Heterogeneity of Factor VIII Antibodies: Further Immunochemical and Biologic Studies

By Mae B. Hultin, Fredda S. London, Sandor S. Shapiro, and William J. Yount

Previous studies using immunoneutralization techniques have shown that many factor VIII inhibitors are IgG antibodies of a single light chain type. We have investigated this apparent homogeneity by immunoneutralization assay and liquid isoelectric focusing of inhibitor fractions from five hemophiliacs and two nonhemophiliacs. By immunoneutralization assay, inhibitors from four hemophiliacs and one nonhemophiliac were exclusively k light chain type: the fifth hemophilic inhibitor was predominantly k, and the second nonhemophilic inhibitor was a mixture of k and l. However, heavy chain subtyping of the six predominantly or exclusively k inhibitors showed all to be mixtures of lgG4 and lgG1. By isoelectric focusing, two inhibitors showed multiple peaks of activity between pH 5 and 9. The remaining five showed predominant peaks of activity, which were solely lgG4, between pH 5.8 and 7, with smaller peaks between pH 7 and 9. The most acidic major peak, focusing at pH 6, was lgG4 in the three cases tested. Two of these acidic peaks neutralized factor VIII more efficiently than other peaks in the same focusing profiles, suggesting greater affinity for factor VIII. These studies demonstrate that factor VIII inhibitors are composed of heterogeneous subpopulations of immunoglobulins which can be separated by isoelectric focusing.

Development of inhibitors to factor VIII in 5% to 10% of patients with hemophilia A and in occasional nonhemophiliacs is an important immunologic response which is not yet well understood. The antibody nature of factor VIII inhibitors has been studied by neutralization of inhibitor activity with antisera specific for human immunoglobulin (Ig) heavy chain classes and light-chain types. When examined by this immunoneutralization technique, factor VIII inhibitors in hemophiliacs appear to be confined to the IgG class and usually to one light-chain type, which has been k in most cases. A mixture of IgGk and IgGa is more common in factor VIII inhibitors in nonhemophiliacs, but occurs occasionally in hemophiliacs as well. Immunoneutralization assays using antisera specific for γ-chain subclasses have been reported for one hemophilic and five nonhemophilic inhibitors, showing IgG4 exclusively or predominantly in five cases and IgG3 in one case. The predominance of the IgG4 subclass in factor VIII inhibitors is unique among human antibodies studied to date and has prompted the present investigation.

The apparent homogeneous nature of many factor VIII antibodies may in...
Table 1. Patients With Factor VIII Inhibitors

<table>
<thead>
<tr>
<th>Patient</th>
<th>Diagnosis</th>
<th>Age*</th>
<th>Duration†</th>
<th>Inhibitor Titer (U/ml in plasma)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Hem. A</td>
<td>33</td>
<td>5 yr</td>
<td>830</td>
</tr>
<tr>
<td>2</td>
<td>Hem. A</td>
<td>37</td>
<td>4 yr</td>
<td>10,400</td>
</tr>
<tr>
<td>3</td>
<td>Hem. A</td>
<td>19</td>
<td>2 wk</td>
<td>400</td>
</tr>
<tr>
<td>4</td>
<td>Hem. A</td>
<td>15</td>
<td>6 mo</td>
<td>280†</td>
</tr>
<tr>
<td>5</td>
<td>Hem. A</td>
<td>33</td>
<td>6½ yr</td>
<td>320</td>
</tr>
<tr>
<td>6</td>
<td>No underlying</td>
<td>71</td>
<td>6 mo</td>
<td>240</td>
</tr>
<tr>
<td>7</td>
<td>Myoclonic epilepsy; recent ampicillin treatment</td>
<td>51</td>
<td>3 wk</td>
<td>90</td>
</tr>
</tbody>
</table>

Hem., hemophilia.

