 μL ortho-phenylenediamine (OPD; Sigma). Each of these steps was separated by four washes with PBS-Tween. The resulting optical density (OD) was read at 492 nm. Hybridomas corresponding to positive supernatants were cloned by limiting dilution and expanded. Antibodies were prepared from the supernatants by salt precipitation and gel filtration chromatography on AcA44 Ultrogel (Pharmacia, Uppsala, Sweden).

Isotypic Distribution of Specific Antibodies

The yield of antibodies obtained by affinity purification and their isotypic distribution were evaluated by ELISA. Polystyrene micro-

Fig 2. Assay for IgG2 antibody subclass. IgG2 antibodies were evaluated by ELISA as described in Materials and Methods. A dose-response curve is presented for each of the four affinity-purified antibody preparations and for a reference sample containing a known concentration of that isotype. The figure shows that each curve is parallel to that of the reference sample.

Fig 3. Dose-response curves for ELISA and chromogenic assay. The capacity of affinity-purified anti-FVIII antibodies to either inhibit the function of FVIII, as measured in a chromogenic assay (□, left vertical axis), or bind to FVIII insolubilized on polystyrene microtitration plates (○, right vertical axis) was compared for each of the four preparations. The concentration of antibodies required to obtain 50% inhibition of FVIII activity was 3, 0.07, 3.5, and 5 μg/mL for patients no. 1, 2, 3, and 4, respectively. An OD signal of 1.0 was obtained with 7, 2, 3.75, and 7 μg/mL.

Specific antibodies (μg/ml)
ANTI-FVIII ANTIBODY CHARACTERIZATIONS

For the determination of IgG1, IgG2, and IgG3 subclasses, polystyrene microtitration plates (Nunc) were coated with 50 µL of anti-mouse IgG1 rat monoclonal IgG (UCL, Brussels, Belgium) at a concentration of 5 µg/mL in GBS, and incubated for 2 hours at RT. The plates were washed five times with PBS-Tween. Fifty microliters of a mouse IgG1 MoAb diluted to 0.5 µg/mL in PBS-BSA and specific for either human IgG1 (Oxoid Ltd, Basingstoke, UK), IgG2, or IgG3 (both from Calbiochem), as appropriate, was then added and incubated for 2 hours at 4°C. The plates were washed as above and 50 µL of the sample or standard reference sample (Jansens, Beersel, Belgium), diluted in PBS-BSA, was added and incubated for a further 2 hours at 21°C. This standard reference sample is a serum in which the concentration of each IgG subclass has been determined; the sample is diluted to cover a range from 1 µg/mL to 25 ng/mL for the subclass under evaluation. After washing, 50 µL of an antihuman Fcγ goat IgG, conjugated with hors eradish-peroxidase (Sigma) and diluted 1/1,000 in Tris-casein, was added to the plates, followed by washing and addition of OPD as described above.

For the evaluation of IgG4, a mouse MoAb specific for that isotype was bound directly onto the plate. Thus, 50 µL of an IgG3 mouse MoAb towards human IgG4 (Calbiochem), diluted 1/2,000 in PBS was added to the plates for 2 hours at 4°C, followed by the sample or reference and the detection system as used for the other subclasses.

