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

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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_1 and V_{ss} 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.

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_r 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.[1,2] 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.[3-7]

Recombinant human factor IX (rFIX) was anticipated as a therapeutic agent for hemophilia B soon after the factor FIX cDNA was cloned in 1982.[8,9] Expression of the rFIX protein in transfected cells in 1985 led to the development of many expression systems[10-14] and became the basis for a technology for producing therapeutic rFIX in a transfected cell line.[15-17] rFIX is expressed by dihydrofolate reductase-deficient and transfected Chinese hamster ovary (CHO) cells.[18] The presence of vitamin K in the medium is required for γ-carboxylation and for production of a functional FIX molecule.[19-22] The initial product had a specific activity of 35 to 75 IU/mg of FIX protein.[18] The rFIX was produced by a serum-free media process[23] and purified to homogeneity by a biochemical process which did not require the use of animal protein.[24] These processes have produced a highly pure protein with a specific activity slightly higher than plasma FIX.[25] These advances in bioengineering made possible limited preclinical testing of rFIX.[26]

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.[27] 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.[28,29]

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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viously 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 comparative immunogenicity of rFIX and pdFIX were ascertained on 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 rFIX 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 monoclonal sandwich ELISA was used to measure FIX plasma concentration, as previously described.[26] FIX coagulant activity was determined by a modified one-stage partial thromboplastin time assay,[30] using kaolin-activated human FIX deficient substrate plasma from a single hemophilia B patient who tested negative for HIV antibody and hepatitis B antigen. Normal human reference plasma consisted of pools from 20 to 30 normal subjects. Partial thromboplastin times (PTT) were determined in the ST4 coagulation instrument (Diagnostica Stago, Asnières, France). For the PTT test, mixtures consisted of equal portions of partial thromboplastin reagent (Thrombosil, Ortho Diagnostics, Raritan, NJ), $CaCl_2$ (0.02 mol/L), and citrated test plasma.[31] Whole blood clotting time (WBCT) was performed by a two-tube procedure at 28°C. One milliliter of whole blood collected with a 1 mL syringe was distributed equally between two siliconized tubes (Vacutainer, #6431; Becton Dickinson, Rutherford, NJ). The first tube was tilted every 30 seconds. After a clot forms, the second tube was tilted and observed every 30 seconds. The endpoint was the clotting time of the second tube. The mean (n = 12) WBCT of normal inbred dogs from the Chapel Hill colony was 8 minutes. The secondary bleeding time test was used for testing the hemostatic effect of infusion of FIX preparations.[32] The primary bleeding time test was performed about 2 hours before infusion and the secondary bleeding time test was performed 15 minutes after infusion. The bleeding time test site was observed until cessation of bleeding or a maximum of 15 minutes, including rebleeding. Normal inbred dogs of the Chapel Hill colony had secondary bleeding times of <5 minutes.[32] Secondary bleeding times of FIX deficient dogs were >15 minutes. FIX antibody assays were performed using two procedures, the Bethesda inhibitor assay[33] and an enzyme-linked immunosorbent assay (ELISA).[26] For the Bethesda inhibitor assay, a patient's plasma with a residual FIX activity of 50% of the normal control was defined as one "Bethesda unit" of inhibitor per milliliter. For ELISA, the titer of each positive sample was given as the log value of the reciprocal of the dilution that generated an optical density (OD) value of equal or greater than two times the negative control OD value. To determine cross-reactivity of the antihuman antibody with canine FIX antigen, the prolongation of the PTT of hemophilia B-canine plasma with neutralizing antibodies mixed with equal amounts of normal canine plasma was compared with controls. Bethesda inhibitor titers were performed with canine FIX antigen.

Determination of FIX recovery. FIX recovery was estimated by dividing the observed value of FIX activity at 15 minutes postinfusion by the expected value. The dose was determined preinfusion and was adjusted for animal studies 4 through 9, 16 through 21, and 25 through 30 on the basis of FIX bioassays on retained samples of the infusate. The expected value was calculated by dividing the infused dose in FIX units by the estimated plasma volume.[34] The latter was calculated using an estimated blood volume of 88 mL/kg and hematocrit.[35,36] With daily doses of FIX, the predose FIX activity level on each day was designated as the trough value. In calculating recovery for repeated doses of FIX in a prophylactic regimen, the observed plasma FIX value was adjusted by subtracting the trough value.

