Intragenic Factor IX Restriction Site Polymorphism in Hemophilia B Variants

By H.J. Hassan, M. Orlando, A. Leonardi, C. Chelucci, R. Guarriero, P.M. Mannucci, G. Mariani, and C. Peschle

This study includes 47 normal subjects and 25 hemophilia B patients without inhibitor(s), showing different factor IX coagulant activity and antigen levels. Genomic DNA, digested with various restriction endonucleases, was hybridized with two different factor IX probes, ie, the cDNA and the subgenomic probe for the intragenic TaqI polymorphic site. cDNA restriction patterns suggest absence of gross rearrangements and/or deletions in all hemophilic patients. The frequency of the X chromosome bearing the TaqI polymorphic site is 0.32 ± 0.09 in hemophilic subjects 0.36 ± 0.06 in normal control subjects, the latter value being comparable to that reported for the normal British population. No association between this polymorphism and hemophilia B variants has been observed, thus indicating that a wide spectrum of mutations underlies this blood-clotting disorder and particularly each of its variants.

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RESULTS

Factor IX C and factor IX antigen values from normal and hemophilia B subjects are summarized in Table 1. Sixty percent of the patients were B'B, with factor IX C levels of <1 U/dL and factor IX antigen undetectable, when assayed with Laurell immunoelectrophoresis. Factor IX antigen levels for eight of 13 B'B patients were also assayed by means of ELISA; low but detectable factor IX antigen levels (0.8 to 7.0 U/dL) were found. Twenty-eight percent of the patients were B'B', with factor IX C levels of <2.7 U/dL and factor IX antigen levels within the normal range; one of these patients was classified as B'B, on the basis of a prolonged prothrombin time, when assayed with an ox brain thromboplastin (Thrombotest [Nyegaard, Oslo] = 65", normal values 35 to 45"). The remaining 12% of the patients were B'B, with factor IX C levels ranging from 1 to 7 U/dL and factor IX antigen levels between 25 and 33 U/dL.

Genomic DNA of hemophiliacs, digested with BamHI, EcoRI, and HindIII endonucleases and hybridized to the cDNA probe, always showed a normal restriction pattern (Fig 1).

Digestion of hemophilic DNA with TaqI and hybridization with the factor IX subgenomic probe (Materials and Methods) yielded a 5.6-kb fragment

Hemophilia B is an inherited bleeding disorder resulting from deficiency or abnormality of blood-clotting factor IX, which is essential for the coagulation process. Three major groups of hemophilia B variants have been described, which differ in the levels of factor IX antigen: (1) B' variant, with normal levels of factor IX antigen; (2) B-reduced (B8) variant, with reduced but detectable levels of antigen; and (3) B variant, with low or undetectable levels of factor IX antigen. Recently, the human factor IX gene has been cloned and characterized in some detail. cDNA and genomic factor IX probes have been developed and used to reveal a TaqI restriction fragment-length polymorphism (RFLP) in the 5'end of the gene, in the intervening sequence downstream from exon “d”. This frequent RFLP can be used for both carrier detection and prenatal diagnosis of hemophilia B.

We have analyzed here the structure of the factor IX gene and the TaqI RFLP in 47 normal and 25 hemophilic subjects (B'B', B'B, B'B8) without factor IX inhibitors.

MATERIALS AND METHODS

Peripheral blood samples from 47 normal control subjects (20 women and 27 men) and 25 hemophilia B patients were collected into 1:10 vol of 3.2% trisodium citrate. Factor IX coagulant (factor IX C) levels were determined on platelet-poor plasma by the one-stage assay, and factor IX antigen levels were determined by Laurell immunoelectrophoresis and enzyme-linked immunosorbent assay (ELISA, Stago, Asnieres/Seine, France). Blood cells, washed with 0.15 mol/L saline, were stored at −20°C until used.

High molecular weight DNA, obtained from peripheral leukocytes by standard techniques, was digested with TaqI, BamHI, EcoRI, and HindIII restriction endonucleases (4 U/μg of DNA) according to the supplier’s specifications (Biotec Inc, Boehringer, Mannheim, West Germany). Restricted DNA was electrophoresed on 0.8% agarose gels, transferred to nitrocellulose filters, and hybridized with 100 ng of 32P-labeled DNA probe (specific activity, 3 to 9 × 106 dpm/μg) as previously described.

Factor IX probes were the subgenomic 2.5 kilobases (kb) (“probe VIII”) and the cDNA fragment (“probe V”), which were kindly provided by G.G. Brownlee, Oxford, England.

