# Correction of hemophilia B in canine and murine models using recombinant adeno-associated viral vectors

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**Hemophilia B, or factor IX deficiency, is an X-linked recessive disorder occurring in about 1 in 25,000 males. Affected individuals are at risk for spontaneous bleeding into many organs; treatment mainly consists of the transfusion of clotting factor concentrates prepared from human blood or recombinant sources after bleeding has started. Small- and large-animal models have been developed and/or characterized that closely mimic the human disease state. As a preclinical model for gene therapy, recombinant adeno-associated viral vectors containing the human or canine factor IX cDNAs were infused into the livers of murine and canine models of hemophilia B, respectively. There was no associated toxicity with infusion in either animal model. Constitutive expression of factor IX was observed, which resulted in the correction of the bleeding disorder over a period of over 17 months in mice. Mice with a steady-state concentration of 25% of the normal human level of factor IX had normal coagulation. In hemophilic dogs, a dose of rAAV that was approximately 1/10 per body weight that given to mice resulted in 1% of normal canine factor IX levels, the absence of inhibitors, and a sustained partial correction of the coagulation defect for at least 8 months.**



Hemophilia is an X-linked recessive bleeding disorder that affects about 1 in 5,000 males and results from a deficiency of clotting factors VIII or IX. Hemophilia B, or factor IX deficiency, represents about 20% of hemophiliacs. Affected individuals are at risk for spontaneous 'bleeds' into many organs, some of which can be life threatening and/or lead to chronic problems such as severe arthritis. Therapy for acute bleeding consists of the transfusion of clotting factors prepared from human blood products. The risks associated with transfusion of these products includes infection with lethal agents that include HIV and hepatitis viruses. As a result, more than 50% of the hemophilia population treated before the early 1980s became infected with HIV, and before 1988–1990, with hepatitis. This and other factors have stimulated the development of recombinant clotting factors now in clinical trials or in clinical use for treatment of bleeding, and in some cases, for prophylaxis against bleeding. There are murine[1–4], canine[5–9], porcine[10] and ovine[11] models of hemophilia that have been used for studying the pathophysiology of the disease as well as new therapeutic formulations of human plasma-derived and recombinant coagulation factors. In addition, these animals are important for developing new gene therapy strategies.

The liver is the native site for factor IX and VIII synthesis, and the successful transfer of vectors to this organ will be important for the eventual cure of hemophilia. Although intramuscular expression of factor IX may be useful for treating hemophilia B (refs. 12–15), it probably will not provide treatment for hemophilia A because of the difficulty the factor VIII protein has in gaining access to the intravascular space and remaining stable[16]. Thus, the demonstration of long-term hepatic gene transfer of factor IX genes into the livers of hemophilia B animal models is important for determining the feasibility of treating hemophilia using gene therapy by this route. In addition, the liver may offer advantages over muscle-derived gene therapy because of the absence of increased immunogenicity and the fact that anaphylaxis and nephrotic syndrome can occur during the onset of an immune response to factor IX (refs. 17,18) . In the past, there has been limited success using adenoviral[12,19–21] and retroviral[22] vectors in hemophilia animal models. Although recombinant retroviruses have resulted in the long-term persistence of factor IX (indicating permanence), the level of gene expression was subtherapeutic. In contrast, adenovirus-mediated therapy resulted in complete correction of the coagulation defect, albeit for a short period of time. Moreover, the adenovirus vectors in these studies were shown to have toxicity and immunogenicity associated with the therapy.

Recombinant adeno-associated viral (rAAV) vectors have been used to deliver the human factor IX gene into hepatocytes

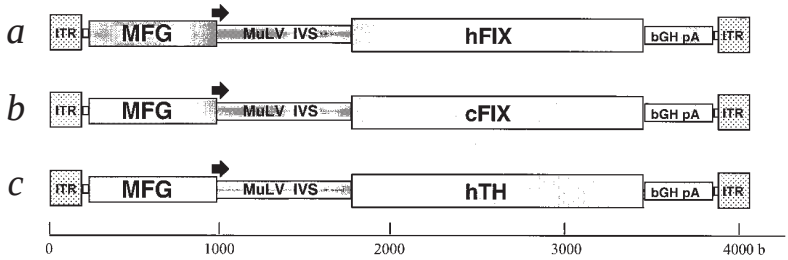


**Fig. 1** Structures of the rAAV vectors. ITR, AAV inverted terminal repeat; MFG promoter, murine Molony virus long terminal repeat; MuLV IVS, mRNA splice donor/splice acceptor; bGH pA, bovine growth hormone polyadenylation site. ***a***, rAAV-MFG-hFIX has been described[23]. hFIX, human factor IX cDNA. ***b***, rAAV-MFG-cFIX. cFIX, canine factor IX cDNA. ***c***, rAAV-MFG-hTH. hTH, human tyrosine hydroxylase cDNA. Scale indicates the length of single-stranded DNA in bases.