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 $t_{1/2\beta}$ and $t_{1/2\alpha}$ were estimated for each 24-hour period using a two-phase linear regression model.[37] On days 2 through 4, the maximum activity levels of plasma FIX and the biphasic intersection points were interpolated.

PKs. 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, $C = Ae^{-\alpha t} + Be^{-\beta t}$, 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 (C_{max}) values, elimination ($t_{1/2\beta}$), and distribution ($t_{1/2\alpha}$) half-lives, initial volume of distribution (V_1), steady state volume of distribution (V_{ss}), clearance (CL), and area under the curve extrapolated to infinity ($AUC_{0-\infty}$). The elimination half-life ($t_{1/2\beta}$) of FIX activity was determined using a two-phase linear regression model.[37]

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

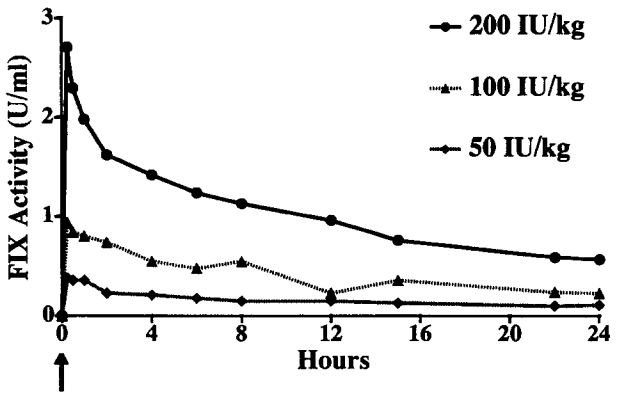

Fig 1. Effect of varying doses of rFIX infused into hemophilia B dogs. The plasma FIX activity values, determined by a modified one-stage PTT test, show the decline of FIX activity over a period of 24 hours in each example. Arrow indicates time of rFIX administration. The 50 IU/kg dose of rFIX is a mean 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 ($t_{1/2\beta}$) 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 the 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

Animal Sex/Age (mo)	FIX Dose (IU/kg)	$t_{1/2\beta}$‡ (h)	WBCT		PTT		Secondary BT
			Pre (min)	Postinfusion* (min)	Pre (s)	Postinfusion* (s)	Postinfusion* (min)
Recombinant human FIX							
F/19	50	17.2	56.0	12.5	>150	96	1.0
M/16	50	21.6	37.0	9.0	145	61	†
M/40	50	18.1	55.5	8.5	136	59	†
M/25	100	16.1	>60	9.0	>150	55	4.5
M/25	200	15.6	—	7.5	—	46	4.0
Plasma-derived human FIX							
F/12	50	15.6	54.5	10.0	140	59	†
F/16	50	18.4	>60	9.0	135	57	2.5
F/12	50	19.7	39.0	10.5	>150	62	2.5

* 15 min postinfusion.

† Not determinable; cuticle arterial bleeding.

‡ Based on FIX activity assays.

Table 2. Plasma Recovery of FIX: Comparison of Different Doses of rFIX and pdFIX

Animal Study No.	Sex/Age (mo)	Dose (IU/kg)	Recovery @ 15 min postinfusion: FIX Activity Expected (U/mL)	FIX Activity Observed (U/mL)	FIX Recovery (%)
Recombinant human FIX					
1	F/19	50	1.03	0.25	24
2	M/16	50	0.98	0.46	47
3	M/40	50	0.95	0.44	47
4*	M/3	50	0.85	0.27	32
5*	M/3	50	0.81	0.35	43
6*	F/3	50	0.85	0.21	24
7*	M/3	50	0.87	0.21	24
8*	M/3	50	0.86	0.26	30
9*	F/3	50	0.85	0.19	23
Mean ± SD					33 ± 10.3
10†	M/25	100	2.32	0.94	41
11	M/3	100	1.65	0.65	40
12	M/3	100	1.58	0.82	52
13	F/3	100	1.65	0.72	44
14	M/3	100	1.67	0.71	42
Mean ± SD					44 ± 4.9
15†	M/25	200	4.64	2.71	57
16†	M/3	200	3.44	1.08	31
17†	M/3	200	3.29	1.78	54
18†	F/3	200	3.50	1.02	29
19†	F/3	200	3.24	1.40	41
20†	F/3	200	3.44	1.04	30
21†	F/3	200	3.39	1.17	34
Mean ± SD					36 ± 15.0
Plasma-derived human FIX					
22	F/12	50	0.87	0.58	66
23	F/16	50	0.95	0.71	75
24	F/12	50	0.96	0.79	82
25*	M/3	50	0.86	0.30	35
26*	M/3	50	0.85	0.36	42
27*	F/3	50	0.80	0.34	43
28*	M/3	50	0.81	0.20	25
29*	M/3	50	0.84	0.26	32
30*	F/3	50	0.82	0.31	37
Mean ± SD					49 ± 20.4

