Immunological Characterization of a Monoclonal \( \gamma G_4, \lambda \) Human Antibody to Factor IX

By Isadore M. Pike, William J. Yount, Elliot M. Puritz, and Harold R. Roberts

A circulating inhibitor specific for factor IX in a patient with hemophilia B was characterized with antisera to human immunoglobulins. Inhibitor-rich plasma was mixed with an excess of specific antisem and then assayed for residual inhibitor activity. Inhibitor activity was completely removed by antisera specific for \( \gamma G_4 \) heavy chains and lambda light chains. Antisera specific for \( \gamma A, \gamma M, \gamma D, \gamma E, \gamma G_1, \gamma G_2, \) and \( \gamma G_3 \) heavy chains and kappa light chains had no effect on the inhibitor. On preparative zone electrophoresis, inhibitor activity was localized in a sharp band in the anodal portion of the \( \gamma G \) peak, an electrophoretic distribution paralleling that of \( \gamma G_4 \). Precise localization of the inhibitor activity on calibrated gel filtration columns revealed a relatively narrow zone of fractions containing the inhibitor, wherein the proteins have an estimated molecular weight of 145,000. The relative homogeneity of the antibody may reflect specificity for a uniform, discrete portion of the human factor IX molecule.

CIRCULATING ANTICOAGULANTS that specifically inhibit fibrinogen, factor V (accelerin), factor VIII [antihemophilic factor, (AHF)], factor IX [plasma thromboplastin component (PTC)], and factor XIII have been described. Most of these inhibitors have been characterized as antibodies. Antibodies to factor VIII have been further characterized as to heavy and light chain types, and some have been found to be restricted in immunoglobulin composition. Other investigators, however, have found antibodies to factor VIII to be heterogeneous and to contain both kappa and lambda light chains. One factor V antibody has been shown to contain a mixture of light chain types.

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Antibodies to factor IX have not been previously characterized in detail. In the present study, a factor IX inhibitor that developed in a patient with hemophilia B was found to be predominantly, if not exclusively, a \( \gamma G4 \) lambda antibody.

**MATERIALS AND METHODS**

**Patient**

Citrated plasma (8 parts blood: 1 part 3.2% sodium citrate), containing a specific inhibitor of factor IX, was obtained from patient P.W.B. with severe hemophilia B. Clinical studies and specificity of the inhibitor for factor IX have previously been described.2

**Immunoglobulin Isolation and Preparation of Antisera**

Purified immunoglobulins were prepared from patients with an established diagnosis of multiple myeloma or macroglobulinemia. Proteins were isolated, and heavy chains, light chains, and enzymatic digestion fragments were prepared as previously described.10

Antisera specific for \( \gamma G, \gamma A, \gamma M, \gamma D, \gamma E \) classes, C heavy-chain subclasses,11,12 kappa and lambda light-chain types, and Gm genetic markers were prepared by immunization of rabbits, goats, cynomolgus monkeys, rhesus monkeys, or baboons with isolated whole immunoglobulins, polypeptide chains or enzymatic digestion fragments.13 Because of the difficulty in preparing antisera specific for the \( \gamma G1 \) subclass, the technique of immunologic tolerance was used in rabbits.14 Five milligrams of purified \( \gamma G2 \) and \( \gamma G3 \) aggregate-free proteins were given i.v. to induce tolerance, followed by footpad immunization with 0.2 mg of \( \gamma G1 \) protein with an equal volume of complete Freund’s adjuvant. The antisera was adsorbed with isolated Bence Jones proteins, myeloma proteins or digestion fragments, Fraction II, isolated normal \( \gamma G \), or selected whole sera in order to obtain the heavy-chain class, light-chain type, \( \gamma G \) subclass, or Gm specificity as has been described previously.13 Quantitation of immunoglobulin was carried out by single, radial immunodiffusion,15 modified as described previously.10 The partial thromboplastin time (PTT) determination16 was found to be affected adversely by normal rabbit and monkey sera, but not by globulin fractions prepared by precipitation with 50% saturated ammonium sulfate. Ammonium sulfate fractions, redissolved in 0.15 M NaCl and dialyzed until no sulfate was detectable in the dialysate by precipitation with BaCl\(_2\), were used in neutralization experiments, in some instances after concentration with polyethylene glycol. The globulin fractions were oxalated (one part 0.1 M sodium oxalate plus five parts of antisera globulin) and absorbed with BaSO\(_4\) (100 mg BaSO\(_4\)/ml globulin fraction). After continuous mixing at room temperature for 45 min, the material was centrifuged, and the supernatant heated at 56°C for 20 min to destroy clotting factor activity.