of immunocompetent normal mice[23]. This resulted in long-term production (mice were 20 months old when killed) of therapeutic, and in some cases, curative concentrations of functionally active factor IX. In addition, the therapy did not induce hepatitis or any type of detectable toxicity. Vector genomes are integrated into chromosomal DNA as multimeric head-to-tail concatamers, resulting in liver-specific gene expression, indicating the potential for cure with a single administration[23,24]. Liver-directed, high-level expression of factor IX in mice has been independently confirmed using a recombinant AAV vector that expresses factor IX from a cellular promoter[25]. Here we describe preclinical data demonstrating the successful treatment of murine and canine hemophlilia B using a single intrahepatic administration of rAAV vectors.

### Vector characterization

To study the utility of AAV vectors in hemophilic animals, we used the recombinant vectors rAAV-MFG-hFIX, rAAV-MFG-cFIX, and rAAV-MFG-TH (Fig. 1). The expression of these vectors was assessed *in vitro* (data not shown).

### rAAV selectively transduces murine liver

Intra-portal infusion of rAAV results in selective transduction of hepatocytes. To determine the bodily distribution of single-stranded vector genomes, we studied normal mice transduced with rAAV-MFG-hFIX through an intraportal infusion at five different time points (1 day to 13 weeks) for the presence of single-stranded stranded DNA in various tissues. Southern analysis showed that the most of the single-stranded vector was in the liver, but small amounts were detected in the spleen and the muscle of one mouse during the first few days only (Fig. 2). Studies in mice have shown a concomitant increase in factor IX gene expression and loss of single-stranded but