* Crossover study (n = 6).

† Double dosing study (n = 4).

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, V_1, and V_{ss} 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 $t_{1/2\beta}$ 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.[26] 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.

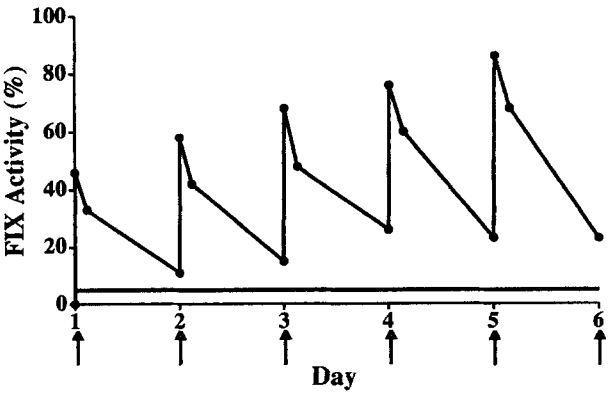

Fig 2. Fluctuations in FIX activity levels following administration of a daily dose, prophylactic regimen of rFIX to hemophilia B dogs. Arrows indicate daily infusions of 50 IU/kg rFIX. Mean rFIX activity values of 3 dogs are plotted (see Table 2, animal studies 1 through 3). FIX trough values for days 2 through 6 were 11%, 15%, 26%, 23%, and 23%.

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

FIX Product	Day	C_{max} (ng/mL)	$t_{1/2\alpha}$ (h)	$t_{1/2\beta}$ (h)	CL (mL/h/kg)	V_1 (mL/kg)	V_{SS} (mL/kg)	$AUC_{0-\infty}$(ng × h/mL)
rFIX	1	1520 ± 150	1.52 ± 1.76	22.5 ± 6.0	8.3 ± 1.7	133.3 ± 28.7	242.3 ± 60.6	25917 ± 342
pdFIX	1	1873 ± 76	2.92 ± 1.09	22.2 ± 6.0	7.1 ± 1.2	138.7 ± 11.0	215.2 ± 61.5	36944 ± 5300
rFIX	5	2520 ± 164	1.10 ± 0.62	21.4 ± 7.1	4.0 ± 1.4	84.4 ± 3.0	109.7 ± 12.8	57073 ± 16214
pdFIX	5	3750 ± 57	1.04 ± 0.66	25.1 ± 10.6	3.9 ± 0.9	68.4 ± 6.3	122.5 ± 20.9	76844 ± 22891

* 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.