**Titration of Factor IX Inhibitor**

Serial dilutions of inhibitor plasma were made with citrated imidazole saline buffer, pH 7.2.17 Each dilution was mixed with equal parts of normal citrated plasma, and the PTT was determined after 1-min incubation at 37°C. Neutralization of Factor IX with this inhibitor had previously been shown to be constant from 1- to 120-min incubation.8 The inhibitor titer was taken as the highest dilution of inhibitor plasma that would prolong the PTT of normal plasma by at least 30 sec.

**Table 1. Neutralization of Factor IX Inhibitor With Specific Antisera**

<table>
<thead>
<tr>
<th>Mixture 1: Neutralization phase</th>
<th>Inhibitor plasma + specific antiserum → precipitate. Ratio, 1:19.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Assay supernatant for residual antigen and antibody to ensure that mixture was in zone of antibody excess.</td>
</tr>
<tr>
<td>Mixture 2: Assay for residual inhibitor</td>
<td>Supernatant from mixture 1 + normal plasma → assay residual factor IX. Ratio 1:1.</td>
</tr>
<tr>
<td></td>
<td>Residual factor IX is directly proportional to amount of inhibitor neutralized.</td>
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</tbody>
</table>
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Factor IX Assay

The specific assay for factor IX was the one-stage test based on the partial thromboplastin time as described previously.\textsuperscript{18}

Neutralization of Factor IX Inhibitor With Specific Antisera

In mixture 1, an attempt was made to neutralize the inhibitor (either in whole plasma or partially purified inhibitor) by using specific antisera (Table 1).\textsuperscript{19} For most experiments, a ratio of one part inhibitor plasma to 19 parts specific antiserum was used. Ratios as low as 1:3 were possible using partially purified inhibitor or highly concentrated antiserum. Controls for each experiment included sera obtained from animals before immunization and processed in a manner identical to the antiserum. The mixtures were incubated for 1 hr at 37°C and then overnight at 4°C. The precipitate was removed by centrifugation at 30,000 g for 30 min, and the supernatant assayed for residual antigen and antibody using Ouchterlony double diffusion and, in some instances, single radial diffusion.\textsuperscript{10} In all experiments reported, the mixtures were in the zone of antibody excess. In mixture 2 (Table 1), the supernatant was assayed for residual factor IX inhibitor by incubating with normal plasma and determining the residual factor IX. Residual factor IX in mixture 2 is directly proportional to the amount of inhibitor neutralized in mixture 1. Additional neutralization experiments included serial dilution of inhibitor plasma in globulins from kappa-specific antiserum and normal rabbit serum.

RESULTS

Heavy-chain Class and Subclass of Factor IX Inhibitor

Initial experiments indicated that the inhibitor was a γG globulin (Table 2). When factor IX inhibitor plasma was mixed with antisera specific for the γA, γD, γE, and γM classes of immunoglobulins in the zone of antibody excess, no inhibitor was neutralized. After mixing with two different antisera specific for heavy chains of the γG class, however, the inhibitor was completely neutralized. If the anti-γG antisera were first adsorbed with purified γG myeloma protein, the effect on the inhibitor was lost, indicating that neutralization was due to the anti-γG specificity. In addition, control globulin fractions obtained from animals prior to immunization had no effect on the inhibitor.

