Recombinant Human Factor IX: Replacement Therapy, Prophylaxis, and Pharmacokinetics in Canine Hemophilia B


Recombinant human factor IX (rFIX) has been expressed in transduced cultured cell systems since 1985. Because there has been limited in vivo testing of rFIX in hemophilia B subjects, this study was undertaken using the severe hemophilia B canines of the Chapel Hill strain. Three groups of hemophilic dogs received either 50, 100, or 200 IU/kg of rFIX. As a control, a fourth group of hemophilic dogs received 50 IU/kg of a high purity, plasma-derived human FIX (pdFIX). The coagulant and hemostatic effects of rFIX and pdFIX were similar with all comparative dosing regimens. Based on activity data, the elimination half-life of rFIX was 18.9 ± 2.3 hours and pdFIX was 17.9 ± 2.1 hours. A prophylactic regimen administering rFIX daily resulted in a continuous therapeutic level of plasma FIX and was accompanied by a two-fold increase in recovery levels by day 5, compared to that observed with administration of a single bolus. The mechanisms of the high to complete recovery of FIX with the prophylactic regimen could depend not only on the degree of saturation of the vascular endothelial binding sites but also on the altered dynamics of the balance of FIX distribution between the intravascular and extravascular compartments. The pharmacokinetic (PK) parameters for rFIX and pdFIX were similar. However, the relative PK values for \( V_c \) and \( V_m \) of both products on day 5 differed greatly from day 1 and may reflect the changing equilibrium of FIX between compartments with elevated levels of plasma FIX. Neutralizing antihuman FIX antibodies resulting from human FIX antigen being administered to FIX deficient dogs were observed beginning at 14 days. The antigenicity of rFIX and pdFIX appeared to be comparable. Despite the very different procedures used for production of rFIX and pdFIX products, in vivo testing in hemophilia B dogs showed the functional behavior of these products is similar; they are highly effective for replacement therapy and for prophylaxis.

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Hemophilia B is an X-linked bleeding disorder caused by an array of mutations in the factor IX gene, resulting in a deficiency of the procoagulant protein, plasma factor IX (FIX). FIX, M, 55,000, is one of a group of vitamin K-dependent procoagulants required for normal coagulation. It is the zymogen of the serine protease, factor IXa, which binds to the factor VIII-lipid complex to activate factor X, an essential step in the coagulation cascade. When given in adequate amounts to hemophilia B subjects, the FIX is activated and corrects the bleeding defect. Historically, therapy of hemophilia B has been by intravenous delivery of plasma factor IX, given initially as fresh-frozen plasma, then by so-called “prothrombin complex” concentrates, and most recently by higher-purity, plasma FIX concentrates. With the plasma concentrates there is risk of two main types of complications, thrombosis and transmission of viral diseases. With the new highly potent plasma FIX concentrates, these risks were minimized.

Recombinant human factor IX (rFIX) was anticipated as a therapeutic agent for hemophilia B soon after the factor FIX cDNA was cloned in 1982. Expression of the rFIX protein in transfected cells in 1985 led to the development of many expression systems and became the basis for a technology for producing therapeutic rFIX in a transfected cell line. rFIX is expressed by dihydrofolate reductase-deficient and transfected Chinese hamster ovary (CHO) cells. The presence of vitamin K in the medium is required for \( \gamma \)-carboxylation and for production of a functional FIX molecule. The initial product had a specific activity of 35 to 75 IU/mg of FIX protein. The rFIX was produced by a serum-free media process and purified to homogeneity by a biochemical process which did not require the use of animal protein. These processes have produced a highly pure protein with a specific activity slightly higher than plasma FIX. These advances in bioengineering made possible limited preclinical testing of rFIX.

In this report, the Chapel Hill strain of hemophilia B dogs was used to determine the therapeutic efficacy of rFIX in an animal model of human hemophilia B. The animals are mixed-breed beagles maintained for over 30 generations. They have a severe hemophilic phenotype comparable to the most severe form of human hemophilia B. Spontaneous hemorrhages and hemarthroses are frequent. The line has been maintained by a transfusion program of prompt replacement therapy using fresh-frozen canine plasma. The affected dogs are negative for cross-reacting material (CRM) with no detectable FIX protein in their plasma, due to a point mutation in the catalytic domain of the molecule, with an amino acid substitution of glutamic acid for glycine. The goal of this report was to determine the therapeutic and prophylactic effect of rFIX along with its pharmacokinetics (PKs) in the hemophilia B dog and to compare rFIX with a highly purified, plasma-derived FIX (Mononine, pdFIX; Armour Pharmaceutical Co, Kankakee, IL). Preliminary reports of some of these findings have been presented.