**Blotting of FVIII and Immunodetection**

A 1-mL solution of rFVIII adjusted at 500 µg/mL in 50 mM L-Tris, 150 mM NaCl, 25 mM CaCl2, and 0.02% sodium azide, pH 7.4, was dialyzed overnight against the same buffer. Two units of bovine thrombin (Roche, Brussels, Belgium) was added to the solution and incubated for 30 minutes at 37°C. Proteolysis was stopped by addition of D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (Calbiochem) at a final concentration of 0.01 mM/L. FVIII was blotted onto nitrocellulose membranes after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using the Phast System (Pharmacia). Two micrograms of native or thrombin-digested rFVIII in 4 µL O’Farrell buffer was applied to a 4% to 15% polyacrylamide gel (4.5 cm × 4.5 cm) for a 30-minute run at 63 V/h. FVIII proteins were electrotransferred to a 0.2 µm porosity nitrocellulose membrane (Machery-Nagel, Düren, Germany) for 30 minutes at 5 V/h. The membranes were then cut in strips, incubated for 30 minutes with 3% gelatine, washed with PBS-Tween, and finally incubated overnight at 4°C with 3 mL of specific antibodies diluted to 3 µg/mL in PBS-BSA. The strips were washed three times in PBS-Tween and incubated for 2 hours at 21°C with 3 mL of biotin-labeled antihuman Fcγ goat IgG (Tago) diluted 1/2,000 in PBS-BSA. After a further wash with PBS-Tween, 3 mL of PBS-BSA containing 1 µg/mL avidin-peroxidase (Sigma) was added and incubated for 1 hour at 21°C. The nitrocellulose strips were then washed twice with PBS-Tween and once with 20 mM/L Tris buffer containing 500 mM/L NaCl, pH 7.5, after which 6 mL of a solution of 20 µg/mL chloronaphtol in methanol and 5 mL of the buffer was added. The strips were finally washed with distilled water.

**Evaluation of the Proportion of Specific Antibodies in Ig Fractions Recovered From the FVIII Immunosorbent**

The proportion of specific anti-FVIII antibodies in the total yield of antibodies recovered by immunoadsorption was evaluated by allowing antibodies to react with soluble FVIII. Hemofil M (Hyland), an affinity-purified preparation of FVIII, was used to this aim because it contains significant amounts of von Willebrand factor...
Fig 5. Evaluation of the functional capacity of anti-FVIII MoAbs. MoAbs were mixed at different concentrations with a standard amount of FVIII and the residual FVIII activity was evaluated in a one-stage coagulation assay. The percentage of FVIII activity was determined by comparison with a sample without antibody that was incubated for the same time interval. MoAbs to FVIII heavy chain (left panel) or light chain (right panel) are presented separately.

Assays for FVIII Antibodies

Coagulation assays. FVIII inhibitor levels were measured according to the Bethesda method when the patients were seen at the Haemophilia Center (Leuven). The residual FVIII activity after incubation with specific MoAbs was measured in a one-stage coagulation test as described. MoAbs that inhibited the FVIII activity by more than 25% were considered as inhibitors.

Chromogenic assay. The presence of inhibitory antibodies in affinity-purified IgG preparations was evaluated by an adaptation of a chromogenic assay performed in our laboratory. In this assay, thrombin-activated FVIII acts as a cofactor to FIXa in the conversion of a colourless substrate by FXa. The concentration of specific antibodies is therefore inversely proportional to OD. A 50-μL aliquot of the sample made in 150 mmol/L NaCl with 10 mmol/L CaCl₂ was mixed for 30 minutes at 37°C with 50 μL of rFVIII adjusted at 50 μg/mL in the same buffer. Reagents, ie, thrombin, phospholipids, FIXa, FX, and enzyme substrate, were then added as recommended (Merz+Dade, Düdingen, Switzerland). Controls included FVIII diluted in buffer. After an incubation of 10 minutes at 21°C, OD was measured at 405 nm.

ELISA. FVIII (Hemofil M) was insolubilized on a microtitration plate using rabbit IgG antibodies to human vWF and the binding of anti-FVIII antibodies was detected by addition of peroxidase-labeled antihuman Fcγ goat IgG. This was performed as follows. A 3 mg/mL solution of rabbit IgG antibodies to human vWF was digested by addition of pepsin at a final concentration of 55 μg/mL in 0.1 mol/L acetate buffer, pH 4.5, containing 60% ethyleneglycol, 0.5% HSA, and 40 mmol/L NaCl. The eluate and flow-through fractions were dialyzed against 150 mmol/L NaCl containing 10 mmol/L CaCl₂ and concentrated by ultrafiltration on a YM10 membrane (Amicon). The IgG content in both fractions was evaluated by ELISA.

(vWF). The mixture of Hemofil M and antibodies was then passed over a column on which anti-vWF antibodies had been insolubilized. The proportion of antibodies retained on the column was taken as a measurement of specificity.