Low recovery of FIX with infused concentrates has been recognized since the earliest use of concentrate therapy in hemophilia B.[38] The extent of recovery has varied over a wide range of values in human studies, from about 22% to 78%.[1,3,39-50] 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.[2] There was no clear evidence of dose dependency in this study. The recovery was 44 ± 4.9% at 100 IU/kg of rFIX and 36 ± 15.0% at 200 IU/kg. Two proposed mechanisms in the pathophysiology of FIX appear to play a role in this initial rapid loss of FIX from the circulation, resulting in low recovery of both rFIX and pdFIX. One mechanism is the dynamic equilibrium of the FIX protein that exists between the intravascular and extravascular compartments, reflected in the volume of distribution in the central compartment (V_1) and at steady state (V_{dss}).[49] Another mechanism is the binding of FIX to the endothelial lining of the vascular system. The binding site on the endothelial surface is both saturable and reversible.[51,52] Several groups have shown that the interaction between FIX and the endothelial cell binding site is specifically mediated by the Gla domain[53-58] with the interaction depending on a proper environment, ie, the hydrophobic stack domain and the first epidermal growth factor domain.[59] Although the recoveries of rFIX and pdFIX are both within the ranges reported for pdFIX products,[1,3,39-50] pdFIX dogs had slightly higher recoveries (Table 2). Factor IX is a complex protein with extensive posttranslational modifications: including propeptide cleavage, carboxylation of glutamic acid residues, N-linked, and O-linked glycosylations sites, sialated N-linked glycans and phosphorylated and sulfonated amino acid residues. Variations in any one or several of these components could alter the distribution and binding of FIX, and thus account for the differences in recovery. The bases for the recovery differences in this study are partially related to the variation in recovery of the pdFIX lots evaluated. pdFIX is purified from pooled human plasma resulting in a product that reflects variations found in the donor population. rFIX is produced in CHO cells under highly controlled conditions, resulting in a product with consistent structural composition. Recovery of the two lots of rFIX evaluated in this study ranged from 24% to 47% (mean 39 ± 10.6%) for one lot and from 23% to 43% (mean 29.3 ± 7.6%) for the other lot. Recovery of one lot of pdFIX ranged from 66% to 82% (mean 74.3 ± 6.6%) whereas the other lot ranged from 25% to 43% (mean 36 ± 6.7%).

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

	Day 14		Day 28	
Animal Sex/Age (mo)	Bethesda Inhibitor (U)	FIX Antibody Titer*	Bethesda Inhibitor (U)	FIX Antibody Titer*
Recombinant human FIX				
F/19	0	2.8	5.0	3.8
M/16	0	Negative	0.9	3.2
M/40	0	2.1	4.0	3.9
Plasma-derived human FIX				
F/12	0.5	3.2	7.0	4.0
F/16	0	3.0	1.0	3.2
F/12	1.0	3.0	2.0	3.3

* 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.[26]

There have been only limited studies on the prophylactic regimen in individuals with hemophilia B.[60,61] Nilsson et al[60] have reported on a prophylactic program in Sweden with 8 hemophilia B subjects up to 32 years of age. This program was very successful in reducing joint damage if started early in life. The trough FIX values ranged from 1% to 5%. However, a maximum dose of 25 to 40 IU/kg administered twice a week did not completely prevent hemorrhages.[60] Manco-Johnson et al[61] found that a FIX dose of 40 IU/kg given semiweekly or triweekly was inadequate to prevent hemorrhage in one subject. A more intense schedule of administration of FIX with higher daily doses has been proposed.[62] In the experimental design of this study, a dose of 50 IU/kg given daily for 14 days was selected. Fluctuations in plasma FIX activity levels during the first five days of the prophylactic regimen of rFIX are illustrated in Fig 2. Higher trough values were observed in the hemophilic dog studies than in the human studies, as would be expected with a more intense dosing schedule. With sequential doses of FIX, there is a

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 $t_{1/2\beta}$ 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_{ss} 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_{ss} values reported in several human hemophilia B patients using plasma FIX concentrations, 62 to 233 mL/kg.[1,3,38,42,47,49] 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.[1,3,42,47,49] The range of elimination half-lives ($t_{1/2\beta}$) in patients with hemophilia B ranged from 17 to 34.6 hours,[1,3,38-46,48-50] 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[26] and in SCID mice.[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.[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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REFERENCES

1. Kim HC, McMillan CW, White GC, Bergman GE, Horton MW, Saidi P: Purified factor IX using monoclonal immunoaffinity technique: Clinical trials in hemophilia B and comparison to prothrombin complex concentrates. Blood 79:568, 1992

2. White GC II, Shapiro AD, Kurczynski EM, Kim HC, Bergman GE, Mononine Study Group: Variability of in vivo recovery of factor IX after infusion of monoclonal antibody purified factor IX concentrates in patients with hemophilia B. Thromb Haemost 73:779, 1995

3. Berntorp E, Bjorkman S, Carlsson M, Lethagen S, Nilsson IM: Biochemical and in vivo properties of high purity factor IX concentrates. Thromb Haemost 70:768, 1993

4. Roberts HR, Eberst ME: Current management of hemophilia B. Hematol Oncol Clin North Am 7:1269, 1993

5. Thompson AR: Factor IX concentrates for clinical use. Semin Thromb Hemost 9:25, 1993

6. Herring SW, Abildgaard C, Shitanishi KT, Harrison J, Gendler S, Heldebrant CM: Human coagulation factor IX: Assessment of thrombogenicity in animal models and viral safety. J Lab Clin Med 121:394, 1993