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associated with either a 2.1-kb (Taq) or a 1.6-kb (Taq') band, related to either absence or presence of RFLP, respectively. The frequency of the X chromosome bearing the polymorphic site in the overall hemophilic sample was 0.32 ± 0.09, which was not significantly different from that of the normal control subjects (ie, 0.36 ± 0.06; P > .5) (Table 2). Obviously, all three bands were present in women heterozygous for this polymorphism (Fig 2).

Interestingly, no significant correlation was observed between frequency of the polymorphic site and levels of factor IX antigen in either of the variants.

**DISCUSSION**

The molecular basis of the factor IX "deficiency" causing hemophilia B is almost unknown. Hemophilia B patients have been subclassified in B, B, and BR groups on the basis of the factor IX antigen level, as evaluated by means of either allogenic or xenogenic antiseraums. Only a few cases have been further characterized on the basis of the biochemical and/or functional properties of the abnormal factor IX protein. An aminoacidic substitution (ie, 145 Arg → His) has been determined only in factor IX Chapel Hill. Nevertheless, a wide spectrum of molecular defects (ie, a number of mutations leading to similar phenotypes) may underlie the abnormal or reduced synthesis of factor IX, as suggested by the heterogeneity of the molecular pathology of thalassemic syndromes.

Partial gene deletions have been observed recently in five British hemophilia B patients, all of whom had developed an inhibitor against factor IX after substitutive therapy. In our patients, gross deletions or rearrangements within the factor IX locus are not apparent, as indicated by gene mapping with three restriction endonucleases, at least within the region analyzed by means of the factor IX cDNA probe. Interestingly, no inhibitor was present in any of the patients reported here. In this regard, hemophilia B cases with factor IX inhibitor are relatively rare (< 1%), while the prevalence of inhibitors in hemophilia A is ~10%. To our knowledge, hemophilia B patients with inhibitor(s) have not been identified in Italy.

RFLPs within or around structural genes are widely used to trace mutant genes by linkage analysis. In the factor IX gene, a TaqI polymorphism, identified within the intervening sequence downstream from exon "d," has been used for segregation analysis in

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**Table 1. Factor IX Levels in Hemophilia B Patients**

<table>
<thead>
<tr>
<th>Variants</th>
<th>No. Cases</th>
<th>Factor IX C Level (U/dL)</th>
<th>Factor IX Antigen Level (U/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>15</td>
<td>&lt;1-7</td>
<td>&lt;10</td>
</tr>
<tr>
<td>B'</td>
<td>4</td>
<td>&lt;1-7</td>
<td>23-33</td>
</tr>
<tr>
<td>B&quot;</td>
<td>6</td>
<td>&lt;1-2.7</td>
<td>86-112</td>
</tr>
<tr>
<td>Normal controls</td>
<td>&gt;100</td>
<td>68-128*</td>
<td>71-119*</td>
</tr>
</tbody>
</table>

*95% confidence limits.

**Table 2. Relative Frequency of TaqI Polymorphism in Normal and Hemophilia B Subjects**

<table>
<thead>
<tr>
<th>Subjects</th>
<th>No. X Chromosomes</th>
<th>Taq* (Frequency ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal men</td>
<td>27</td>
<td>0.26 ± 0.08</td>
</tr>
<tr>
<td>Normal women</td>
<td>40</td>
<td>0.43 ± 0.08</td>
</tr>
<tr>
<td>Total</td>
<td>67</td>
<td>0.36 ± 0.06</td>
</tr>
<tr>
<td>Patients</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B' variants*</td>
<td>7</td>
<td>0.29 ± 0.17</td>
</tr>
<tr>
<td>BR variants</td>
<td>3</td>
<td>0.33 ± 0.27</td>
</tr>
<tr>
<td>B variants</td>
<td>15</td>
<td>0.33 ± 0.12</td>
</tr>
<tr>
<td>Total patients</td>
<td>25</td>
<td>0.32 ± 0.09</td>
</tr>
</tbody>
</table>

*including one patient classified as B" variant (Table 1).
several families with factor IX deficiency. In our normal cases, the frequency of the TaqI polymorphic site did not differ significantly from that reported for the British population, thus indicating absence of geographical selection. We have also observed that the frequency of TaqI polymorphism is similar for both hemophilic and normal factor IX X chromosomes. More important, no preferential association of the polymorphism has been detected in three immunologic variants of hemophilia B. In this regard, a tight linkage may be assumed to exist between mutations in the factor IX locus and the TaqI intragenic site, as observed between mutations in the globin gene domains and polymorphic sites within these regions. It follows that the random distribution of TaqI polymorphism in hemophilia B variants strongly indicates that a wide spectrum of mutations underlies this blood clotting disorder and particularly each of its variants.

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REFERENCES

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