Diagnostic Role of an Immunoassay-Detected Polymorphism of Factor IX for Potential Carriers of Hemophilia B

By Arthur R. Thompson, Shi-Han Chen, and Kenneth J. Smith

In hemophilia B, assays based on a monoclonal antifactor IX specific for the Thr-148 variant of an exonic polymorphism have diagnosed carriers in selected families by either establishing linkage or by indicating the presence or absence of a given normal factor IX. The sensitivity of the immunoassays for detecting heterozygous women was explored by comparing results from immunoassays with solid-phase polyclonal vs the monoclonal antifactor IXs. Factor IX with the normal Ala-148 variant gave a flat dilution curve, qualitatively distinct from factor IX with the Thr-148 variant in the monoclonal assay. The two were indistinguishable in the polyclonal assay. Mixtures of equal amounts of the two types gave an intermediate result, about half as reactive in the monoclonal as compared with the polyclonal assay system. Whereas mixtures with 10% Ala-148 and 90% Thr-148 factor IXs could not readily be distinguished from Thr-148 factor IX plasma, as little as 1% of the Thr-148 protein was detected in Ala-148 factor IX plasma. The frequency of the Ala-148 variant varied in individuals with different ethnic backgrounds: it was found in 29% of white, 12% of black, and none of Asian blood donors’ factor IX genes in Seattle. Only 4% of samples from South African black men were nonreactive (ie, Ala-148). The Thr/Ala-148 dimorphism is in strong linkage disequilibrium with TaqI restriction fragment length polymorphisms (RFLPs). Three recombinations were noted in normal white genes and one in a normal black factor IX gene (<2% of those examined). In 34 white families with at least one woman being a possible carrier, genetically, the immunoassay results were informative in 18. RFLP analyses were informative in eight of the 15 families tested. In five families each, assignment of carrier status was made to a woman by only DNA or only immunoassay results, whereas the other approach was noninformative. The immunoassays provide a rapid, inexpensive screening test and complement DNA analysis in white women who are potential carriers of hemophilia B.

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run as negative controls in all batches of monoclonal IRMAs. In all immunoassays, nonspecific binding using plasmas without detectable factor IX, either as congenital defects with undetectable factor IX antigen levels or immunoaffinity-depleted plasma, was <1% and there were no dose-response or dilution effects. The monoclonal IRMA was sensitive to 2 ng/ml (0.04 U/dL). Coefficients of variation on day-to-day determinations of the standard curve were <10%.

Leukocyte DNA was prepared, digested with TaqI, electrophoresed, blotted, and hybridized with 32P-factor IX-cDNA as previously described.13

Subjects' plasma samples. Normal white, black, or Asian subjects were consenting blood donors drawn during 9 months at the Puget Sound Blood Center in Seattle. They were distinct from those reported previously10 and those in a recent series of RFLPs.9 Repeat donors were excluded. Subjects were assumed to be unrelated although relatives were not specifically identified. Asian samples included persons of Chinese, Japanese, and Filipino ancestry. Samples from African black men were from a previous series of plasmas drawn into acid-citrate-dextrose in South African black health care facilities for genetic marker studies and had been stored at 15°C for 8 years. These subjects represented ten distinct tribes, and samples were provided by Dr Eloise Gibblett.

Samples from hemophilia B patients and their family members were as drawn or referred for diagnosis or carrier testing in Seattle. Consent was obtained as approved by the University of Washington’s Human Subjects Review Committee. Plasmas were rendered platelet poor and stored at 15°C, although most samples were tested by immunoassays before freezing. For several normal and hemophilic individuals, 15 to 30 mL venous blood in heparin or EDTA was simultaneously collected for leukocyte DNA preparations.

RESULTS

Detection of Thr/Ala-148 variants by immunoassays. The distinction of two types of factor IX by immunoassays is shown in Fig 1. Two male subjects each had levels of factor IX antigen just above that of the normal pool (Fig 1A), but only the one with Thr-148 factor IX was at the same level when the solid-phase monoclonal IRMA was used (Fig 1B). When factor IX contained Ala-148, the monoclonal IRMA showed minimal reactivity and a nearly flat dilution curve. Of 17 different normal men with markedly reduced reactivity in the monoclonal IRMA examined for this study (not previously published), all were associated with the same, nearly flat dose-response curve as the one in Fig. 2B (not shown), as were white women designated as homozygous for Ala-148 factor IX.