Rabbit IgG antibodies to human vWF (Dakopatts, Copenhagen, Denmark) were oxidized with NaIO₄ and allowed to react with Affigel-HZ essentially as described above for rFVIII. Ten micrograms of antibodies were used per milliliter of packed gel. Four aliquots containing 2, 4, 8, or 20 μg antibodies per milliliter were prepared in PBS containing 10 mmol/L CaCl₂ and mixed with an equal volume of 80 ng/mL Hemofil M diluted in the same buffer; the mixture was incubated for 1 hour at RT. The lowest concentration of antibodies that completely neutralized FVIII activity, as detected by a chromogenic assay (see below), was used for adsorption. Thus, complexes of Hemofil M and anti-FVIII antibodies were prepared as described above at a 1/50 (wt/wt) ratio. One milliliter containing 2 μg antibodies and 40 ng FVIII was passed over a 6-mL column of anti-vWF immunosorbent. The column was washed with PBS-CaCl₂, and bound complexes eluted by passage of 25 mL acid buffer (50 mmol/L imidazole, pH 4.5, containing 60% ethyleneglycol, 0.5% HSA, and 40 mmol/L CaCl₂). The eluate and flow-through fractions were dialyzed against 150 mmol/L NaCl and concentrated by ultrafiltration on a YM10 membrane (Amicon). The IgG content in both fractions was evaluated by ELISA.
anti-vWF F(ab')² fragments. The plates were washed four times with PBS-Tween and saturated during 30 minutes at 21°C with 100 µL Tris-casein. The plates were then washed as above before the addition of 50 µL Hemofil M diluted to 2 µg/mL in PBS-BSA. After 2 hours at 21°C, the plates were washed and 50 µL of sample containing a dilution of anti-FVIII antibodies was added to each well. A further incubation of 2 hours at 21°C was followed by the addition of 50 µL peroxidase-labeled antihuman Fcγ goat IgG (Sigma) diluted 1,000-fold in Tris-casein. The plates were then washed again, 50 µL OPD added, and the resulting OD read at 492 nm. Negative control samples included multidonor pooled gammaglobulins with no anti-FVIII activity as a substitute for specific antibodies. Peak OD values reached 1.0, whereas negative controls were less than 0.05.

*Competition between murine monoclonal and human anti-FVIII antibodies.* Polystyrene microtiter plates were coated with rabbit anti-vWF F(ab')² fragments and Hemofil M as described above. Fifty microliters of an anti-FVIII MoAb diluted to 2 µg/mL in PBS-BSA was mixed with 50 µL of a sample containing a dilution of human anti-FVIII antibodies in the same buffer. Fifty microliters of the mixture was added to FVIII-coated plates for 2 hours of incubation at 21°C. The plates were washed as above before addition of 50 µL biotin-conjugated antimouse Fcγ goat IgG (Tago) diluted 1/4,000 in Tris-casein. The plates were incubated for a further 2 hours at 21°C and then washed four times before the addition of 50 µL avidin-peroxidase (Sigma) at 1 µg/mL in PBS-BSA. After 30 minutes at 21°C, OPD was added and the OD read at 492 nm. Control experiments included substitution of human anti-FVIII antibodies by a preparation of multidonor pooled gammaglobulins (Gammagard; Hyland).

**RESULTS**

*Yield and Determination of Specific Antibodies in Ig Fractions Recovered by Immunoaffinity.*

The total amount of IgG antibodies in the fraction eluted from the FVIII immunosorbent was evaluated by ELISA. Results are presented in Table 1 and show an average yield of 140 µg per 10 mg of IgG in the initial plasma sample. The percentage of specific antibodies in the eluates was measured by determining the proportion of IgG that could bind soluble FVIII using an anti-vWF immunosorbent and an FVIII preparation containing vWF; this proportion was 77%, 77%, 56%, and 64% for eluates obtained from plasmas 1 through 4, respectively. In no case was it possible to identify specific IgM antibodies in the 19S fraction obtained by gel filtration chromatography. The serum of each of the four patients contained detectable amounts of IgE antibodies but none was found in affinity-purified antibodies.

