Polymorphism of normal factor IX detected by mouse monoclonal antibodies

(immunoradiometric assay/hybridoma/factor IX coagulant activity/factor IX antigen)

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ABSTRACT Hemophilia B is an X-chromosomal recessive disease due to deficiency of coagulation factor IX. Three monoclonal antibodies against factor IX were prepared and used to develop immunoradiometric assays (IRMAs) of factor IX antigen (IX-Ag). IX-Ag was measured in 65 normal individuals with one IRA based on polyclonal anti-IX antibodies and two IRMAs based on three monoclonal anti-IX antibodies. One of the monoclonal antibodies differed in specificity since it neutralized ≤50% of the clotting activity of factor IX (IX-C), whereas the other two monoclonal antibodies neutralized 80–95%. When the former antibody was used as the solid phase in IRMA, two groups of normal individuals were distinguished: group A with measurable IX-Ag, and group B without detectable IX-Ag. There were no differences between the groups either in IX-C or in IX-Ag measured with polyclonal antibodies. A subgroup comprising only women could be distinguished in group A, in whom intermediate IX-Ag concentrations were found. Family studies showed the group B variant of normal factor IX to be transmitted according to the pattern of X-linked recessive inheritance. The allelic frequency of group A was 0.66, and that of group B was 0.34.

Hemophilia B is due to a deficiency of factor IX, a coagulation protein involved in the intrinsic phase of blood coagulation. The disease has a recessive X-linked mode of inheritance and can be classified as severe, moderate, or mild, according to the biological activity of factor IX (IX-C) in plasma (1, 2). Molecular variants of factor IX in hemophilia B were detected after it had become possible to measure factor IX antigen (IX-Ag) by using different immunological assays (3–5). The two other monoclonal antibodies were directed against the active site of the protein (3–6). Factor IX antigen concentrations varied widely from family to family, partly owing to differences in the specificity of the various antibodies used.

The structure of normal human factor IX has been determined by isolation and characterization of a cDNA, and was found to be a polypeptide chain composed of 416 amino acids (8). The polypeptide chain is activated by cleavage of two internal peptide bonds and transformed to a serine protease composed of a light and heavy chain (9, 10), the active site being located in the heavy chain (8). Recently, a restriction fragment length polymorphism has been observed in the factor IX gene by means of cDNA probes derived from mRNA (11–13).

In this work, molecular variants of the normal human factor IX are demonstrated by measuring IX-Ag in normal individuals with immunoradiometric assays (IRMAs) based on three monoclonal antibodies and one polyclonal antibody. When one of the monoclonal antibodies was used as solid phase in the assays 80 of the 29 men (34%) investigated and 5 of the 36 women (14%) had no detectable IX-Ag. A subgroup comprising at least 6 females with intermediate concentrations of IX-Ag was distinguished among the remaining 31 females. However, no differences in IX-C were found, despite the differences in IX-Ag. Analyses of normal females showed the variant with undetectable IX-Ag to be transmitted according to the pattern of X-linked recessive inheritance.

MATERIALS AND METHODS

Subjects. Blood was collected in 3.8% sodium citrate solution (9:1, vol/vol) from 65 normal individuals (male patients and laboratory staff, 29 men, 36 women), their mean age being 34 years (range 18–59). In addition, 35 propositi were selected for family studies, which led to the addition of 27 subjects to the study. Platelet-poor plasma was prepared by centrifugation at 2000 × g for 20 min. The plasma samples were stored at −80°C until analyzed. Plasma was also obtained from a patient with severe hemophilia B, who had been defined in earlier studies as cross-reacting material negative (CRM−) (6).

Immunization, Cell Fusion, and Growth of Clones. Factor IX was purified as described (14, 15), and was activated by purified human factor Xla. Both factor IX and factor IXa were kindly supplied by W. Kiesie (Department of Biochemistry, University of Washington, Seattle). Sixty microliters of purified factor IX was emulsified in complete Freund's adjuvant (Difco) and injected subcutaneously into 3-month-old BALB/c mice. After 25 days the mice received subcutaneous booster injections of 60 μg of protein without adjuvant. A final intravenous injection of 60 μg was given 9–10 weeks after priming. Four days later the spleen cells of the mice were harvested, fused with Sp 2/0-Ag 14 mouse myeloma cells, and seeded in 96-well culture plates as described (16). Culture supernatants were screened for specific antibodies 10–20 days after fusion. Selected positive hybridomas were cloned four times by using the limiting dilution technique (17) and preserved in liquid nitrogen or grown as ascites tumors in BALB/c mice.