A mixture of equal volumes of plasma from two normal men (one with Thr and the other with Ala-148 factor IX) gave intermediate reactivity (with a slightly lower slope in its dilution curve, Fig 1B). In the polyclonal IRMA, the mixture gave a parallel dilution curve, slightly above the normal curve (not shown), as observed for the two individuals by themselves. By comparison of results of these two assays and assumption of parallel curves, the mixture is essentially half as reactive in the IRMA with the solid-phase monoclonal antibody as opposed to the polyclonal antifactor IX. In separate experiments, samples in which 5% and 10% of the factor IX had Ala-148 (ie, 90% and 95% Thr-148 protein) could not be reproducibly distinguished from the normal individual or 100% Thr-148 factor IX pooled plasmas, whereas the dilution curve of a mixture with 20% Ala-148 factor IX was clearly lower. At the other extreme, mixtures containing only 1%, 2%, and 5% of Thr-148 factor IX could all be readily distinguished from citrated plasma with a 100% Ala-148 factor IX variant.

Ethnic frequencies of the Thr/Ala-148 dimorphism. Table 1 shows the frequency of the less common Ala-148 variant in samples from subjects from different ethnic groups. Together with previous samples from whites,10,12 57 of 217 normal and hemophilic white men had Ala-148 factor IX for an overall incidence of 26%. The percentage was the same in the subset of 75 hemophilic men as in the larger group of normal men. Among black donors in Seattle, the gene for the Ala-148 factor IX variant was only about one-half as prevalent as in whites and it was uncommon in the South African male samples. Samples from the Asian donors in Seattle had no detectable factor IX with Ala-148. The possibility of alteration of the epitopes on factor IX during years of storage at only 15°C exists, but the mean polyclonal IRMA levels of factor IX antigen in South African blacks was 103.6 U/dL, suggesting no significant deterioration. The 49 South African samples reactive in the
To define further the strong linkage disequilibrium between Thr-148 and TaqI polymorphisms noted between these polymorphisms, all five Ala variants detected in black women were in heterozygotes. Numbers in parentheses are genes coding for Ala-148 factor IX. All five Ala variants detected in black women were in heterozygotes.

**Table 1. Ethnic Frequencies of Ala-148 Factor IX**

<table>
<thead>
<tr>
<th>No. of Donors, Ethnic Group</th>
<th>No. of Factor IX Genes</th>
<th>Ala-148 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>35 Whites (Seattle)</td>
<td>35 (10)</td>
<td>35 29</td>
</tr>
<tr>
<td>47 Blacks (Seattle)</td>
<td>26 (3)</td>
<td>52 12</td>
</tr>
<tr>
<td>51 Blacks (South Africa)</td>
<td>51 (2)</td>
<td>51 4</td>
</tr>
<tr>
<td>64 Asians (Seattle)</td>
<td>33 (0)</td>
<td>95 0</td>
</tr>
</tbody>
</table>

Numbers of genes are from unrelated donors from nonhemophilic families in the current series. Numbers in parentheses are numbers of genes coding for Ala-148 factor IX. All five Ala variants detected in black women were in heterozygotes.

Monoclonal IRMA had levels in the normal range and the correlations between their two factor IX antigen levels was high (r = .87).

**Association of the Thr/Ala-148 and TaqI polymorphisms.** To define further the strong linkage disequilibrium noted between these polymorphisms,28 of the white, five of the black, and six of the Asian male donors shown in Table 2 were also tested for TaqI RFLPs. Results of 12 unrelated, nonaffected white men and 11 additional hemophilic men were also tested for both polymorphisms in the course of screening families for carrier testing. Results on these 62 men were pooled with previously published results from 67 men28 and are included in Table 2. Two exceptional factor IX genes were noted in this series, each coding for the Thr-148 variant associated with the 1.3-kilobase (kb) TaqI fragment. Of 47 unrelated women tested, including ten normal black and six normal Asian donors (the rest represented one each from 31 different families screened for carrier testing of hemophilia B), two recombinations of Ala-148 factor IX with the 1.8-kb TaqI were noted (Table 2). In 24 additional women from these 31 families, only the common associations were found (not shown).