MATERIALS AND METHODS

Factor IX preparations. rFIX was prepared by Genetics Institute, Inc (Andover, MA, Lots RB2455-069 and 0715H01) as pre-

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vously described.23-25 The specific activity of the rFIX lots was 243 IU/mg and 280 IU/mg of FIX, respectively. The protein concentration of the rFIX solution was approximately 1.64 mg/mL. The rFIX was stored at −80°C in its vehicle formulation buffer (glycine, sucrose, histidine, Tween 80, pH 6.8) until infused.26 pdFIX was an immunoaffinity chromatography purified commercial preparation (Mononine, Lots M87203 and P98401). The package insert indicated a specific activity of not less than 150 IU/mg of FIX. The lyophilized protein was reconstituted according to manufacturer’s directions and promptly infused.

Hemophilia B animals. Hemophilia B dogs came from the inbred colony maintained since 1966 at the Francis Owen Blood Research Laboratory (University of North Carolina at Chapel Hill). Some characteristics of these dogs are given (see Tables 1 and 2). The Bethesda inhibitor assay and FIX antibody titer analysis for neutralizing anti-FIX antibodies of all animals were negative.

Administration of FIX preparations and blood sampling. Hemophilia B dogs were infused with rFIX and pdFIX preparations via the cephalic vein. A total of 108 doses of 50, 100, or 200 IU FIX/kg were administered. 60 doses of rFIX and 48 of pdFIX. Six of the dogs were employed for 12 PK analyses with a dose of 50 IU/kg. Three of the animals received rFIX and 3 received pdFIX daily for 14 consecutive days. PK analyses were performed on blood samples collected on days 1 and 5. Blood samples for PK analyses were collected preinfusion and postinfusion at the following time intervals: 5, 15, and 30 minutes, 1, 2, 4, 6, 8, 12, 15, 22, and 24 hours. Before each reinfusion, samples were collected daily for trough value determinations of FIX. Prophylactic administration of FIX and comparisons of FIX and pdFIX were ascertained in this same group of animals. All animals were monitored for clinical signs of reaction to the human FIX products for the 14 days of infusions. The remaining 14 hemophilic animals were given one bolus (n = 4) or two boluses (n = 10) of FIX, the second bolus being provided on day 5 or 7. The hemophilic animals given one bolus all received 100 IU/kg of rFIX. The hemophilic animals given two boluses were on three separate regimens. In a cross-over regimen, 6 animals received 50 IU/kg of either rFIX or pdFIX followed by 50 IU/kg of the opposite product. The remaining two regimens were double dose studies; in the first, one animal received 100 IU/kg of FIX followed by 200 IU/kg of rFIX; in the second, 3 animals received two separate doses of 200 IU/kg of rFIX.

Coagulant and hemostatic testing. A monoswab lancet ELISA was used to measure FIX plasma concentration, as previously described.26 FIX coagulant activity was determined by a modified Bethesda inhibitor assay and FIX antibody titer analysis for neutralizing anti-FIX antibodies of all animals were negative.

Prophylactic regimen of FIX administration. The pattern of fluctuation in plasma FIX levels following daily doses of rFIX was depicted in schematic graphs. For each 24-hour period, the preinfusion and postinfusion trough values were the minimum values and the 5-minute postinfusion level of FIX was the maximum value. In plotting the biphasic pattern of FIX decline, the points of intersection of t1/2 and t1/2 were estimated for each 24-hour period using a two-phase linear regression model.27 On days 2 through 4, the maximum activity levels of plasma FIX and the biphasic intersection points were interpolated.