*Age of patient when inhibitor sample was obtained for IEF.
†Duration of positive inhibitor assay (in hemophiliacs) or bleeding diathesis (in nonhemophiliacs) prior to obtaining sample.
†Titer in serum.

part reflect the limitations of the immunoneutralization technique itself, which may be disproportionately sensitive to high-affinity antibodies in inhibitor samples containing mixtures of antibodies of different affinities. For this reason we have used liquid isoelectric focusing (IEF) to investigate the relative heterogeneity of the factor VIII antibody population in seven patients. We have also performed γ-chain subclass typing on six of these inhibitors and on isolated IEF peaks from three cases.

MATERIALS AND METHODS

Patient Samples

High-titer factor VIII inhibitors were obtained from five male patients with hemophilia A and two male patients with spontaneously acquired inhibitors (Table 1). Blood samples were collected by venipuncture into one-ninth volume 3.2%, or 3.8%, sodium citrate or by plasmapheresis into acid-citrate-dextrose (ACD). Platelet-poor plasma was obtained by centrifugation for 15 min at 1200 g or greater and was stored at temperatures between −60°C and −90°C for periods ranging from 2 wk to 9 yr. The inhibitor titer and specificity were confirmed by appropriate specific assays.

Factor VIII and Factor VIII Inhibitor Assays

Factor VIII activity was measured by a manual one-stage kaolin-activated modified partial thromboplastin time assay using Thrombofax (Ortho Diagnostics, Raritan, N.J.) and single-donor hemophilic plasma (factor VIII < 0.01 U/ml). In a few cases an automated assay was employed (Coagulyzer, Sherwood Industries, St. Louis, Mo.), using ellagic acid-activated Thrombofax (Ortho) instead of the kaolin Thrombofax mixture. Inhibitor activity was measured by the Bethesda assay with two modifications: (1) plasma from a single normal donor was used for all studies as the factor VIII substrate; and (2) for all samples from the same patient, inhibitor units were calculated using a curve constructed from the same patient’s plasma, rather than from the published theoretical standard curve. The inhibitor plasma dilution was plotted arithmetically versus the logarithm of residual factor VIII activity, with 50% residual factor VIII designated as 1 inhibitor unit/ml. At least three points between a residual factor VIII of 0.25 and 0.75 U/ml were obtained to construct each curve.

Preparation of Serum and 40% Saturated Ammonium Sulfate Fractions

Each plasma sample was thawed at 37°C and clotted with purified human thrombin at a final concentration of 10 U/ml. After 20 min incubation at 37°C, the clot was removed by centrifuga-
tion at 5000 g for 20 min at 4°C. An IgG-rich inhibitor fraction was precipitated from the serum by addition of saturated ammonium sulfate (SAS), pH 6.5, at 4°C to a final concentration of 40% (v/v). The precipitate was washed twice with 40% SAS redissolved in 0.01 M phosphate buffer, pH 6.5, to 0.2–0.5 of the original serum volume, and dialyzed at 4°C against three changes of the same buffer over 24 hr. Material precipitated during dialysis was removed by centrifugation at 5000 g for 20 min and the supernatant was assayed for inhibitor titer and absorbance at 280 nm (A$_{280}$). Protein concentration was estimated using an E of 14.0. The yield of inhibitor units in the final fraction compared to the original plasma was determined in four cases and was 65%–85%.

**Sephadex G-150 Gel Filtration of Inhibitor Sera**

Each inhibitor serum was subjected to Sephadex G-150 gel filtration at 4°C to determine the elution volume of the inhibitor. A 1.4-ml serum sample was applied to a 2.5 x 80-cm column and eluted with a buffer consisting of 0.025 M imidazole, 0.15 M NaCl, pH 7.2. Fractions of 1.5 ml were collected, and A$_{280}$ and inhibitor titer measured in each fraction.