Screening of Antibodies Against Factor IX. Hybridoma supernatants were screened for specific antibodies by using a solid-phase radioimmunoassay with purified factor IX coated to the wells of polyvinylchloride microtiter plates and radiolabeled staphylococcal protein A as developing reagent, essentially as described by Buchanan et al. (18).

Abbreviations: IX-C, factor IX coagulant activity; IX-Ag, factor IX antigen; IRMA, immunoradiometric assay.
Purification and Radiolabeling of the Monoclonal Antibodies. The total immunoglobulin G (IgG) was prepared from ascites fluid by ammonium sulfate precipitation, overnight dialysis, and DEAE-Sephaloc ion-exchange chromatography (16). The IgG was further purified by affinity chromatography on factor IX-Sepharose 4B, using a batch procedure (19). The eluted IgG was assayed and stored in Tris-buffered saline, pH 7.5, at 4°C in the presence of 0.1% (15 mM) sodium azide. The purified antibodies were labeled with 125I by the lactoperoxidase method (20). Specific activities obtained were about 1.0 MBq/µg of IgG.

Neutralization Test. The anti-IX-C effect of the monoclonal antibodies was tested as described (19). The IgG preparations in various dilutions (60–1000 µg/ml) were incubated for 60 min at 37°C with 5 units/ml purified factor IX (=35 µg/ml) or factor IXa (=0.9 µg/ml), and the residual IX-C was measured (see below). Reference curve was constructed of serial dilutions of purified factor IX, incubated with 3 µg/ml of an unrelated monoclonal antibody (R2) against factor VIII-related antigen (21).

Monoclonal Anti-IX-Sepharose. Purified monoclonal antibodies 9.1 and 9.9 (see below) were coupled to cyanogen bromide-activated Sepharose 4B (5 mg of IgG per ml of gel) as recommended by the manufacturer (Pharmacia). Pooled plasma (4 ml) from two normal individuals was assayed for various clotting factors (see below) both before and after passage through the column. The results were compared with those obtained when purified normal human IgG was coupled to Sepharose 4B by using the same procedure.

Analyses. The protein content of the IgG preparations was measured with Bradford’s method (22), and the IgG content was determined immunochemically by using Laurell’s rocket technique (23) and commercial antisera (Sigma). The IgG samples were carbamoylated before electrophoresis. Non-DetSO4/polyacrylamide gel electrophoresis of the purified monoclonal antibodies was performed in 8.5% polyacrylamide gels both with and without a reducing agent (24). The IgG subclasses and the light chains of the monoclonal antibodies were determined by using the same technique as in the screening procedure, specific antisera to mouse IgG1, IgG2a, IgG2b, IgG3, 8 chain, or 6 chain (Miles) being used as second layer.

Polyclonal Antibodies Against Factor IX. Polyclonal antibodies were isolated from a hemophilia B patient who had an inhibitor titer of 50 Malmö units/ml (corresponding to 150 Bethesda units/ml). The IgG was prepared and radiolabeled as described (25).

IX-C Assay. IX-C was measured in all subjects by using a one-stage recalcification assay as described by Nilsson (26). Citrated pooled plasma from 20 normal individuals was used as reference plasma. The activity was expressed as units/dl. One unit was defined as the activity present in 1 ml of normal plasma. The normal range was 60–140 units/dl.

Other Coagulation Assays. Factor XI, XII, fibrinogen, factor VIII coagulant activity (VIII-C), and factor VIII-related antigen (VIII-R-Ag) were assayed as described (26, 27). Antithrombin III and a2-antiplasmin were determined amidolytically, using S-2238 and S-2251 (KabiVitrum, Stockhlom, Sweden), respectively.