PK parameters were analyzed from plasma FIX antigen concentration versus time data for individual dogs. Initial estimates of PK parameters were determined for each profile using the curve stripping program (JANA; SCI Software, Apex, NC). These preliminary estimates were then used in the PK modeling program (PCNONLIN v4.2; SCI Software). A biexponential equation in the form, C = Ae−αt + Be−βt, was fit to the data (where C is the concentration of FIX in the plasma at time t, A and B are the ordinate intercepts, α and β are the first-order rate constants). The PK parameter estimates included maximum concentration (Cmax) values, elimination (t1/2α), and distribution (t1/2β) half-lives, initial volume of distribution (V1), steady state volume of distribution (Vss), clearance (CL), and area under the curve extrapolated to infinity (AUC∞). The elimination half-life (t1/2α) of FIX activity was determined using a two-phase linear regression model.27

Experimental design. The response of hemophilia B dogs to rFIX was evaluated by the following analyses: (1) coagulant response and recovery after a single bolus injection of 3 different doses of rFIX on day 1, including hemostatic testing, FIX recovery, half-life, and decline of FIX concentration. (2) coagulant response to daily injections of 50 IU/kg rFIX and pdFIX, which is analogous to a prophylactic regimen for prevention of spontaneous or trauma-induced hemorrhage in surgery, (3) pharmacodynamics of both FIX
RECOMBINANT FACTOR IX IN CANINE HEMOPHILIA B

The plasma FIX activity values, determined by a modified one-stage PTT test, show the decline of FIX activity over a period of 24 hours in each example. Arrow indicates time of rFIX administration. The 50 IU/kg dose of rFIX is a mean of 3 dogs (animal studies 1 through 3) on day 1. An illustrative example is given for the 100 IU/kg dose of rFIX (animal study 10) and the 200 IU/kg dose of rFIX (animal study 15).

products, (4) PK analyses and the comparison of rFIX and pdFIX on days 1 and 5 following administration of 50 IU/kg of rFIX or pdFIX, and (5) comparison of the antigenicity of the recombinant and native FIX protein in the hemophilia B dog.

RESULTS

Coagulant response of rFIX infused into hemophilia B dogs. Figure 1 illustrates the decay of rFIX activity following infusions of three different doses of the recombinant product into hemophilia B dogs. The FIX activity levels declined in a biphasic manner. The hemostatic and coagulant defect was corrected at each dose level. The baseline values and pharmacodynamic data at 15 minutes postinfusion of rFIX are given in Table 1. For comparison, similar data is shown for pdFIX. The WBCT, which is greatly prolonged in the hemophilia B animals, was shortened to normal or nearly normal levels. The PTT values were likewise shortened considerably at 15 minutes postinfusion in comparison to the baseline values. The prolonged secondary bleeding time was normalized regardless of dose. The elimination half-life (t1/2) values were similar for the two products.

Plasma recovery of infused FIX. The dose and type of human FIX preparation infused into the hemophilia B dogs are indicated in Table 2. The expected and observed levels of plasma FIX activity and the plasma FIX recovery at 15 minutes postinfusion are also shown. A total of 30 animal studies were divided into 4 groups: three groups receiving different doses of rFIX and one group receiving a 50 IU/kg dose of pdFIX. The mean recovery was 33% with a range of 23% to 47% in 9 animal studies with 50 IU/kg rFIX, 44% with a range of 40% to 52% in the 5 animal studies with 100 IU/kg rFIX, and 36% with a range of 29% to 57% in the 7 animal studies with 200 IU/kg rFIX. There is an overlap in recovery between the three groups given rFIX. A mean recovery of 49% with a very wide range of values (25% to 82%) was observed in the 9 animal studies with pdFIX; the values overlapped the values observed in all of the rFIX dose groups.