**Liquid Isoelectric Focusing**

IEF was performed at 4°C using Ampholines and a 110-ml IEF column (LKB-Produkter, Bromma, Sweden) with the anode at the bottom of the column in each case. A 50°, 5°, (g/dl) linear gradient of aqueous sucrose was poured using an LKB gradient maker and a nonpulsatile pump at a rate of 1.5 ml/min. Ampholines of a chosen pH range were mixed with the sucrose solution, 0.75 of the total volume in the dense solution and the remainder in the light solution, to give a final concentration of 1°. Pouring of the sucrose gradient was interrupted at the halfway point to apply the 40°, SAS fraction. Samples applied were 1.0 ml or less and contained 350–3000 units of inhibitor, 6.42 mg of protein, and in most cases 25°, sucrose (g/dl). IEF was performed for 65–72 hr at 300 V for the pH 3.5–10 range and at 600 V for narrower pH ranges, using a constant-voltage power source. The column was then emptied by gravity, collected in 1.5-ml fractions at the rate of 1.2 ml/min, and the pH and A$_{280}$ were measured immediately. All fractions were then quick-frozen in acetone dry ice and stored at −70°C. Focusing was essentially complete as judged by the linearity of the pH gradient and the number of protein peaks measured at 280 nm.

To establish the range of Ig focusing and the conditions necessary for factor VIII inhibitor assay of IEF fractions, IEF was performed using a 40°, SAS fraction from normal single-donor human plasma. The presence of IgG, IgA, and IgM in collected fractions was assessed by Ouchterlony double diffusion in agarose, using class-specific antisera (Meloy Laboratories, Springfield, Va.). Over the pH range of 3.5–9.5, a 1:10 dilution of IEF fractions in 0.05 M imidazole, 0.15 M NaCl buffer, pH 7.3 (IBS), was adequate to eliminate interference with the factor VIII inhibitor assay. Alternatively, inhibitor assays could be performed without interference on 1:2 dilutions of IEF fractions if these fractions were dialyzed against 100 volumes of 0.01 M phosphate, 0.2 M NaCl, pH 7.2, for 4 hr, followed by three dialyses against 0.01 M phosphate, 0.15 M NaCl, pH 7.2, over 20 hr.

Each 40°, SAS inhibitor fraction was first focused at pH 3.5–10 and the distribution of anti-

![Fig. 1. Patient 1. Sample applied: 1400 units factor VIII inhibitor, 42 mg protein.](image)
Fig. 2. Patient 2. Sample applied: 3000 units factor VIII inhibitor, 6 mg protein.

Fig. 3. Patient 3. Sample applied: 1800 units factor VIII inhibitor, 34 mg protein.

Fig. 4. Patient 4. Sample applied: 700 units factor VIII inhibitor, 40 mg protein.

Fig. 5. Patient 5. Sample applied: 800 units factor VIII inhibitor, 27 mg protein.
body activity located by inhibitor assay of each IEF fraction using serial dilutions starting at 1:10. A second focusing of each 40", SAS fraction (Figs. 1-7) was performed using a narrower pH range, chosen on the basis of the pH 3.5-10 inhibitor profile, either pH 5.8 or pH 5.9 (the latter made with equal volumes of Ampholines pH 5.7 and 7.9). The wide-range and narrow-range IEF profiles for each inhibitor were similar, generally with better resolution using the narrower range. Inhibitor yields were 50\% 90\% of the units applied. After inhibitor assay, IEF fractions were immediately refrozen in acetone dry ice and stored at -70°C. Subsequently, selected IEF fractions were subjected to dialysis as outlined above and then assayed for inhibitor titer and Ig class, IgG subclass, and light-chain type. IEF inhibitor profiles obtained on undialyzed and dialyzed fractions were similar.

To compare factor VIII neutralizing ability of separate peaks, inhibitory activity was measured, usually in duplicate, on serial dilutions of appropriate dialyzed IEF fractions. The log-log plot of inhibitor concentration versus residual factor VIII activity was linear. Slopes were negative: i.e., the higher the concentration of inhibitor, the lower the residual factor VIII activity. Dilution curves and slopes of 40\", SAS fractions were obtained in a similar fashion (Tables 2 and 3).