IX-Ag Assays. IX-Ag was determined in all subjects with three different two-site solid-phase IRMAs. IRMA I was based on the polyclonal human hemophilic antibody, which was used both for the immobilization of IX-Ag and (radio-)labeled for the detection of bound IX-Ag (25). The normal range was 60–160 units/dl. In IRMA II and IRMA III, the monoclonal antibody 9.9 (see below) was used as the solid phase, and the radiolabeled monoclonal antibodies 9.1 (IRMA II) or 9.5 (IRMA III) were used for detecting bound factor IX. Assay technique: Styrene microtiter wells (Removastrips; Dynatech, Lidingö, Sweden) were coated with IgG by incubation of 300 µg of 0.1 M sodium carbonate buffer, pH 9.6, containing 15 µg of IgG per ml (IRMA I) or 30 µg of IgG per ml (IRMA II and III) for 18 hr (IRMA I) or 36 hr (IRMA II and III) at 4°C. The wells were washed three times with 300 µl of phosphate-buffered saline/0.1% bovine serum albumin, pH 7.4. The plasma samples to be tested, serially diluted in phosphate-buffered saline/6% bovine serum albumin, were added to the wells in volumes of 200 µl and incubated for 18 hr at room temperature. After washing, 200 µl (20,000 cpm) of labeled antibody solution was added and the wells were again incubated for 18 hr at room temperature. The wells were then washed three times with 300 µl of distilled water and their radioactivities were measured with a 1260 Multigamma (LKB). A standard curve was constructed from serial dilutions of the same reference plasma as used for IX-C. Maximal binding of radioactivity was 20–25% in both IRMA II and IRMA III. A linear dose–response curve was obtained for dilutions 1:32–1:1024 for both IRMAs. The sensitivity limit of these methods was 0.10 unit/dl. Coefficients of variation within assay at different plasma dilutions were 10% and between assays they were 15%.

RESULTS

Monoclonal Antibodies Against Factor IX. Ninety-six wells showed hybridoma growths and initially there were 12 positive cultures when the supernatants were tested by means of the screening assay. Three of these (9.1, 9.5, and 9.9) were recloned four times and grown in BALB/c mice for ascites production. The monoclonal antibodies were prepared from ascites fluid, and the purity of the fractions was confirmed by SDS/polyacrylamide gels, which showed only one band in the IgG region, two bands when a reducing agent was present. Protein content in the pooled fractions was 0.5–2.0 mg/ml, and all three monoclonal antibodies were found to belong to the subclass IgG1 with light chains.

Neutralization Test. The residual IX-C after incubation of purified factor IX or factor IXa with each of the three monoclonal antibodies and human polyclonal anti-IX antibodies is shown in Fig. 1. The 9.5 antibody inhibited both factor IX and IXa most efficiently, even more than the polyclonal antibodies. The 9.9 antibody showed weaker

![Fig. 1](image-url)
inhibitory activity than either 9.1 or 9.5, never exceeding 50% inhibition, even at a concentration of 1000 μg of IgG per ml.

Monoclonal Anti-IX-Sepharose. Coupled to Sepharose 4B, antibodies 9.1 and 9.9 removed ~95% of IX-C in plasma passed through the columns. No such effect was found with normal human IgG similarly coupled to Sepharose. Other coagulation factors investigated in the passed plasma were unaffected (not shown).

IX-C. Normal IX-C concentrations were obtained in all 65 normal individuals and in all 27 members in the three families investigated (Table 1).

IX-Ag. When IRMA I, in which the same polyclonal human antibodies were used for coating and detection, was used, all normal individuals and family members investigated had IX-Ag concentrations within the normal range. In IRMAs II and III, the monoclonal antibody 9.9, which neutralized <50% of IX-C, was used for coating. For detecting bound IX-Ag, the radiolabeled monoclonal antibody 9.1 (IRMA II) or 9.5 (IRMA III) was used. Two distinct groups emerged when IX-Ag was measured with these two methods: group A, with measurable IX-Ag (19 men, 31 women), and group B, without measurable IX-Ag (<0.1 unit/dl) (10 men, 5 women). There was no difference in IX-C or IX-Ag (IRMA I) between the groups according to two-way analysis of variance (between-groups F (1/61) = 0.07 and 0.22; between-sex F (1/61) = 0.01 and 0.50; interaction F (1/61) = 0.08 and 0.05, respectively). Linear regression analysis showed a significant correlation between the results obtained by IRMAs II and III (r = 0.89). When plasma from a patient with severe hemophilia B was tested by using the different IRMAs, no binding of radioactivity was found (Fig. 2). Fig. 2 also shows the differences in bound radioactivity obtained when pooled plasma from 6 men belonging to group A was used instead of the pooled plasma from 20 normal individuals described earlier. Fig. 3 demonstrates the distribution of IX-Ag in group A when measured with IRMAs II and III, using the reference curve based on pooled group A plasma. As shown in Fig. 3, a subgroup of at least six women was found to have intermediate IX-Ag concentrations (9-60 units/dl). IX-Ag was also measured in 20 normal individuals (10 belonging to group A and 10 to group B) with an IRMA based on the monoclonal antibody 9.9 as solid phase and the polyclonal anti-IX antibody as radiolabeled tracer. Results were comparable to those of IRMAs II and III.