Prophylactic regimen for management of hemophilia B. The daily administration of 50 IU/kg FIX for the first 5 days was considered as a model for the analysis of high dose prophylaxis for prevention of hemorrhage. All dogs maintained plasma FIX values above 10% at all times. The mean trough activity ranged from 11% to 23% with rFIX and 17% to 45% with pdFIX. The maximum mean level of plasma FIX was 86% with rFIX and 156% with pdFIX. The pattern of FIX fluctuations is illustrated in Fig 2 for the group of animals receiving rFIX. For the animals given pdFIX, the pattern was similar to that in Fig 2. Plasma FIX recovery on day 5 was compared to recovery on day 1 for both rFIX and pdFIX. Recovery for rFIX ranged from 64% to 82% on

Table 1. Coagulant and Hemostatic Response of Hemophilia B Dogs to Administration of rFIX and pdFIX

<table>
<thead>
<tr>
<th>Animal Sex/Age (mo)</th>
<th>FIX Dose (IU/kg)</th>
<th>t1/2&lt;sup&gt;+&lt;/sup&gt; (h)</th>
<th>WBCT Pre (min)</th>
<th>Postinfusion&lt;sup&gt;+&lt;/sup&gt; (min)</th>
<th>PTT Pre (s)</th>
<th>Postinfusion&lt;sup&gt;+&lt;/sup&gt; (s)</th>
<th>Secondary BT Postinfusion&lt;sup&gt;+&lt;/sup&gt; (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recombinant human FIX</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F/19</td>
<td>50</td>
<td>17.2</td>
<td>56.0</td>
<td>12.5</td>
<td>&gt;150</td>
<td>96</td>
<td>1.0</td>
</tr>
<tr>
<td>M/16</td>
<td>50</td>
<td>21.6</td>
<td>37.0</td>
<td>9.0</td>
<td>145</td>
<td>61</td>
<td>t</td>
</tr>
<tr>
<td>M/40</td>
<td>50</td>
<td>18.1</td>
<td>55.5</td>
<td>8.5</td>
<td>136</td>
<td>59</td>
<td>t</td>
</tr>
<tr>
<td>M/25</td>
<td>100</td>
<td>16.1</td>
<td>&gt;60</td>
<td>9.0</td>
<td>&gt;150</td>
<td>55</td>
<td>4.5</td>
</tr>
<tr>
<td>M/25</td>
<td>200</td>
<td>15.6</td>
<td>—</td>
<td>7.5</td>
<td>—</td>
<td>46</td>
<td>4.0</td>
</tr>
<tr>
<td>Plasma-derived human FIX</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F/12</td>
<td>50</td>
<td>15.6</td>
<td>54.5</td>
<td>10.0</td>
<td>140</td>
<td>59</td>
<td>t</td>
</tr>
<tr>
<td>F/16</td>
<td>50</td>
<td>18.4</td>
<td>&gt;60</td>
<td>9.0</td>
<td>136</td>
<td>57</td>
<td>2.5</td>
</tr>
<tr>
<td>F/12</td>
<td>50</td>
<td>19.7</td>
<td>39.0</td>
<td>10.5</td>
<td>&gt;150</td>
<td>62</td>
<td>2.5</td>
</tr>
</tbody>
</table>

* 15 min postinfusion.
† Not determinable; cuticle arterial bleeding.
‡ Based on FIX activity assays.

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day 5, an average of nearly twofold higher than the day 1 range of 24% to 47% (animal studies 1 through 3). Recovery for pdFIX on day 5 was approximately 1.5 times higher than recovery for pdFIX on day 1 (animal studies 22 through 24).

**PK analysis of rFIX and pdFIX.** The estimated PK parameters for rFIX and pdFIX are given in Table 3 for both day 1 and day 5. The PK estimates obtained with rFIX and pdFIX were similar for each day. The mean maximum plasma FIX concentration on day 5 was approximately twofold higher than the mean maximum plasma FIX concentration on day 1 for both products, suggesting that the daily dose was greater than the daily usage of FIX, reaching a steady state. The CL, Vt, and Vm on day 5 are approximately half those of day 1, whereas the mean maximum plasma FIX concentration for pdFIX was slightly higher than that of rFIX. The rate of elimination of FIX concentration appears to have been constant throughout, judging from lIC50 values for days 1 and 5.