**Hemophilic carrier detection.** As previously reported,10 the immunoassay can be diagnostic for the carrier state in two ways. The first is by linkage. Owing to strong linkage disequilibrium, ~98% of such families should also be informative by TaqI RFLPs. The second way is particularly useful in families in which plasmas of affected members have little or no detectable factor IX antigen. The presence or absence of normal phenotypes can be diagnostic in this type of result, irrespective of linked RFLPs, as illustrated in Fig 2. In two additional families with sporadic cases of severe hemophilia B associated with factor IX antigen levels of <1 U/dL, the mother was homozygous for common RFLPs. Absence of the hemophilic gene in the sister of one family and a maternal cousin in the other was indicated by their heterozygous IRMA results which demonstrated two forms of normal, circulating factor IX antigen; results were 86 and 41 U/dL in the first and 135 and 105 U/dL in the second woman when IRMAs with polyclonal antibody and the monoclonal antibody used in solid-phase binding, respectively, were compared. In five of these six pedigrees, the hemophilic and normal factor IX genes could not be distinguished by DNA analyses owing to homozygosity of RFLP alleles in the patients' mothers.

**Table 2. Linkage Disequilibrium Between Thr/Ala-148 and TaqI Polymorphisms**

<table>
<thead>
<tr>
<th>n/sex</th>
<th>Factor IX Genes</th>
<th>Common Association</th>
<th>Uncommon Association*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of</td>
<td>Thr/1.8 Ala/1.3</td>
<td>Thr/1.8 Ala/1.8</td>
</tr>
<tr>
<td>129 M</td>
<td>129</td>
<td>97 30</td>
<td>2 0</td>
</tr>
<tr>
<td>47 F</td>
<td>94</td>
<td>72 20</td>
<td>0 2</td>
</tr>
</tbody>
</table>

Polymorphisms are expressed as Thr or Ala for residue 148 or as 1.8 or 1.3 as kilobases of the polymorphic fragment following Taq1 digestion.

*The two exceptional men were white, one from the current series of 35 normal donors (Table 1) and one in family B, Fig 2. The 14 women heterozygous for both polymorphisms were assumed to have the common associations. Of the two women homozygous for the 1.8-kb TaqI allele but heterozygous for Thr/Ala-148, one was a normal black donor with factor IX antigen levels by polyclonal and monoclonal IRMAs of 74 and 38 U/dL, respectively. The other is in family D, Fig 2. The uncommon associations in subjects shown in Fig 2 were confirmed on a second sample from each.

**Fig 2.** Four pedigrees (A through D) of patients with hemophilia B. Arrows indicate possible carriers for which immunoassays are (or would be) informative. Men (squares) and women (half-circles) factor IXs are as Thr-148 (T), Ala-148 (A), low antigen level hemophilic (h) or uncertain (?) variants by IRMAs; hatching indicates clinical hemophilia or proven carrier status. Factor IX antigen levels are given above symbols by polyclonal IRMAs (left) and monoclonal IRMA (right) in units per deciliter. Results from TaqI RFLPs, where performed, appear beneath symbols as the more common 1.8-kb allele, indicating the absence of the restriction site and 1.3 kb, the presence of this site in intron 4. (A) The cousin of the affected man had IRMA results typical for homozygosity, excluding the carrier state. Subsequent DNA-RFLP analyses of five family members was informative. (B) The daughter of an obligate carrier of hemophilia B has an affected brother yet no trace of her mother's normal Thr-148 factor IX in her plasma. Her deceased father must have had a normal Ala-148 variant. Thus, she either preferentially inactivated her mother's normal factor IX gene (to >99%) or she inherited her mother's hemophilic factor IX gene; the latter is compatible with her clotting activity of 40 U/dL. The carrier state in her half-sister is excluded by the RFLP analyses alone. (C) This patient's carrier sister was homozygous for RFLP analyses. Because her daughter's father has a different normal factor IX, immunoassays are predicted to be informative in their infant daughter. Any evidence of her mother's circulating normal Thr-148 factor IX would exclude the carrier state. (D) The mother of three moderately severe hemophilic sons is homozygous by RFLPs but heterozygous for a Thr-148 hemophilic and an Ala-148 normal factor IX. Her daughter's homozygous pattern for Ala-148 excludes the carrier state, barring extreme (<99%) inactivation of the X-chromosome with the hemophilic factor IX gene.
Data in Table 3 summarize the diagnostic value of immunoassays v DNA-RFLP approaches in 16 families with at least one possible carrier when both types of tests were performed. Numbers indicate families to avoid bias introduced when several potential heterozygotes in a given family are either heterozygous or homozygous. Three symptomatic carriers in these pedigrees were excluded from analysis because their low factor IX clotting activities were sufficient with the inheritance pattern to diagnose the carrier state. In five families each, either the immunoassay-detected variants or DNA analyses were informative when the other approach was noninformative. In nine of 18 other families, the immunoassay-detected variants were informative; DNA analyses (not performed) would likely be informative in these nine and, probably, in some of the other nine families.