**Immunoneutralization Assays**

Determinations of Ig class and light-chain type were performed as previously described\(^7\)\(^10\) on every 40\", SAS fraction in duplicate and on selected dialyzed IEF fractions, with the following modifications. Bovine serum albumin (BSA), 1 g/dl, in IBS was the diluent for inhibitor controls, and an appropriate dilution of the inhibitor fraction was chosen so that the inhibitor control gave a residual factor VIII of 0.25 ± 0.10 U/ml. Over this range of factor VIII measurement (clotting times of 75-82 sec), the error of the clotting time was ± 1.5 sec (2 SD) or less. Clotting times of IBS controls (factor VIII 0.50 U/ml) were 65.9 ± 1.3 sec (2 SD). Antisera to IgG, IgA, and IgM classes and \(a\) and \(\lambda\) light-chain types were obtained commercially (Meloy Laboratories: Behring Diagnostics, Somerville, N.J.; Atlantic Antibodies, Westbrook, Me.). Antisera to IgG\(_1\), IgG\(_3\), and IgG\(_4\) subclasses were prepared as previously described\(^10\). The IgG subclass and the Behring \(\lambda\) antisera were used as 50\", SAS fractions. For experiments with
### Table 2. Characteristics of Hemophilic Inhibitors

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sample</th>
<th>Ig Class</th>
<th>γ-Chain Subclass</th>
<th>Light Chain Type</th>
<th>Slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>40% SAS fx</td>
<td>G</td>
<td>1 and 4</td>
<td>κ</td>
<td>-1.6, -1.7</td>
</tr>
<tr>
<td>37,* pH 6.1</td>
<td>G</td>
<td>4</td>
<td>κ</td>
<td>-2.9, -2.6</td>
<td></td>
</tr>
<tr>
<td>48, pH 6.7</td>
<td>G</td>
<td>4</td>
<td>κ</td>
<td>-1.7, -1.3</td>
<td></td>
</tr>
<tr>
<td>61, pH 7.4</td>
<td>G</td>
<td>-</td>
<td>-</td>
<td>-1.3, -1.3</td>
<td></td>
</tr>
<tr>
<td>64, pH 7.5</td>
<td>G</td>
<td>-</td>
<td>κ</td>
<td>-0.9</td>
<td></td>
</tr>
<tr>
<td>70, pH 7.7</td>
<td>G</td>
<td>-</td>
<td>-</td>
<td>-0.7, -0.5</td>
<td></td>
</tr>
<tr>
<td>73, pH 7.8</td>
<td>G</td>
<td>-</td>
<td>-</td>
<td>-1.4, -1.2</td>
<td></td>
</tr>
<tr>
<td>76, pH 7.9</td>
<td>G</td>
<td>-</td>
<td>κ</td>
<td>-1.4, -1.2</td>
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</tr>
</tbody>
</table>

### Table 3. Characteristics of Nonhemophilic Inhibitors

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sample</th>
<th>Ig Class</th>
<th>γ-Chain Subclass</th>
<th>Light Chain Type</th>
<th>Slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>40% SAS fx</td>
<td>G</td>
<td>1 and 4</td>
<td>κ</td>
<td>-1.1, -0.9</td>
</tr>
<tr>
<td>23,* pH 5.9</td>
<td>G</td>
<td>-</td>
<td>κ</td>
<td>-1.0</td>
<td></td>
</tr>
<tr>
<td>25, pH 6.2</td>
<td>G</td>
<td>-</td>
<td>κ</td>
<td>-1.0</td>
<td></td>
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<tr>
<td>28, pH 6.6</td>
<td>G</td>
<td>-</td>
<td>κ</td>
<td>-0.7</td>
<td></td>
</tr>
<tr>
<td>30, pH 6.7</td>
<td>G</td>
<td>-</td>
<td>κ</td>
<td>-1.0</td>
<td></td>
</tr>
<tr>
<td>35, pH 7.1</td>
<td>G</td>
<td>-</td>
<td>κ</td>
<td>-1.1</td>
<td></td>
</tr>
</tbody>
</table>