**Immune response to human FIX.** Neutralizing antibody testing was negative on all dogs on either day 5 or day 7, and all dogs were positive by day 28 (Table 4). Most of the hemophilia B dogs developed antihuman neutralizing FIX antibodies after daily infusions for 14 days of either rFIX or pdFIX, as detected by both the Bethesda inhibitor assay and ELISA. In vivo neutralization of infused rFIX on day 14 occurred promptly. The mean plasma FIX antigen concentration fell to below detectable levels by 2 hours postinfusion in a group of 3 dogs given rFIX. The antihuman FIX antibody was cross-reactive with the canine FIX antigen, as shown by prolongation of the PTT of normal canine plasma mixed with an equal volume of plasma from the hemophilia B dogs on day 28, following treatment with either rFIX or pdFIX. A value of 4.0 Bethesda units was obtained using normal and FIX deficient canine plasma in the Bethesda inhibitor assay, the same as with normal and FIX deficient human plasma on day 28 (Table 4: experiment 3, day 21).

The administration of the FIX products was completely innocuous during the first 9 days of treatment of the hemophilia B subjects. However, during the period between days 10 and 14, some animals developed a transient, generalized reaction immediately following the infusions of the human FIX preparations. With rFIX, these episodes occurred following 8 of 30 injections (27%), and with pdFIX, 10 of 15 injections (67%).

**DISCUSSION**

This report examined a new rFIX designed for clinical use in hemophilia B by comparing it to a highly purified pdFIX in hemophilia B dogs. Coagulant data from both a single therapeutic dose and a multidose, prophylactic regimen of infused FIX were available for analysis.
Low recovery of FIX with infused concentrates has been recognized since the earliest use of concentrate therapy in hemophilia B. The extent of recovery has varied over a wide range of values in human studies, from about 22% to 78%. Similar results were obtained in this study. At 50 IU/kg, the mean expected plasma activity was 0.89 ± 0.07 IU/mL for rFIX and 0.86 ± 0.06 IU/mL for pdFIX, whereas the mean observed activity was 0.29 ± 0.10 IU/mL for rFIX and 0.43 ± 0.21 IU/mL for pdFIX. These values resulted in a mean recovery of 33 ± 10.3% for rFIX and 49 ± 20.4% for pdFIX. In human studies with administration of pdFIX, it has been suggested that FIX recovery was partly dose-dependent. There was no clear evidence of dose dependency in this study. The recovery was 44% at 50 IU/kg given daily. The day 1 data are based on the analyses of the plasma samples collected between 120 and 144 hours. Each PK parameter is a mean with standard deviation of 3 individual PK values. Values for rFIX are from animal studies 1 through 3 and values for pdFIX are from animal studies 22 through 24.

Table 3. Pharmacokinetic Analysis of 6 Hemophilia B Dogs Given rFIX or pdFIX

<table>
<thead>
<tr>
<th>FIX Product</th>
<th>Day</th>
<th>Cmax (ng/mL)</th>
<th>t1/2 (h)</th>
<th>tmax (h)</th>
<th>CL (ml/kg/h)</th>
<th>V1 (ml/kg)</th>
<th>Vss (ml/kg)</th>
<th>AUCl (ng x h/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rFIX</td>
<td>1</td>
<td>1520 ± 150</td>
<td>1.52 ± 1.76</td>
<td>22.5 ± 6.0</td>
<td>8.3 ± 1.7</td>
<td>133.3 ± 28.7</td>
<td>242.3 ± 60.6</td>
<td>25917 ± 342</td>
</tr>
<tr>
<td>pdFIX</td>
<td>1</td>
<td>1873 ± 76</td>
<td>2.92 ± 1.09</td>
<td>22.2 ± 6.0</td>
<td>7.1 ± 1.2</td>
<td>138.7 ± 11.0</td>
<td>215.2 ± 61.5</td>
<td>36944 ± 5300</td>
</tr>
<tr>
<td>rFIX</td>
<td>5</td>
<td>2520 ± 164</td>
<td>1.10 ± 0.62</td>
<td>21.4 ± 7.1</td>
<td>4.0 ± 1.4</td>
<td>84.4 ± 3.0</td>
<td>108.7 ± 12.8</td>
<td>57073 ± 16214</td>
</tr>
<tr>
<td>pdFIX</td>
<td>5</td>
<td>3750 ± 57</td>
<td>1.04 ± 0.86</td>
<td>25.1 ± 10.6</td>
<td>3.9 ± 0.9</td>
<td>68.4 ± 6.3</td>
<td>122.5 ± 20.9</td>
<td>78644 ± 22891</td>
</tr>
</tbody>
</table>

* Each animal was given 50 IU FIX/kg daily. The day 1 data are based on the analyses of the plasma samples collected in the first 24 hours of the dosing regimen. The day 5 data are based on the analyses of the plasma samples collected between 120 and 144 hours. Each PK parameter is a mean with standard deviation of 3 individual PK values. Values for rFIX are from animal studies 1 through 3 and values for pdFIX are from animal studies 22 through 24.