7. Kasper CK, Lusher JM: Recent evolution of clotting factor concentrates for hemophilia A and B: Transfusion Practices Committee. Transfusion 33:422, 1993

8. Choo KH, Gould KG, Rees DJG, Brownlee GG: Molecular cloning of the gene for human anti-hemophilic factor IX. Nature 299:178, 1982

9. Kurachi K, Davie EW: Isolation and characterization of a cDNA coding for human factor IX. Proc Natl Acad Sci USA 79:6461, 1982

10. Brinkhous KM: Gene transfer in the hemophilias: Retrospect and prospect. Thromb Res 67:329, 1992

11. Thompson AR: Molecular biology of the hemophilias. Prog Hemost Thromb 10:175, 1991

12. Thompson AR, Palmer TD, Lynch CM, Miller AD: Gene transfer as an approach to cure patients with hemophilia A or B. Curr Stud Hematol Blood Transfus 58:59, 1991

13. Limentani SA, Roth DA, Furie BC, Furie B: Recombinant blood clotting proteins for hemophilia therapy. Semin Thromb Hemost 19:62, 1993

14. Lozier JN, Brinkhous KM: Gene therapy and the hemophilias. JAMA 271:47, 1994

15. Anson DS, Austen DEG, Brownlee GG: Expression of active human clotting factor IX from recombinant DNA clones in mammalian cells. Nature 315:683, 1985

16. Busby S, Kumar A, Joseph M, Halfpap L, Insley M, Berkner K, Kurachi K, Woodbury R: Expression of active human factor IX in transfected cells. Nature 316:271, 1985

17. de la Salle H, Altenburger W, Elkaim R, Dott K, Dieterle A, Drillien R, Cazenave J-P, Tostoshev P, Lecocq J-P: Active γ-carboxylated human factor IX expressed using recombinant DNA techniques. Nature 316:268, 1985

18. Kaufman RJ, Wasley LC, Furie BC, Furie B, Shoemaker CB: Expression, purification, and characterization of recombinant γ-carboxylated factor IX synthesized in Chinese hamster ovary cells. J Biol Chem 261:9622, 1986

19. Jorgensen MJ, Cantor AB, Furie BC, Brown CL, Shoemaker CB, Furie B: Recognition site directing vitamin K-dependent γ-carboxylation resides on the propeptide of factor IX. Cell 48:185, 1987

20. Galeffi P, Brownlee GG: The propeptide region of clotting factor IX is a signal for a vitamin K dependent carboxylase: Evidence from protein engineering of amino acid -4. Nucleic Acids Res 15:9505, 1987

21. Rabiet MJ, Jorgensen MJ, Furie B, Furie BC: Effect of propeptide mutations on post-translational processing of factor IX: Evidence that β-hydroxylation and γ-carboxylation are independent events. J Biol Chem 262:14895, 1987

22. Soute BA, Balland A, Faure T, de la Salle H, Vermeer C: In vitro carboxylation of a blood coagulation factor IX precursor pro-

duced by recombinant-DNA technology. Thromb Haemost 61:238, 1989

23. Harrison S, Clancy B, Brodeur S, Oakes P, Miller D, Drapeau D, Hamilton M, Charlebois T, Leonard M, McCarthy M, Zollner R, Adamson SR: Development of a serum-free process for recombinant factor IX expression in Chinese hamster ovary cells. Thromb Haemost 73:1222, 1995

24. Foster WB, Anagnostopoulos A, Bonam D, Costigan RJ, Knight A, Sterl KS, Switzer MB, Walsh RE: Development of a process for purification of recombinant human factor IX. Blood 86:870a, 1995 (abstr, suppl 1)

25. Rodriques H, Giles K, Sefton L, Griffon V, Duxbury M, Letwin B: Analytical characterization of recombinant human factor IX (rhFIX). Thromb Haemost 73:1206, 1995

26. Keith JC Jr, Ferranti TJ, Misra B, Frederick T, Rup B, McCarthy K, Faulkner R, Bush L, Schaub RG: Evaluation of recombinant human factor IX: Pharmacokinetic studies in the rat and the dog. Thromb Haemost 73:101, 1994