To validate the antibody purification system used here, ie, to determine whether specific antibodies were representative of the anti-FVIII immune response, the number of inhibitory units present in the flow-through fraction was measured by the chromogenic assay and subtracted from that measured before immunoadsorption; the result was then compared with the number of units recovered in the eluates. The recovery of inhibitory antibodies was 77%, 42%, and 40% for patients no. 1, 2, and 3, respectively, which was considered significant, because complete depletion of anti-FVIII activity could be obtained after repeated passage over the immunosorbent. However, for patient no. 4, the recovery was only 6%, even though the total amount of specific anti-FVIII antibodies was 125 µg, in keeping with the three other samples (Table 1). The most likely explanation for this low recovery is therefore that a functional antibody with high affinity and strong inhibitory potential was retained on the column. However, because the yield of antibodies and their isotypic distribution did not differ significantly from that of the first three patients, results for the four patients are presented. That affinity-purified anti-FVIII antibodies were representative of specific antibodies in plasma is further supported by the good concordance between values obtained by measuring the capacity to inhibit FVIII activity in plasma and that obtained with purified antibodies (Fig 1).

The IgG subclass distribution, also shown in Table 1, indicates that the immune response towards FVIII is not restricted to the IgG4 and IgG1 isotypes, because almost one-third of affinity-purified antibodies were of IgG2 isotype; IgG1 accounted for 50% and IgG4 for 20%, although great variations were observed among patients for this latter isotype. Because these findings departed from published results, we carefully verified assay performances (a representative dose-response curve for IgG2 is given in Fig 2); it can be seen that IgG2 is readily detectable down to 60 ng/mL and that the slope of the curves obtained by diluting the assay
samples is almost parallel to that of the reference curve. Taken together, these results show that the distribution of specific antibodies is close to normal with, however, an increased proportion of IgG4.

Comparison Between Assays for Anti-FVIII Antibodies

The capacity of affinity-purified antibodies to inhibit the function of FVIII was measured in a chromogenic assay and compared with values obtained in a direct binding ELISA. The results are presented in Fig 3 for each of the four patients. It can be seen that the functional assay is at least as sensitive as the detection of antibodies by direct binding. However, the ratio of antibody concentrations required to obtain either a predetermined OD value of 1,000 or a 50% end-point inhibition of FVIII activity varies greatly from one patient to the other (2.3, 28, 1.1, and 1.4, for patients no. 1 through 4, respectively. Most of the variation in these ratios is due to results of the functional assay, indicating that the level of antibodies towards nonfunctional FVIII determinants is very comparable among the four patients. Moreover, the slope of the dose-response curve for the chromogenic assay differs from that of the ELISA. These findings therefore suggest that the ELISA allows the detection of antibodies directed towards FVIII determinants that are not involved in the functional activity of the molecule.

Immunoblotting

All four purified antibody preparations recognized the whole FVIII molecule as well as fragments generated by thrombin cleavage, ie, the 92-Kd heavy chain, the 44-Kd heavy chain fragment, and the 72-Kd light chain. Fragments of lower molecular weight resulting from further digestion of the heavy chain (30 and 20 Kd) were not recognized by specific antibodies. A representative result is shown in Fig 4 for patient no. 1.

Competition Between Human and Monoclonal Anti-FVIII Antibodies

Eleven MoAbs were obtained from a single fusion. MoAbs 1 and 6, 2 and 5, and 15 and 20 recognized identical or closely related epitopes as determined by cross-competition assays: MoAbs 1, 2, and 15 were selected for further experiments. Five commercially available ESH MoAbs were added to this panel: one of them (ESH5) cross-reacted partly with MoAb 15. Altogether, a total of 12 nonoverlapping epitopes were identified on the FVIII molecule.

Seven antibodies were directed towards the heavy chain (nos. 1, 2, 7, 12, 15, 19, and ESH5). All recognized the intact heavy chain on immunoblots; MoAb 15 also recognized the 44-Kd fragment generated by thrombin digestion of the heavy chain. The other six MoAbs (nos. 13, 18, ESH1,
ESH3, ESH6, and ESH7) were directed towards the light chain.

Figure 5 shows the functional characteristics of these MoAbs measured in a coagulation assay. Two antibodies (15 and ESH5) of the seven directed towards the FVIII heavy chain and four (ESH1, ESH3, ESH6, and ESH7) of the six light chain-specific antibodies were inhibitory in this assay.