Table 4. Antihuman Neutralizing FIX Antibodies in Hemophilia B Dogs Receiving 50 IU/kg of rFIX or pdFIX

<table>
<thead>
<tr>
<th>Animal Sex/Age</th>
<th>Day 14</th>
<th>Day 28</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bethesda Inhibitor</td>
<td>FIX Antibody Titer*</td>
</tr>
<tr>
<td>Recombinant human FIX</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F/19</td>
<td>0</td>
<td>2.8</td>
</tr>
<tr>
<td>M/16</td>
<td>0</td>
<td>Negative</td>
</tr>
<tr>
<td>M/40</td>
<td>0</td>
<td>2.1</td>
</tr>
<tr>
<td>Plasma-derived human FIX</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F/12</td>
<td>0.5</td>
<td>3.2</td>
</tr>
<tr>
<td>F/16</td>
<td>0</td>
<td>3.0</td>
</tr>
<tr>
<td>F/12</td>
<td>1.0</td>
<td>3.0</td>
</tr>
</tbody>
</table>

* Titer for each sample is the log of the reciprocal of the dilution of that sample, which generated an optical density (OD) value of ≥ two times the negative control OD value shown in an ELISA.
significant increase in recovery. The recovery of FIX was nearly twice as great on day 5 as on day 1 with this regimen. The increased recovery with repeated doses of FIX could be related to the reduced FIX binding capacity of endothelium and the attainment of steady state FIX levels. The increased $C_{max}$ on day 5 would have been predicted to be 1.9-fold higher for rFIX and 2.1-fold higher for pdFIX based on the day 1 $t_{1/2}$ and the dosing interval. The actual values of 1.67-fold for rFIX and twofold for pdFIX were similar to these predicted values (Table 3).

The PK estimates for rFIX were similar to those observed with pdFIX in this study as well as to those in several human studies. For example, in the case of FIX concentration data, the range of $V_\text{a}$ values with rFIX and pdFIX was 143 to 328 mL/kg and 167 to 296 mL/kg, respectively (Table 3), which are comparable to $V_\text{a}$ values reported in several human hemophilia B patients using plasma FIX concentrates, 62 to 233 mL/kg.\textsuperscript{13,38,42,47,49} The range of CL values with rFIX and pdFIX in this study was 5.98 to 9.58 mL/hr/kg and 6.10 to 8.68 mL/hr/kg, respectively (Table 3), compared to a wider range for human values, 1.9 to 9.2 mL/hr/ kg.\textsuperscript{13,38,42,47,49} The range of elimination half-lives ($t_{1/2}$) in patients with hemophilia B ranged from 17 to 34.6 hours,\textsuperscript{13,38,46-50} which is analogous to parameter estimates in our hemophilia B dogs (15.6 to 21.6 for rFIX and 15.6 to 19.7 for pdFIX). Comparable results were observed in normal dogs\textsuperscript{56} and in SCID mice.\textsuperscript{63} The use of the hemophilia B dog as a model appears to be predictive of FIX behavior in humans.

In this study with daily administration of human rFIX or pdFIX in the hemophilia B dog, no adverse reactions were observed until the 9th through 14th day. The data on the coagulant and hemostatic effect of rFIX were all obtained before day 9. Both the rFIX and pdFIX showed similar antigenicity in the dogs. The cross-reactivity of the antihuman FIX antibody with canine FIX is in concordance with an earlier study in which it was found that a high degree of identity at the molecular level existed between human and canine FIX; 91% of nucleotides and 85% of amino acids are the same.\textsuperscript{64}

Pure human FIX, free of both human plasma proteins and viral contaminants, has been produced by recombinant technology. The recombinant protein has been shown to be as safe and effective in correcting the coagulation deficiency of hemophilia B dogs as pdFIX.

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