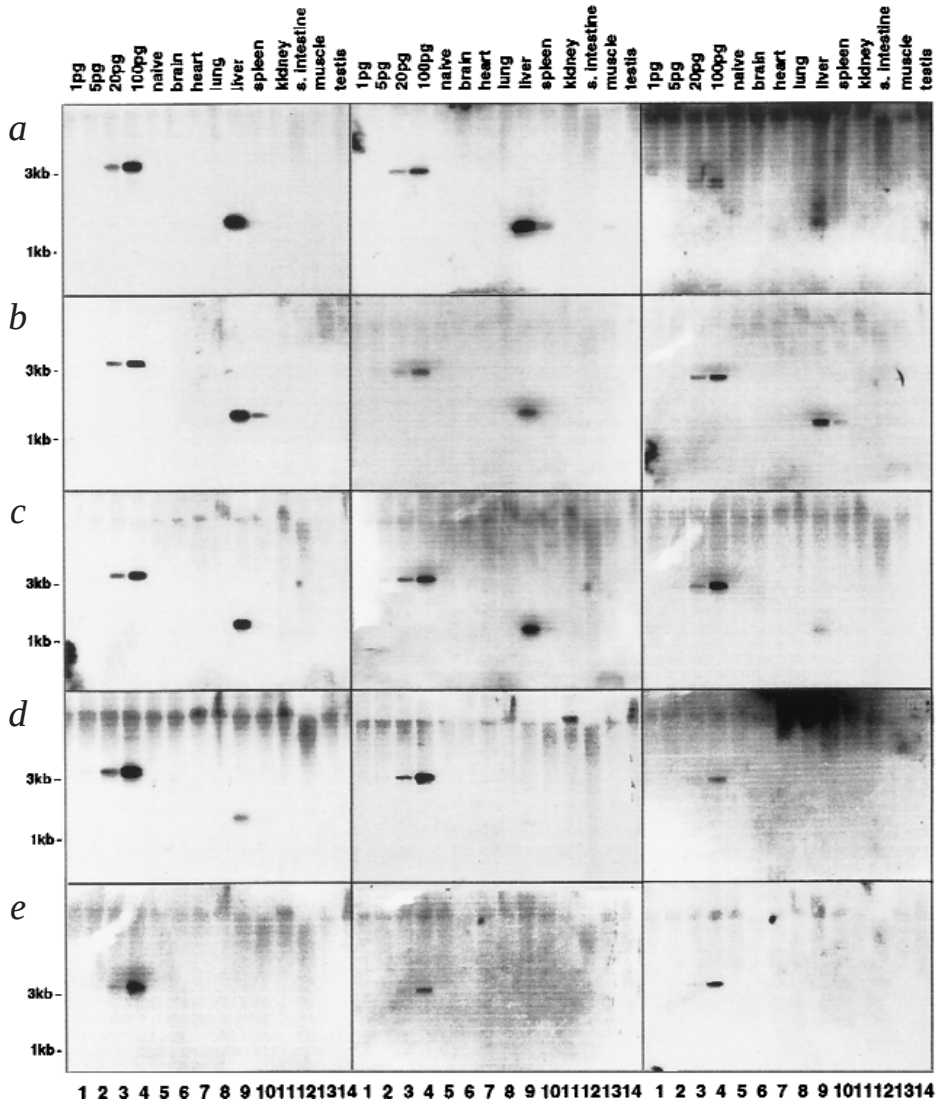


**Fig. 2** Tissue distribution of the vector genome over time after gene transfer in mice. Total cellular DNA was isolated from different organs of five groups of mice ($n$ = 3 per group) that had received $4.2 \times 10^{10}$ particles of rAAV-MFG-hFIX. Each panel represents an individual mouse; each group was killed at a different time point (***a***, day 1; ***b***, day 3; ***c***, day 7; ***d***, day 35; ***e***, day 91). DNA was analyzed by Southern blot with a radiolabeled human factor IX probe. Lanes 1–4: 1, 5, 20 and 100 pg of pSSV9-MFG-hFIX plasmid mixed with total DNA isolated from a naive mouse; lane 5: liver DNA from a naive mouse; lanes 6–14: DNA from different organs from AAV-treated mice: lane 6, brain; lane 7, heart; lane 8, lung; lane 9, liver; lane 10, spleen; lane 11, kidney; lane 12, small intestine; lane 13, skeletal muscle; lane 14, testis. The double-stranded DNA migrates with the genomic DNA at the top of the gel.

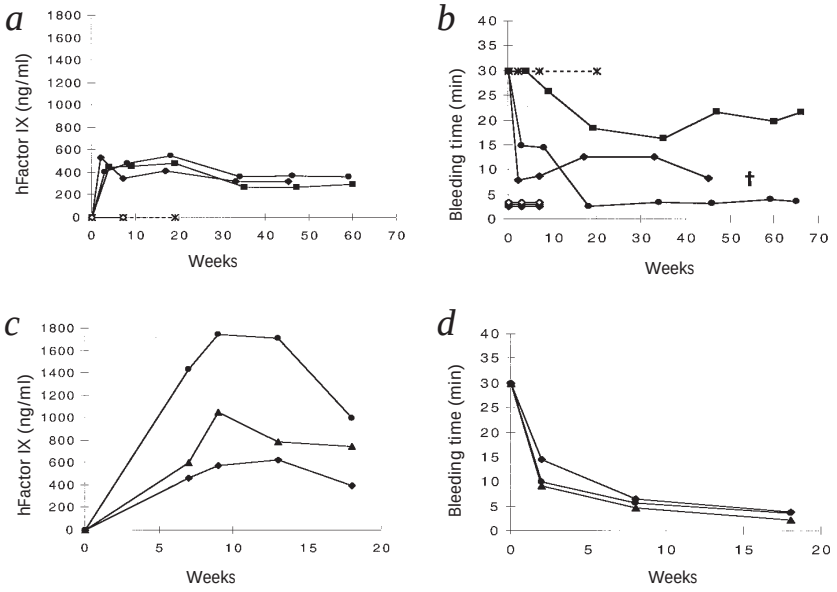


**Fig. 3** Gene therapy in hemophilia B mice. Recombinant rAAV-MFG-hFIX or rAAV-MFG-TH was delivered to the liver of hemophilia B mice 5–6 weeks of age as a single intra-portal or tail vein administration of $4.2 \times 10^{10}$ ($n = 3$) (***a*** and ***b***) or $6.3 \times 10^{10}$ ($n = 3$) (***c*** and ***d***) particles in a volume of 200–300μl. Mice were bled at the indicated number of weeks after AAV administration (horizontal axis); the expression of hFIX was determined by an ELISA (*a* and *d*), and bleeding times were determined by transecting the tail and measuring the time required to clot (*c* and *d*). For mice whose blood did not clot, blood was collected for 30 min or until 300 μl had been collected, at which time the tails were cauterized. Asterisks (*a* and *b*), hemophilia B mice infused with rAAV-MFG-TH; diamonds, non-transduced normal mice (*a* and *b*); squares, a mouse given a tail vein infusion of vector; all others were infused through the portal vein. The mice in panels *c* and *d* were treated the same through intra-portal injection. One mouse died at age 54 weeks for reasons that were not known. None of the treated mice suffered from spontaneous hemorrhages. Most of the untreated hemophilia B mice die of bleeding within the first few months of life unless housed in separate cages.

an increase in double stranded integrated rAAV genomes during the first 5 weeks after vector infusion, whereas the spleen did not contain double-stranded rAAV DNA at the same or later time points in identical experimental conditions[23,24].