In an attempt to characterize more precisely the epitopes recognized by human anti-FVIII antibodies, and in particular to identify antibodies to nonfunctional sites of FVIII, an ELISA was used in which different concentrations of human antibodies competed with specific MoAbs for the binding to insolubilized FVIII. Figure 6 shows that the degree of inhibition is proportional to the concentration of human antibodies added, whereas multidonor pooled gammaglobulins used at the same concentrations do not displace MoAbs from the plate. Based on these results, and considering that the ratio of monoclonal versus human antibody concentrations was only 1 to 5, an inhibition ≥10% was considered as significant.

Typical results are given in Fig 7 for the four patients and the complete panel of MoAbs, using 10 μg/mL human antibodies and 2 μg/mL MoAbs. It can be seen that the capacity to inhibit the binding of a given MoAb varies to a large extent from one patient to another, but in every case epitopes are recognized on both the heavy (left panel) and light chains (right panel), in keeping with results obtained by immunoblot analysis. Further, human antibodies were capable of inhibiting the binding of MoAbs directed towards FVIII determinants that are not involved in the functional activity of the molecule, supporting the results shown in Fig 3. Because five of the seven MoAbs directed toward the heavy chain do not inhibit the procoagulation function of FVIII, one can conclude that human antibodies are frequently directed towards nonfunctional FVIII epitopes. On the light chain, there are at least two sites, identified by MoAbs 13 and 18, that do not participate in FVIII activity and are well recognized by human antibodies.

Moreover, there was no relationship between Bethesda units and the number of functional epitopes recognized by human antibodies on FVIII; antibodies from patient no. 1 with a titer of 160 Bethesda units (see Fig 1) competed significantly only with one of the MoAbs to a functional epitope located on the heavy chain (ESH 5) and with two of those directed towards the light chain. On the other hand, antibodies from patient no. 4, with the lowest number of Bethesda units, competed with two of the MoAbs to heavy chain functional epitopes (no. 15 and ESH5) and with three of six MoAbs towards the light chain.

**DISCUSSION**

Anti-FVIII antibodies were prepared by specific immunoadsorption of the plasma of four hemophiliacs with functional inhibitors. The evaluation of these anti-FVIII antibodies shows that (1) the immune response towards FVIII is not isotypically restricted, insofar as the antibody distribution closely follows the normal pattern; (2) specific antibodies are frequently directed towards nonfunctional parts of the FVIII molecule; (3) each patient exhibits a unique profile of antibody specificity; and (4) the routine determination of anti-FVIII antibodies by the Bethesda method does not provide an accurate representation of the antibody response towards FVIII.

The method used to prepare specific anti-FVIII antibodies in the present study resulted in a high yield of antibodies. Special care was therefore taken to ensure that nonspecific antibody retention and elution from the immunosorbent was kept to a minimum. This was verified by measuring the proportion of eluted antibodies capable of binding to soluble FVIII. One factor that could be a determinant for this high antibody yield is the method used for coupling FVIII on the solid phase. Thus, we compared several methods, including cyanogen bromide and carbodiimide activation. In every case it resulted in an efficient coupling of FVIII but a very poor capacity to retain specific antibodies (data not shown). Coupling FVIII by its carbohydrate moieties apparently preserves antigenic determinants, as confirmed by the absence of residual anti-FVIII activity in the antibody fractions. The fact that very few epitopes are located on the FVIII B domain,1,6,19 which carries most of polysaccharidic determinants, favors this type of insolubilization procedure, not to mention that conditions required to couple FVIII are mild enough to maintain the molecule integrity. To our knowledge, there is only one report in the literature in which a quantitative evaluation of anti-FVIII antibodies was performed after direct adsorption of plasma on cyanogen-bromide-activated FVIII-Sepharose; 40 μg/mL of specific antibodies was obtained.25

The difficulty of insolubilizing FVIII while maintaining its full capacity to react with antibodies was also encountered in immunoassays. An alternative to the direct adsorption of FVIII onto polystyrene plates is to interpose vWF. The reasoning was that the FVIII molecule, which is completely bound to vWF in its circulating form, would be presented to specific antibodies in a more physiologic conformation. We realized that, by doing so, antibodies that are specific for the vWF-binding site of FVIII26 would not be detected; however, this would represent a marginal underestimation of the antibody yield, well compensated by a substantial improvement of immunoassays.