Competitive Test. To ascertain whether an unspecific factor present in group B plasmas interfered with the binding of IX-Ag to antibody 9.9, the following test was performed: IX-Ag in a group A plasma (measurable IX-Ag with monoclonal IRMA) was mixed with phosphate-buffered saline/6% bovine serum albumin at four different dilutions (1:50, 1:100, 1:200, 1:400). Each dilution was then mixed with equal volumes of a serially diluted group B plasma (unmeasurable IX-Ag with monoclonal IRMA) or buffer/6% albumin and IX-Ag was measured with IRMA III. The results obtained at dilution 1:100 are shown in Table 2 (similar results were obtained at the other dilution levels). No interfering factor in group B plasma could be demonstrated.

Family Studies. To evaluate the hereditary pattern of the observed variants of factor IX, three families were investigated. In family I (Fig. 4a) none had IX-Ag measurable by using the monoclonal IRMAs (i.e., all tested belonged to group B). In family 2 (Fig. 4b) individual I-1 belonged to group A, whereas I-2 had intermediate values. Their daughter, II-2, had the same low values as the mother, whereas her son, II-3, had unmeasurable IX-Ag (i.e., he belonged to group B). II-3 had three sons; the two tested belonged to group A like their mother. In family 3 (Fig. 4c) I-3 belonged to group B, whereas I-4 belonged to group A. Their sons, II-2 and II-3, belonged to group A. The grandsons, III-2 and III-4,

Table 1. IX-C and IX-Ag measured with three different IRMAs in 65 normal individuals

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>IX-C, units/dl</th>
<th>IRMA I</th>
<th>IRMA II</th>
<th>IRMA III</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Men</td>
<td>19</td>
<td>103 ± 16</td>
<td>99 ± 24</td>
<td>145 ± 44</td>
<td>145 ± 31</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>104 ± 23</td>
<td>106 ± 28</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Women</td>
<td>31</td>
<td>104 ± 23</td>
<td>108 ± 37</td>
<td>105 ± 52</td>
<td>108 ± 54</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>101 ± 22</td>
<td>110 ± 27</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Total</td>
<td>50</td>
<td>104 ± 20</td>
<td>104 ± 32</td>
<td>120 ± 53</td>
<td>122 ± 50</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>102 ± 22</td>
<td>107 ± 27</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
</tr>
</tbody>
</table>

IRMA I is based on polyclonal human hemophilic antibodies. IRMAs II and III are based on monoclonal antibodies. All results are mean ± SD.
belonged to group B and A, respectively, although the IX-Ag concentrations of their mothers were similar to each other.

**DISCUSSION**

Both allogenic and xenogenic antibodies against factor IX have been used in various forms of immunologic assays and a genetically determined heterogeneity has been discovered among hemophilia B patients (3-6). A polymorphism of the normal factor IX has never, however, been found with these polyclonal antisera. Owing to its exquisite specificity for a structural conformation or epitope, a monoclonal antibody is capable of detecting small structural differences between proteins. The anti-IX antibodies produced were judged to be monoclonal because (i) the hybridoma lines were recloned four times before intraperitoneal injections, (ii) each of the isolated antibodies represented one defined isotype—\(\text{IgG1 k}\), and (iii) each antibody migrated as a single band on sodium dodecyl sulfate/polyacrylamide gels. They may be regarded as being specific for IX-Ag since they bind to purified human factor IX and to factor IX present in normal pooled plasma but do not react with plasma from a patient with severe hemophilia B. When immobilized on Sepharose 4B, the antibodies remove factor IX-C selectively from normal plasma. Antibodies 9.1 and 9.5 had similar characteristics and neutralized 80–95% of IX-C, whereas the third monoclonal antibody (9.9) had distinctly different properties and neutralized <50% of IX-C. Presumably antibody 9.9 is directed against an immunoreactive site remote from the part of the molecule associated with the clotting activity, and structural differences in this part of the molecule are less prone to affect the clotting activity of the protein.