### Notes:
- Abbreviations: fx, fraction; Ig, immunoglobulin.
- *Refers to IEF fraction number as shown in corresponding patient figure (Figs. 1–5).
Table 4. Specificity of IgG4 and IgG1 Antisera: Patient 2 Inhibitor Fraction

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Clotting Time (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IBS-BSA control</td>
<td>66.5</td>
</tr>
<tr>
<td>Inhibitor + IBS-BSA</td>
<td>78.4</td>
</tr>
<tr>
<td>Inhibitor + antiserum</td>
<td>68.6</td>
</tr>
<tr>
<td>Inhibitor + absorbed antiserum</td>
<td>77.9</td>
</tr>
<tr>
<td>Inhibitor + absorbed antiserum</td>
<td>72.8</td>
</tr>
<tr>
<td>IgG4 antiserum</td>
<td></td>
</tr>
<tr>
<td>IBS-BSA control</td>
<td>66.5</td>
</tr>
<tr>
<td>Inhibitor + IBS-BSA</td>
<td>77.6</td>
</tr>
<tr>
<td>Inhibitor + antiserum</td>
<td>65.8</td>
</tr>
<tr>
<td>Inhibitor + antiserum</td>
<td>68.0</td>
</tr>
<tr>
<td>IgG1 antiserum</td>
<td></td>
</tr>
<tr>
<td>IBS-BSA control</td>
<td>66.0</td>
</tr>
<tr>
<td>Inhibitor + IBS-BSA</td>
<td>65.8</td>
</tr>
<tr>
<td>Inhibitor + antiserum</td>
<td>78.0</td>
</tr>
<tr>
<td>Inhibitor + absorbed antiserum</td>
<td>66.5</td>
</tr>
</tbody>
</table>

*Clotting times in final factor VIII measurement of immunoneutralization assay. Clotting times were averages of two determinations, which were within 1 sec of each other.

IgG4 and IgG1 experiments were done on different days, using different concentrations of inhibitor. Standard factor VIII dilution curves were determined separately for each experiment.

The specificity of neutralization of factor VIII inhibitor activity by IgG1 and IgG4 antisera was shown in the following manner. Each antiserum was incubated with IgG, IgA, IgM, κ, and λ antisera, a ratio of one part antiserum to one part inhibitor fraction was sufficient to attain antiserum excess. An antiserum-to-inhibitor ratio of 3:1 was necessary for the IgG3 and IgG1 antisera, and 29:1 for the IgG4 antisera. For all experiments reported, antiserum excess was proved by Ouchterlony double diffusion in agarose.

The specificity of neutralization of factor VIII inhibitor activity by IgG1 and IgG4 antisera was shown in the following manner. Each antiserum was incubated with IgG1 or IgG4 purified myeloma protein at a final concentration of 1 mg/ml for 1 hr at 37°C and overnight in the cold. The absorbed antisera were compared to unabsorbed antisera in the immunoneutralization assay (Table 4).

RESULTS

Isoelectric Focusing (Figs. 1–7)

The distribution of immunoglobulins, as determined by Ouchterlony analysis, was similar for normal and patient samples, with IgA focusing from pH 4.4 to 6.8 and IgG from pH 5.0 to 9.5. The 40% SAS fraction which was subjected to IEF contained little IgM, most of which had precipitated during dialysis in the cold against 0.01 M phosphate buffer, preparatory to IEF. Loss of IgM was not important for the focusing of the factor VIII inhibitors studied, since Sephadex G-150 gel filtration of each inhibitor serum showed a single peak of inhibitor activity eluting simultaneously with the second protein peak, with no inhibitor present in the void volume peak where IgM eluted.

The IEF profiles of patients 1, 2, 4, and 6 showed major peaks in the pH 6–7 range, with minor peaks in the more alkaline range. Patients 3, 5, and 7 showed inhibitor more uniformly distributed over the entire pH 5–9 range, although patient 3 showed a major acidic peak of inhibitor activity.