In 23 other families with affected members whose factor IX antigen was >1 U/dL, obligate heterozygotes or mothers of sporadic cases were tested. Eight were heterozygous for Thr/Ala-148 factor IXs. Of the other 15 women, 14 had only Thr-148 factor IX detectable and one had only the Ala-148 variant as shown by IRMAs.

**DISCUSSION**

**Sensitivity of specific immunoassays.** In selected families, immunoassays have indicated carrier status by linkage of hemophilia B with Thr or Ala at residue 148 or by demonstrating the presence or absence of normal Thr or Ala-148 factor IXs. To be generally useful, however, the sensitivity of an assay must be defined. By using a normal pool of citrated plasmas from men with only the Thr-148 variant in the present study, the mean value of 81 men were the same for specific (monoclonal) and non-specific (polyclonal) IRMAs (r = .86). A false assignment of homozygosity for Thr-148 in women who are actually heterozygous should have occurred in ~2.5% of women in the present study. From data on mixtures of the two types of factor IX, the present IRMAs will also classify an occasional heterozygote as homozygous for Thr-148 factor IX when her Ala-148 variant is >80% to 90% inactivated. They will rarely misclassify a heterozygote as homozygous for Ala-148 factor IXs, since the slope of the curve is qualitatively different. For families in which the plasmas of affected members have intermediate levels of factor IX antigen (e.g., 10 to 50 U/dL), it would take less of an imbalance of inactivation of a hemophilic gene with a linked Ala-148 residue, for example, to be misclassified as homozygous Thr-148.

Wallmark et al. using a monoclonal antifactor IX which is also specific for Thr-148 factor IX, found a strong correlation between IRMAs with this v another monoclonal antifactor IX (r = .89). In their more recent series, the results from their two IRMAs illustrated poor correlation when a normal pool containing both variants of factor IX was used. This creates sufficient variability in their IRMAs to preclude being able to distinguish heterozygous women from those homozygous for Thr-148. Thus, for recognition of heterozygous women, a pool of reactive male donors is needed as the standard, as used in this study and earlier ones.

**Ethnic frequencies.** Among different ethnic groups, the frequency of Ala-148 factor IX was highest in whites. A lower frequency (14%) has been reported in whites, and an absence of Ala-148 has been reported in blacks in North Carolina, but IRMAs (see above) apparently did not distinguish heterozygous women in their series. Thus, only homozygous Ala-148 women would have been detected. This would underestimate the incidence of the Ala-148 variant in their samples. The parallel frequencies of the Thr/Ala-148 variants (Table 1) with TaqI alleles suggest a strong degree of linkage disequilibrium. Only four of >200 white or black factor IX genes were identified in which recombinations between the more and less common forms were noted (Table 2).

For Americans blacks, BamHI RFLPs are more frequently polymorphic, as are MspI RFLPs, and these polymorphisms would be very useful for carrier detection of hemophilia B. Among Asians, only the linked extragenic DXS99 polymorphic site appears to be frequent. The Thr/Ala-148 polymorphism will thus be most useful in families with hemophilia B patients when they have white ancestry. A 25% frequency of the Ala-148 variant was detected in normal men, and heterozygosity in normal women was within the predicted frequency limits.