The antibody was further characterized using antisera specific for the γG subclasses (Table 3). The normal percentage and standard deviation for each subclass, as previously determined in a large number of normal adult controls,\textsuperscript{10,26} are shown. The γG4 subclass usually accounts for only 4.2 ± 2.6% of the total γG. In patient P.W.B., the total γG was 7.81 mg/ml, and the γG4 0.45 mg/ml or 5.8% of the total γG. γG1, γG2, and γG3 were also present in normal proportions. Antisera specific for γG1, γG2, and γG3 had no effect on the factor IX inhibitor. However, antisera specific for γG4 neutralized 81

### Table 2. Immunoglobulin Class of Factor IX Inhibitor

<table>
<thead>
<tr>
<th>Anti-γ</th>
<th>Residual Factor IX (% Control)</th>
<th>Residual Inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td>γA</td>
<td>&lt;1</td>
<td>Present</td>
</tr>
<tr>
<td>γD</td>
<td>&lt;1</td>
<td>Present</td>
</tr>
<tr>
<td>γE</td>
<td>&lt;1</td>
<td>Present</td>
</tr>
<tr>
<td>γG</td>
<td>123,104</td>
<td>Undetectable</td>
</tr>
<tr>
<td>γG (adsorbed with purified γG)</td>
<td>&lt;1</td>
<td>Present</td>
</tr>
<tr>
<td>γM</td>
<td>&lt;1</td>
<td>Present</td>
</tr>
</tbody>
</table>
Fig. 1. Ouchterlony analysis of γG4-specific antiserum before and after neutralization of factor IX inhibitor. Well A contains antiserum to γG4 H-chains prepared in a rhesus monkey. There is no precipitin line between purified γG1, γG2, and γG3 myeloma proteins, nor do these proteins inhibit the lines with purified γG4 myeloma proteins or P.W.B. inhibitor plasma. Supernatant after neutralization of inhibitor plasma (S) retains γG4 specificity, indicating that mixture was in zone of antibody excess. In addition, no γG4 antigen is detected in (S) by antiserum (A). There is complete fusion of precipitin lines with inhibitor plasma and purified γG4 myeloma proteins.

and 90% of inhibitor activity in two experiments. The factor IX antibody, therefore, was composed of γG4 heavy chains. The neutralization was completely blocked only by prior adsorption of the antiserum with purified γG4 myeloma proteins, not with those of other subclasses. The specificity of the γG4 antiserum before and after mixing with inhibitor plasma is shown in Fig. 1.

Light-chain Type of Factor IX Inhibitor

Neutralization experiments with antisera to light chains indicated that the inhibitor molecules were composed exclusively of lambda light chains (Table 4). Two antisera specific for kappa determinants had no effect on the inhibitor. On the other hand, three different antisera specific for lambda chains completely neutralized all the factor IX inhibitor activity (Table 4). When lambda specificity was adsorbed with purified lambda Bence Jones protein, the capacity to neutralize inhibitor was lost. Several approaches were employed to look for small amounts of kappa chains among inhibitor molecules. Serial dilutions of inhibitor plasma were made in both rabbit antikappa antiserum and preimmunization rabbit globulin. At each dilution, the effect on factor IX level of normal human plasma was determined (Fig. 2). As can be seen, identical curves were obtained, indicating that kappa antiserum had no detectable effect at any dilution. A second approach, also using whole inhibitor plasma,