Immunoneutralization Assays (Tables 2–4)

Immunoneutralization assays of the 40% SAS fractions of patients 1, 2, 3, and 6 showed complete neutralization of each inhibitor by IgG and by κ antisera, with no neutralization by IgA, IgM, and λ antisera. Assays of several
dialyzed IEF inhibitor peaks from each of these patients also showed neutral- 
ization solely with IgG and κ antisera. In patient 4, assay of the 40₀₉ SAS 
fraction showed exclusively IgG and predominantly κ inhibitor with traces of 
λ inhibitor as well. One of the IEF peaks (No. 68) isolated from this patient 
contained a mixture of IgGκ and IgGλ inhibitor, while all others tested were 
exclusively IgGκ. In patient 5 some λ activity was detected in one IEF peak 
(No. 30) despite the inability to detect any λ in the 40₀₉ SAS fraction. Inhibitor 
7 was clearly a mixture of κ and λ types by assay of the 40₀₉ SAS fraction, and 
assays of selected IEF peaks supported this finding.

Typing of the γ-chain subclass of 40₀₉ SAS fractions from patients 1–6 
showed that each inhibitor was partially neutralized by IgG₄ and IgG₁ anti-
sera. No neutralization was obtained with antiserum to IgG₃. Neutralization 
with IgG₂ antiserum was not carried out, and therefore the presence of a minor 
population of IgG₂ inhibitor was not absolutely excluded. However, the degree 
of neutralization obtained with IgG₄ and IgG₁ antisera could account for all 
of the inhibitor activity in each case. The neutralization of factor VIII inhibitors 
by IgG₄ and IgG₁ antisera was completely blocked by prior absorption of each 
antiserum with purified IgG₄ and IgG₁ myeloma proteins, respectively, as illus-
trated in Table 4. The major acidic peak, around pH 6.0, was completely neu-
tralized by IgG₄ antiserum in the three cases tested (patients 1–3), while the 
second major peak in patient 1 (No. 48, pH 6.7) was not neutralized by the 
IgG₄ antiserum.

Dilution Curves (Tables 2 and 3)

Dilution curves of the 40₀₉ SAS fractions and of selected dialyzed IEF frac-
tions were performed for all seven inhibitors. Although insufficient data were 
obtained to calculate the range of error of the method, duplicate determinations 
were generally in good agreement. Slopes of the curves of the 40₀₉ SAS frac-
tions of patients 2–6 were quite similar, while that of patient 1 was steeper, and 
patient 7 flatter than the others. In each of four patients (3, 4, 6, and 7), dilu-
tion curves of dialyzed IEF peaks from the same profile showed similar slopes. 
In contrast, in patients 1 and 2 the slope of the major acidic peak, which ap-
peared to be solely IgG₄, was considerably steeper than the slopes of the other 
peaks in the same profile. The two nonhemophilic inhibitors appeared to differ 
from each other; the slopes of the 40₀₉ SAS fraction and IEF peaks in case 6 
were steeper than in case 7 and more similar to the slopes of the hemophilic 
inhibitors.

DISCUSSION

The development of factor VIII inhibitors in hemophiliacs following factor 
VIII infusion or in nonhemophiliacs spontaneously is an unfortunate immune 
response which often precludes effective factor VIII replacement therapy. Pre-
vious studies have suggested that some of these antibodies are monoclonal, 
since they are confined to the IgG class and to one light-chain type. The 
present studies confirm those findings but nevertheless demonstrate that these 
inhibitors are actually mixtures of immunoglobulin populations, as shown by 
IEF profiles, by γ-chain subclasses, and by factor VIII neutralizing activity
FACTOR VIII ANTIBODIES