27. Evans JP, Brinkhous KM, Brayer GD, Reisner HM, High KA: Canine hemophilia B resulting from a point mutation with unusual consequences. Proc Natl Acad Sci USA 86:10095, 1989

28. Brinkhous KM, Sigman J, Read MS, Stewart P, Rup B, Frederick T, McCarthy K, Timony G, Keith J, Schaub R: Recombinant human factor IX restores normal hemostasis in a canine model of hemophilia B. Blood 84:64a, 1994 (abstr, suppl 1)

29. Brinkhous KM, Sigman J, Read MS, Stewart P, Timony G, McCarthy K, Leppanen S, Rupp B, Keith JC, Schaub RG: The hemostatic and pharmacokinetic response of recombinant human factor IX is similar to plasma-derived human factor IX in the hemophilia B dog. Thromb Haemost 73:428a, 1995

30. Barrow EM, Bullock WR, Graham JB: A study of the carrier state for plasma thromboplastin component (PTC, Christmas factor) deficiency, utilizing a new assay procedure. J Lab Clin Med 55:936, 1959

31. Langdell RD, Wagner RH, Brinkhous KM: Effect of antihemophilic factor on one-stage clotting tests. J Lab Clin Med 41:745, 1953

32. Brinkhous KM, Sandberg H, Garris JB, Mattsson C, Palm M, Griggs T, Read MS: Purified human factor VIII procoagulant protein: Comparative hemostatic response after infusions into hemophilic and von Willebrand disease dogs. Proc Natl Acad Sci USA 82:8752, 1985

33. Kasper CK, Aledort LM, Counts RB, Edson JR, Fratantoni J, Green D, Hampton JW, Hilgartner MW, Lazerson J, Levine PH, McMillan CW, Pool JG, Shapiro SS, Shulman NR, van Eys J: A more uniform measurement of factor VIII inhibitors. Thromb Diathes Haemorrh 34:869, 1975

34. Feldschuh J, Enson Y: Prediction of the normal blood volume: Relation of blood volume to body habitus. Circulation 56:605, 1977

35. Pratt PW: Medical Nursing for Animal Health Technicians (ed 1). Santa Barbara, CA, American Veterinary Publications, 1985, p 329

36. Fraser CM: The Merck Veterinary Manual: A Handbook of Diagnosis, Therapy, and Disease Prevention and Control for the Veterinarian (ed 6). Rahway, NJ, Merck, 1986, p 32

37. Lee ML, Poon W-Y, Kingdon HS: A two-phase linear regression model for biologic half-life data. J Lab Clin Med 115:745, 1990

38. Hoag MS, Johnson FF, Robinson JA, Aggeler PM: Treatment of hemophilia B with a new clotting factor concentrate. N Engl J Med 280:581, 1969

39. Zauber PN, Levin J: Factor IX levels in patients with hemophilia B (Christmas disease) following transfusion with concentrates of factor IX or fresh frozen plasma (FFP). Medicine 56:213, 1977

40. Heldebrant CM, Gomperts ED, Kasper CK, McDougal JS, Friedman AE, Hwang DS, Muchmore E, Jordan S, Miller R, Sergis-Davenport E, Lam W: Evaluation of two viral inactivation methods for the preparation of safer factor VIII and factor IX concentrates. Transfusion 25:510, 1985

41. Menache D: Coagulation factor IX concentrate: Properties and clinical investigation. Thromb Haemost 54:282a, 1985

42. Longo G, Cinotti S, Filimberti E, Giustarini G, Messori A, Morfini M, Ferrini PR: Single-dose pharmacokinetics of factor IX evaluated by model-independent methods. Eur J Haematol 39:426, 1987

43. Kohler M, Seifried E, Hellstern P, Pindur G, Miyashita C, Morsdorf S, Fasco F, Wenzel E: In vivo recovery and half-life time of a steam-treated factor IX concentrate in hemophilia B patients: The influence of reagents and standards. Blut 57:341, 1988

44. Kim HC, McMillan CW, White GC, Bergman GE, Saidi P: Clinical experience of a new monoclonal antibody purified factor IX: Half-life, recovery, and safety in patients with hemophilia B. Semin Hematol 27:30S2, 1990