<table>
<thead>
<tr>
<th>Anti-</th>
<th>Residual Factor IX (% of control)</th>
<th>Residual Inhibitor</th>
<th>Normal % of Total γG ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>γG1</td>
<td>&lt;1</td>
<td>Present</td>
<td>66 ± 8</td>
</tr>
<tr>
<td>γG2</td>
<td>&lt;1</td>
<td>Present</td>
<td>23 ± 8</td>
</tr>
<tr>
<td>γG3</td>
<td>&lt;1</td>
<td>Present</td>
<td>7.3 ± 3.8</td>
</tr>
<tr>
<td>γG4</td>
<td>81, 90</td>
<td>Undetectable</td>
<td>4.2 ± 2.6</td>
</tr>
<tr>
<td>γG4 (adsorbed with purified γG4)</td>
<td>&lt;1</td>
<td>Present</td>
<td></td>
</tr>
</tbody>
</table>
Fig. 2. Lack of kappa molecules in factor IX inhibitor from patient P.W.B. Serial dilutions of P.W.B. inhibitor plasma in kappa-specific rabbit globulin and preimmunization normal rabbit globulin. All dilutions were in zone of antibody excess. Per cent IX inhibited at each dilution was virtually identical with kappa antiserum and normal rabbit globulin.

was the search for low titers of residual inhibitor after neutralization with lambda antiserum concentrated 30-fold. Using the usual dilution of one part inhibitor plasma to 19 parts anti-\(\lambda\) antiserum in mixture 1 (Table 1), small amounts of kappa-type inhibitor might not be detected. With 30-fold concentrated lambda antiserum, complete inhibitor neutralization was achieved with an inhibitor: antiserum ratio of 1:3, whereas preimmunization globulin concentrated 30-fold had no effect. Inhibitor titer in unneutralized plasma was 1:64, and traces of inhibitor could be detected at 1:300. This indicated that at least 98–99% of the inhibitor was neutralized by the lambda antiserum. Using partially purified inhibitor (titer 1:32), a 1:2 ratio of inhibitor:\(\lambda\)-antiserum was sufficient for antibody excess. The lambda antiserum completely neutralized the partially purified inhibitor.

Partial Isolation and Further Characterization of Factor IX Antibody

Inhibitor plasma was fractionated by preparative zone electrophoresis. The Factor IX inhibitor was found in a sharp peak in three fractions (Fig. 3). These were localized to the extreme anodal portion of the \(\gamma G\) peak, where \(\gamma G4\) normally migrates, and were found localized to the anodal half of the \(\gamma G\) peak determined by radial immunodiffusion. The three fractions with peak inhibitor activity were pooled, concentrated, and passed over a calibrated Sephadex G-200 column in Tris-HCl NaCl pH 8.0 (Fig. 4). The exclusion volume, \(V_o\), was determined simultaneously with a dextran blue marker, and total volume, \(V_t\), was determined with barbital buffer. A narrow zone (fractions 64–68) showed inhibition of the PTT corresponding to peak II OD 280 mm. The elution volume of inhibitor was identical to that of \(\gamma G\) as determined by radial immunodiffusion of column fractions and gave an estimated molecular weight of 145,000. The partially isolated inhibitor was completely neutralized by lambda antiserum, as described above.

<table>
<thead>
<tr>
<th>Anti-</th>
<th>Residual Factor IX (% control)</th>
<th>Residual Inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\kappa)</td>
<td>&lt;1, &lt;1</td>
<td>Present</td>
</tr>
<tr>
<td>(\lambda)</td>
<td>102, 99</td>
<td>Undetectable</td>
</tr>
<tr>
<td>(\lambda) (Higher ratio Inhibitor: IX)</td>
<td>103</td>
<td>Undetectable</td>
</tr>
<tr>
<td>(\lambda) (Adsorbed with purified (\lambda) Bence Jones)</td>
<td>&lt;1</td>
<td>Present</td>
</tr>
</tbody>
</table>
DISCUSSION

The studies show that the factor IX inhibitor from patient P.W.B. is an antibody consisting predominantly, if not exclusively, of \( \gamma G_4 \) heavy chains and lambda light chains. It is of note that no \( \gamma G_4 \) monoclonal band was found on preparative zone electrophoresis, and careful immunoelectrophoretic studies of whole plasma, with particular emphasis on \( \gamma G_4 \) and lambda antisera, gave no indication of a monoclonal band. Thus, this factor IX antibody resembles antibodies to factor VIII.\(^8\)\(^9\) Five of the six antibodies to Factor VIII charac-