**Carrier detection in hemophilia B.** For a woman at risk of being a carrier of hemophilia B, a probability can be calculated from her factor IX clotting activity. Occasionally, carriers will have levels sufficiently low to be recognized by this test alone; they may even be asymptomatic. Clotting assays, however, are not always reproducible, and normal levels have a wide distribution. The presence of factor IX antigen in marked excess over clotting activity can be diagnostic in some possible carriers. As more distant relatives are considered, however, results are even more likely to be normal by either approach. Because X-chromosome inactivation is random, normal circulating levels of factor IX do not exclude the carrier state. Only occasionally does the hemophilic defect itself serve as a specific marker on DNA analyses. Of known DNA polymorphisms in the factor IX gene, linkage should be informative in two-thirds to three-fourths of white women. The likelihood of recombination between a given polymorphism and a mutation within the factor IX gene should be between one in 1,000 and one in 10,000, assuming 1% recombination per centimorgan (1,000
EXONIC POLYMORPHISM IN HEMOPHILIA B CARRIERS

When informative, linkage patterns within families can therefore be far more definitive than the clotting assays.

An exonic polymorphism distinguished by immunoassays can also establish linkage.\(^{16,17}\) Strong linkage disequilibrium between the Thr/Ala-148 dimorphism and TaqI RFLPs, for example, implies that either could establish linkage in white families in which affected members have circulating factor IX antigen at levels near normal; the DNA approach would be more definitive because immunoassays fail to recognize all heterozygous women. The immunoassay approach is far more rapid and requires only a small volume of any blood sample, even those stored for long periods of time. When the hemophilic gene results in low or undetectable levels of factor IX antigen, however, only the DNA-RFLPs can establish linkage with the mutant gene. Regardless of the level of defective factor IX antigen, however, the immunoassays can be informative other than by direct linkage (ie, by indicating the type(s) of normal factor IX proteins present) (Fig 2).\(^{10,14}\)

If an affected member has low or undetectable factor IX antigen, a possible carrier can be excluded when she is heterozygous for Thr and Ala-148 normal factor IXs at normal levels. This occurred in family A (Fig 2), in which the hemophilic defect, a new stop codon number 86,\(^{24}\) precludes expression of the codon for the epitope including residue 148. The cousin's mother's carrier status could be determined only by use of oligonucleotide probes including 19 bases centered on the single-based deletion and the 18 bases including the deletion that led to the new stop codon; she and her sister, but not her daughter, were carriers by oligonucleotide hybridization on Southern blots (A.F. Weinmann, S-H. Chen, B.G. Schach, and A.R. Thompson, unpublished observations, 1987). In families B and D, the immunoassays demonstrated lack of the mother's normal or hemophilic Thr-148 factor IXs, respectively. Although nonrandom X chromosome inactivation was the basis for hemophilic symptoms in one hemophilic carrier,\(^{25}\) values in one mother (family D) indicate that normal random inactivation is operative in her X chromosome pair. In family C, both the patient and nephew have severe hemophilia B with high titer inhibitors and no gross deletion in their factor IX genes.\(^{26}\) Immunoassays alone should be diagnostic in the infant daughter.

A strategy for carrier detection in white women at risk of being carriers of hemophilia B is to begin with the immunoassay and, when it is informative by linkage, to confirm it with TaqI RFLPs. The latter is necessary for early prenatal diagnosis. If the defect is associated with circulating factor IX antigen and immunoassays are noninformative, other polymorphic sites (eg, XmnI, MspI, or DXS99) should be evaluated. In families in which the hemophilic defect has low or undetectable circulating factor IX antigen, results of the immunoassays are less likely to be informative by linkage than RFLP testing. In one-fourth of such families studied, immunoassays were nevertheless informative by indicating absence of a normal variant or presence of both types, even when RFLP analyses were not helpful (Table 3) and even when specific DNA probes for the codon 148 polymorphisms should be noninformative. Thus, each approach, independently, can be informative. Similar immunoassay-detected polymorphisms could be applied to inheritance of other X-linked or autosomal congenital defects.

ACKNOWLEDGMENT

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ADDITIONAL

Since submission of this manuscript, Graham et al\(^{27}\) published their data confirming that the Swedish monoclonal antifactor IX is also specific for the Thr-148 variant. Relevant to linkage disequilibrium between Thr/Ala-148 and TaqI RFLPs, there were three exceptional associations in a group of 43 Swedish men.

REFERENCES

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