45. Kim HC, Matts L, Eisele J, Czachur M, Saidi P: Monoclonal antibody-purified factor IX: Comparative thrombogenicity to prothrombin complex concentrate. Semin Hematol 28:15S6, 1991

46. Kasper CK, Abramson SB, Goldsmith JC, Herring S: In vivo recovery, half-life and safety of affinity purified solvent-detergent coagulation factor IX. Blood 78:58a, 1991

47. Morfini M, Longo G, Cinotti S, Filimberti E, Messori A, Rafanelli D, Mannucci PM: Comparative single-dose pharmacokinetic study of alphanine vs. prolinine. Thromb Haemost 65:2424a, 1991

48. Goldsmith JC, Kasper CK, Blatt PM, Gomperts ED, Kessler CM, Thompson AR, Herring SW, Novak PL: Coagulation factor IX: Successful surgical experience with a purified factor IX concentrate. Am J Hematol 40:210, 1992

49. Bjorkman S, Carlsson M, Berntorp E: Pharmacokinetics of factor IX in patients with haemophilia B: Methodological aspects and physiological interpretation. Eur J Clin Pharmacol 46:325, 1994

50. Poon M-C, Aledort LM, Anderle K, Kunschak M, Morfini M, Group FIS: Comparison of the recovery and half-life of a high-purity factor IX concentrate with those of a factor IX complex concentrate. Transfusion 35:319, 1995

51. Rimon S, Melamed R, Savion N, Scott T, Nawroth PP, Stern DM: Indentification of a factor IX/IXa binding protein on the endothelial cell surface. J Biol Chem 262:6023, 1987

52. Cheung WF, Hamaguchi N, Smith KJ, Stafford DW: The binding of human factor IX to endothelial cells is mediated by residues 3-11. J Biol Chem 267:20529, 1992

53. Heimark RL, Schwartz SM: Binding of coagulation factors IX and X to the endothelial cell surface. Biochem Biophys Res Comm 11:723, 1983

54. Stern DM, Drillings M, Nossel HL, Hurlet-Jensen A, LaGamma KS, Owen J: Binding of factors IX and IXa to cultured vascular endothelial cells. Proc Natl Acad Sci USA 80:4119, 1983

55. Ryan J, Wolitzky B, Heimer E, Lambrose T, Felix A, Tam JP, Huang LH, Nawroth P, Wilner G, Kisiel W, Nelsestuen GL, Stern DM: Structural determinants of the factor IX molecule mediating interaction with the endothelial cell binding site are distinct from those involved in phospholipid binding. J Biol Chem 264:20283, 1989

56. Astermark J, Stenglo J: The epidermal growth factor-like domains of factor IX: Effect on blood clotting and endothelial cell binding of a fragment containing the epidermal growth factor-like domains linked to the γ-carboxyglutamic acid. J Biol Chem 266:2438, 1991

57. Cheung WF, Straight DL, Smith KJ, Lin S-W, Roberts HR, Stafford DW: The role of the epidermal growth factor-1 and hy-

drophobic stack domains of human factor IX in binding to endothelial cells. J Biol Chem 266:8797, 1991

58. Toomey JR, Smith KJ, Roberts HR, Stafford DW: The endothelial cell binding determinant of human factor IX resides in the γ-carboxyglutamic acid domain. Biochemistry 31:1806, 1992

59. Mayhew M, Handford P, Brownlee GW: The binding of natural variants of human factor IX to endothelial cells. FEBS Lett 341:74, 1994

60. Nilsson IM, Berntorp E, Lofqvist T, Petterson H: Twenty-five years' experience of prophylactic treatment in severe haemophilia A and B. J Intern Med 232:25, 1992

61. Manco-Johnson MJ, Nuss R, Geraghty S, Funk S, Kilcoyne R: Results of secondary prophylaxis in children with severe hemophilia. Am J Hematol 47:113, 1994

62. Furie B, Limentani SA, Rosenfield CG: A practical guide to the evaluation and treatment of hemophilia. Blood 84:3, 1994

63. Yao S-N, Smith KJ, Kurachi K: Primary myoblast-mediated gene transfer: Persistent expression of a human factor IX in mice. Gene Therapy 1:99, 1994

64. Evans JP, Watzke HH, Ware JL, Stafford DW, High KA: Molecular cloning of a cDNA encoding canine factor IX. Blood 74:207, 1989