Fig. 3. Preparative zone electrophoresis of plasma from patient P.W.B. Sharp peak of inhibitor activity was found in anodal portion of \( \gamma G \) peak and was restricted to anodal portion of \( \gamma G_4 \) subgroup as determined by radial immunodiffusion. There was no overt evidence of a monoclonal band using either total protein, \( \gamma G \), or \( \gamma G_4 \) quantitation, indicating that monoclonal antibody represented a minor population of total immunoglobulin.

Fig. 4. Gel filtration of pooled and concentrated zone electrophoresis fractions 17, 18, and 19 (Fig. 3) on calibrated Sephadex G-200 column equilibrated with Tris-HCl 0.1 \( M \), NaCl 0.5 \( M \), pH 8.0. Sharp inhibitor peak corresponded in elution volume to peak II and coincided with \( \gamma G \) peak. Exclusion volume (\( V_0 \)) was determined with dextran blue, and total volume (\( V_t \)) determined with barbital buffer. Peak III did not contain inhibitor and was not further identified.
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terized as to γG subclass have been composed primarily of γG4 heavy chains. In the experiments reported here, the γG4 antiserum produced 81 and 90% neutralization. Thus, not all inhibitor was neutralized. However, γG antiserum produced complete neutralization and antisera to γG1, γG2, and γG3 had no effect. The γG4 subclass-specific antisera are highly adsorbed and are relatively weakly precipitating. The two γG4 antisera utilized had specificity for γG4 Fc determinants with no affinity for Fab. Although no experimental proof is available, we feel the most likely explanation for the presence of residual inhibitor in the γG4 neutralization experiments is the presence of small amounts of soluble inhibitor-anti-γG4 complexes that retain inhibitor activity.

It is of interest that the most minor γG subclass should predominate among antibodies to both factors VIII and factor IX. This represents the fourth known biologic activity for the γG4 subclass: antibodies to factor VIII, factor IX, glomerular basement membrane,21 and antinuclear antibodies.22 The γG4 subclass is known to be biologically unlike other subgroups in that it does not bind to C1q,23-24 although γG2 binds poorly. The relationship between this property and the propensity for antibodies to factors VIII and IX is uncertain. Despite the presence of circulating antibody and periodic administration of factor IX i.v. with subsequent rise in antibody titer,2, the patient with the factor IX inhibitor manifested neither serum sickness nor evidence for immune complex disease, particularly nephritis. This may relate to the relatively small amount of circulating antibody or to the small amount of factor IX administered, but it may also reflect the lack of complement fixation by antigen antibody complexes. To date, antibodies to clotting factors other than VIII and the presently reported antibody to factor IX have not been characterized as to subclass.

All experiments indicated that the factor IX antibody contained predominantly lambda light chains. Some investigators have found that factor VIII antibodies have a single light-chain type5-9 When minor populations of lower affinity antibodies were sought, however, other investigators found that most antibodies to factor VIII contain a mixture of light-chain types.4 One of the two factor V antibodies recently reported could be characterized as to light-chain content and was found to be heterogeneous.3 To explore this point with the factor IX antibody, several approaches were utilized: neutralization experiments using whole plasma rather than isolated factor IX antibody; concentration of antisera, so that minor populations of antibody containing kappa light chains could be detected; confirmatory studies with partially isolated inhibitor, so that higher concentrations of inhibitor could be used in neutralization experiments; and serial dilutions of inhibitor plasma in kappa-specific antiserum. The availability of higher titer antisera plus high titer factor IX inhibitor in patient P.W.B. permitted the estimate that at least 98% of his inhibitor molecules contained lambda light chains.