of separate IEF peaks. This heterogeneity is probably representative of the total factor VIII antibody population in patient plasma, in view of the good yield of inhibitor in the 40°, SAS fraction and following IEF. The seven inhibitors show varying degrees of heterogeneity. The IEF patterns of patients 1, 2, 4, and 6 were partially restricted to one or more broad peaks between pH 6 and 7, in contrast to the single narrow IEF peak reported for human myeloma protein and human rheumatoid factor. A number of structural variables might cause differences in net electrical charge and IEF patterns of factor VIII antibodies, including γ-chain subclass, subtype of κ chains, the nature of the carbohydrate attached to the IgG heavy chain, and the amino acid sequence in hypervariable or antigen-combining site regions of both heavy and light chains. It is nevertheless possible that many of the factor VIII antibody subpopulations separated by IEF have similar specificity and, therefore, closely similar combining sites for factor VIII despite differences in other structural determinants.

γ-chain subclass typing was performed on six of our seven inhibitors. All six 40°, SAS inhibitor fractions were mixtures of IgG4 and IgG1, a finding which probably explains at least part of the heterogeneity shown by IEF. The most acidic major IEF peak in the three inhibitors tested (Table 2, patients 1-3) was composed of IgG4, consistent with the known acidic pH of this IgG subclass. The major acidic peaks in inhibitors 4 and 6 were not assayed for γ-chain subclass because of limited antiserum but may also have consisted of IgG4, since IgG4 was demonstrated in both 40° SAS inhibitor fractions. The demonstration of IgG4 in all six inhibitors tested provided strong evidence that this subclass was an important component of most factor VIII inhibitors, in hemophiliacs as well as nonhemophiliacs. This minor subclass, comprising less than 4% of the IgG in plasma, was the sole component of one factor IX inhibitor, although a second was a mixture of IgG subclasses. Similar immuno-neutralization assays of three factor XIII inhibitors showed solely IgG1κ in one case, IgG1 with both light-chain types in another, and a mixture of IgG1, and IgG3, with κ and λ light chains in the third. The reasons for the apparent restriction of some inhibitors of coagulation factors to one light-chain type or one γ-chain subclass are not known.

The results of dilution curves of individual IEF peaks must be regarded as preliminary, but the differences in slopes observed suggest that IEF can separate inhibitor subpopulations with differing factor VIII neutralizing ability. Such a deduction is based on the observation that the increment in factor VIII neutralization achieved by doubling inhibitor concentrations varies for different peaks. It might be expected that the more avid or specific an antibody for factor VIII, the greater the effect of such doubling of concentration. Thus, the steeper slopes of two acidic IgG4κ IEF peaks may actually reflect greater affinity for factor VIII. Previous studies of the kinetics of factor VIII inactivation have suggested that some nonhemophilic inhibitors have lower affinity for factor VIII than most hemophilic inhibitors. Dilution curves of 40° SAS fractions and of separated IEF peaks from one of our nonhemophilic inhibitors (patient 6) are similar to those of most hemophilic inhibitors, while curves from the second nonhemophilic inhibitor (patient 7) are more typical of the nonhemophilic group. Some of these differences may reflect variations in the combining sites of these antibodies, although no direct evidence for such a com-
clusion is currently available. Studies of the idiotypic determinants of factor VIII antibodies for cross-specificity could lend further support to this hypothesis.

Our studies demonstrate that factor VIII inhibitors show varying degrees of heterogeneity both in terms of structure and biologic activity. IEF appears to be one technique suitable for separating inhibitor subpopulations. Such separation may be related to differences in factor VIII antibody combining sites or to differences among IgG heavy- and light-chain subtypes, or both. The number and properties of the inhibitor subpopulations found in our patients may be characteristic for an individual patient or may reflect the particular time in the natural history of the inhibitor at which the sample is obtained for study. Further analysis of these subpopulations may be useful in understanding the genesis and natural history of factor VIII inhibitors. In addition, separated antibody subpopulations may provide immunologic probes for the study of factor VIII structure.

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Heterogeneity of factor VIII antibodies: further immunochemical and biologic studies

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