Our results are at variance with the reported isotypic restriction of anti-FVIII antibodies to IgG1 and IgG4 subtypes.15,27 In fact, the distribution of IgG follows closely the physiologic profile of IgG subclasses. The relative increase in IgG4, also found in the present study, could be related to long-term immunization of these hemophiliac patients who have received infusions of FVIII on occasions, but over long periods of time. Interestingly, patient no. 2, with the highest concentration of specific IgG4, was the only one to be on regular FVIII transfusions as part of a treatment to reduce the production of FVIII inhibitors. Contrasting with this situation, recently induced anti-FVIII immune responses do not show a relative increase in IgG4 (Gilles et al, manuscript submitted). Nevertheless, the most remarkable observation made here is the presence of substantial amounts of specific IgG2. This isotype is preferentially, though not exclusively, produced towards carbohydrate moieties,28 and
we are currently verifying whether this is the case with FVIII. That the coupling procedure does not seem to hinder the adsorption of IgG2 on the immunosorbent might argue against this possibility; however, up to 20% of carbohydrate moieties are located on heavy and light chains. The reasons why specific IgG2 antibodies have not been detected previously could be related to the difficulty encountered in designing a reliable assay for this isotype; only recently have a limited number of specific MoAbs been made available. A second reason could stem from the presence of anti-idiotypic antibodies; the use of whole plasma instead of purified specific antibodies can lead to an underestimation of anti-FVIII antibodies. This was indeed the case with the four samples analyzed here. Specific anti-idiotypic antibodies were present in the plasma samples but were eliminated by the procedure used to prepare specific antibodies (Gilles et al, manuscript in preparation).

Antibodies towards sites of FVIII that are not directly involved in the function of the molecule were found in each of the four plasma samples analyzed here. Considering the number of MoAbs to nonfunctional FVIII determinants that we used in the present study and whose binding was significantly inhibited by patients' antibodies, it appears that a majority of the FVIII determinants recognized by human antibodies are nonfunctional. The clinical relevance of these antibodies is poorly understood. They have been detected in patients treated with high doses of FVIII in an attempt to reduce the level of functional antibodies. Their presence was already suspected in plasma samples analyzed by FVIII immunoblots. Such antibodies can possibly interfere with the clearance rate of infused FVIII, which might explain why the t½ of FVIII is reduced in some patients with undetectable levels of functional inhibitors.

MoAbs were used to map the FVIII epitopes recognized by human antibodies. We used here a panel of MoAbs directed towards different epitopes on both the heavy and light chains. Given the relatively low number of known FVIII epitopes,29 such a panel should provide a reliable representation of epitope location on FVIII. A potential difficulty inherent to antibody competition assays is the relative affinity of competing molecules. This was circumvented here by using a constant amount of MoAb and different dilutions of polyclonal anti-FVIII antibodies.

Taken together, our data show that the immune response towards FVIII varies greatly from one patient to another, particularly with regard to epitope specificity. This latter point is in keeping with published data16,30 and with the absence of relationship between the capacity to mount an anti-FVIII immune response and expression of certain HLA class II determinants.31 However, it should be stressed that the analysis presented here represents the antibody response at a single time point. It might be that epitope specificity29 and/or isotopic distribution of anti-FVIII antibodies vary with time, either spontaneously or as a result of treatment.

Nevertheless, we suggest that, in addition to the currently used Bethesda method, anti-FVIII antibodies should be evaluated by a method detecting both functional and nonfunctional antibodies. These two types of antibodies are to be taken into account when determining the immunogenicity of current or novel FVIII preparations and in the design of novel therapeutic approaches of antibody suppression.

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Anti-factor VIII antibodies of hemophiliac patients are frequently directed towards nonfunctional determinants and do not exhibit isotypic restriction

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