The monoclonal nature of the factor IX antibody, both regarding heavy-chain content and light-chain type, suggests further study regarding other properties found in myeloma proteins, i.e., allelic exclusion and individual antigenic specificity. Two genetic markers for γG4 have been described recently.25
but typing requires separation of \( \gamma G4 \) from other \( \gamma G \) subclasses. The donor P.W.B. was Gm \( a+g+f+b+n+ \) and therefore was heterozygous for genetic markers of the \( \gamma G1 \) and \( \gamma G3 \) loci. Thus, he may well be heterozygous for the 4a and 4b markers of the \( \gamma G4 \) locus. Demonstration of allelic exclusion will require purified antibody; such studies are in progress with glutaraldehyde-insolubilized factor IX is an immunoadsorbent. With isolated antibody, further characterization of the lambda light chains regarding presence or absence of \( O_2 \) antigen can be completed. \( O_2 \) antigen relates to a single, amino acid substitution in the constant portion of lambda chains at position 190.\(^{26,27}\) All normal persons contain both \( O_2 (+) \) (lys) and \( O_2 (-) \) (arg) molecules, indicating that \( O_2 \) is not allotypic, but myeloma lambda chains are either (+) or (−). The variable regions of lambda chains, based on sequence studies of myeloma light chains or Bence Jones proteins, are composed of five prototype sequences,\(^{28}\) and individual monoclonal lambda chains are restricted to one of the prototype sequences or variable region subgroups. If truly monoclonal, the lambda chains of the isolated antibody, when characterized, should be either \( O_2(+) \) or \( O_2(-) \), and the variable region should conform to one of these prototype sequences.

Antisera to myeloma proteins and homogeneous antibody populations exhibit the property of individual antigenic specificity, probably reflecting antigenic uniqueness of sequences related to the combining site.\(^{28}\) Antibodies of the same or very closely related specificity exhibit the phenomenon of cross-specificity, probably related to sequence similarities in the combining site regions, e.g., human cold agglutinins.\(^{29}\) The kappa chains of purified human \( \gamma G \) monoclonal rheumatoid factors found in hypergammaglobulinemic purpura have exhibited sequence homologies in the hypervariable regions of heavy and light chains.\(^{30-33}\) The factor IX antibody reported in the present study appears to have the same specificity for factor IX as that from patient R.T.J.\(^2\) Unfortunately high-titer antibody from R.T.J. was not available and thus, could neither be characterized in the present study, nor its individual specificity compared with that of P.W.B. antibody. Ten per cent of males with hemophilia B possess cross-reactive material (CRM*) that is biologically inactive but combines with antibody from either of these two patients.\(^{34}\) Family studies of CRM* patients have shown that all affected members are CRM* as well. These antibodies appear to have specificity for antigenic determinants shared by normal factor IX and CRM* biologically inactive factor IX. The monoclonal nature of these factor IX antibodies may relate to the discrete nature of the antigenic site recognized on the factor IX molecule and may reflect very limited structural difference between normal factor IX and the factor IX found in CRM* patients.

Immunization with complex antigens, such as blood group substances, diphtheria, and tetanus toxoids, usually produces a heterogeneous antibody response in which \( \gamma G4 \) subgroup occurs in its normal minor proportions.\(^{35}\) Isolated human antibodies to the carbohydrate polymers, dextrans, levan, and teichoic acid have been shown to be restricted to the \( \gamma G2 \) subgroup and restricted in light-chain content when further fractionated as to oligosaccharide
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In two heterozygous donors, the antibodies exhibited the property of allelic exclusion so characteristic of myeloma proteins. Several had previously been shown to exhibit individual antigenic specificity. The monoclonal nature of the presently reported factor IX antibody is indicated by heavy- and light-chain homogeneity in neutralization experiments. Isolation of antibody will permit many additional studies exploring the questions of allelic exclusion, binding affinity, and antigenic and structural studies relating to the variable region.

ACKNOWLEDGMENT

It is a pleasure to acknowledge the technical assistance of Mr. Randall Fuller and Mr. Ronald Neal.

REFERENCES


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