In the present investigation 65 normal individuals were examined for IX-Ag with different IRMAs. When the monoclonal antibody 9.9 was used for coating, and another monoclonal or polyclonal anti-IX antibody for detecting, two distinct groups emerged: group A (66% of the men and 86% of the women) with measurable IX-Ag, and group B (34% of the men and 14% of the women) without demonstrable IX-Ag. Despite the differences in IX-Ag, there was no difference in IX-C between the two groups, indicating that there exist two structurally different factor IX proteins with normal function. Subjects belonging to group B lack the epitope against which antibody 9.9 is directed and which is present in group A. The observed gene frequency in group B agreed well between sexes (men 0.34 and women 0.37). Since similar results were obtained when either polyclonal or monoclonal antibodies were used as a tracer, we conclude that the differentiation between group A and B was dependent on the properties of the monoclonal antibody 9.9. A subgroup comprising only women could be distinguished in group A, in whom intermediate IX-Ag concentrations were found. This finding suggested a heterozygous state of the allele responsible for the factor IX protein present in group A. Pedigree analysis clearly showed group B variant of factor IX to be inherited in the X-linked recessive way. Family 1 (Fig. 4a) obviously has only one of the alleles for factor IX. Families 2 and 3 (Fig. 4b and c) have both alleles for factor IX. Members I-2 and II-2 in family 2 have IX-Ag concentrations in accordance with their heterozygous state. Member II-1 in family 3 has normal concentrations despite a presumed genetically heterozygous state, which can be explained as the effect of an extreme inactivation of one X chromosome (28). Since member II-4 has a son belonging to group A it is impossible to decide if she is a heterozygote or belongs to group A.

One explanation of the results could be that an unspecified factor in some normal plasmas interfered with the IRMAs based on monoclonal antibodies. This possibility was ruled out since the results obtained for persons belonging to group A were unaffected when their plasmas were mixed with those of patients belonging to group B (Table 2).

The mean values of IX-Ag and the standard deviations were greater when measured with the IRMAs based on monoclonal antibodies than with the IRMA based on polyclonal antibodies (Table 1). Increased mean values were to be expected since the reference plasma was a mixture of 20 normal plasmas (both men and women), in which ~20% may be assumed to have IX-Ag unmeasurable with the monoclonals, thus giving a false reference curve (Fig. 2). As shown in Fig. 3, the lowest IX-Ag concentrations in group A were found in females. If the frequency of males belonging to group A is assumed to represent an accurate estimate of the gene frequency of group A, 16 heterozygote females should have been expected according to the Hardy–Weinberg law. However, it was not possible to distinguish unequivocal heterozygotes because a considerable overlapping between heterozygotes and homozygotes can be assumed by analogy with
the overlapping found in IX-C between carriers of hemophilia B and normal individuals.

Several presumably heterozygous females were included in group A, which accounts for the increased range in standard deviations.

Earlier studies with monoclonal antibodies against factor IX did not reveal polymorphism in the normal population (29–35). The monoclonal antibody used in the study by Thompson (30) was directed against structures on the heavy chain, where the coagulation active site is situated. Two recent studies of DNA in normal individuals have revealed a restriction fragment length polymorphism of the factor IX gene (11, 13). It is noteworthy that the allelic frequencies found with the Taq I restriction enzyme in the two studies (11, 13), 0.29/0.71 and 0.35/0.65, respectively, are of magnitude comparable to those found in our study for men belonging to group B or A (0.34/0.66). However, the two normal factor IX proteins demonstrated in our study cannot be the products of the two alleles shown by the Taq I restriction enzyme since the Taq I polymorphic site is within an intron of the gene (12, 36). Differences have been found in the nucleotide sequence of the human factor IX mRNA at position 609 in two studies (36, 37). Such a difference would alter the amino acid sequence in the activation peptide. However, this cannot be the polymorphic site in our study since antibody 9.9 reacts with both factor IX and factor IXa.

An inherited variation in protein structure without the function being affected is recognized in several other plasma proteins, such as haptoglobin, transferrin, immunoglobulins, albumin, and α1-antitrypsin (38–43). The structural basis for the allelism of factor IX—whether it is a substitution, deletion, or insertion in the molecule—has yet to be established.

Our conclusion is that there exist at least two genetically determined molecular variants of normal human factor IX, and we propose that they be designated IX-1 (group A) and IX-2 (group B). Studies with other monoclonal antibodies together with further knowledge of the factor IX gene may reveal a yet more complex polymorphism. The polymorphism may also prove useful as a marker of the X chromosome in carrier detection.

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