### Correction of the bleeding disorder in hemophilia B mice

To determine if the bleeding disorder in factor IX-deficient, hemophilia B mice could be corrected, we infused $4.2 \times 10^{10}$ or $6.3 \times 10^{10}$ particles of rAAV-MFG-hFIX into the livers of these mice through the portal vein. However, because of severe bleeding diathesis, gene transfer into the hemophilia B mice required pre-infusion of normal mouse plasma from normal donor C57Bl/6 mice. The serum human factor IX (hFIX) levels were assessed by ELISA (Fig. 3*a* and *c*); the concentrations achieved in these hemophilia B mice were similar to levels obtained in normal transduced mice[23], ranging from 250 ng/ml to 1,800 ng/ml. No hFIX was detected in control hemophilia B mice infused with rAAV-MFG-TH or in normal untreated mice.

Bleeding times were assessed in these transduced mice after transection of their tails (Fig. 3*b* and *d*). Normal mice had bleeding times of 3–5 minutes, whereas the naive mice or hemophilia B mice treated with rAAV-MFG-TH had bleeding times of greater than 30 minutes, the time allotted before cauterization (without tail cauterization they would exsanguinate). After infusion of rAAV-MFG-hFIX, the hemophilia B mice showed a correction in their bleeding times ranging from 3 to 20 minutes. Between 17 and 20 weeks, the human factor IX biological activity was determined to be 7%, 8% and 25% (Fig. 3*a* and *b*) and 16%, 52% and 110% (Fig. 3*c* and *d*), which correlated with the degree of correction of the bleeding times. With 16–25% or more activity, the bleeding times were in the normal range, indicating that AAV-mediated delivery of human factor IX can cure murine hemophilia B.

### Detection of anti-hFIX antibodies in mice

To determine whether a humoral response directed against hFIX was generated in the hemophilia B mice, we did a western analysis using sera from mice treated with rAAV-MFG-hFIX. Anti-hFIX antibodies were generated in rAAV-treated non-hemophilic C57Bl/6 mice and hemophilic mice, but not C57Bl/6-scid mice (Fig. 4). No endogenous anti-hFIX antibodies were present in naive hemophilic mice whether or not they were treated with normal mouse plasma.

### Correction of the bleeding disorder in hemophilia B dogs

We tested the rAAV-MFG-cFIX vector in C57Bl/6 mice before studying it in dogs. With a dose of $3.5 \times 10^{10}$ rAAV particles, the canine factor IX (cFIX) levels varied between 100 ng/ml and 300 ng/ml (data not shown) and was similar to that obtained with the rAAV-MFG-hFIX vector in normal mice[23]. To determine if the bleeding disorder could be corrected in a large-animal model of hemophilia B, we infused two factor IX-deficient dogs with $2 \times 10^{12}$ particles of rAAV-MFG-cFIX through the portal vein. This dose was about 10% of that used in the mouse (lower dose) on a per weight basis.

Injection into the portal vein required that the hemophilia B dogs be infused with plasma from normal donor dogs before surgery[21]. Four recipient hemophilia B and twelve normal donor dogs were screened for the presence of anti-AAV neutralizing antibodies before surgery and vector administration. No

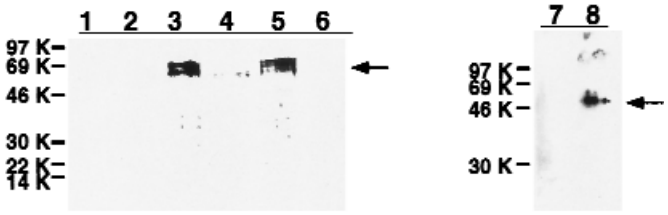


**Fig. 4** Western blot analysis for anti-hFIX antibodies in rAAV treated mice. Purified factor IX protein was transferred to nitrocellulose blots and incubated with diluted serum samples collected between 7 and 10 weeks after rAAV therapy and then with peroxidase-labelled goat anti-mouse IgG. The serum samples were diluted: lanes 1–6, 1:2,000; lanes 7–8, 1:1,000. All sera were from mice that received rAAV except lanes 2 and 7. Lane 1, Secondary antibody only (control); lane 2, naive hemophilia control that received mouse plasma only; lanes 3 and 4, Hemophilic, AAV-HFIX treated mice; lane 5, Non-hemophilia littermate; lane 6, C57Bl/6 scid control; lane 7, naive control; lane 8, C57Bl/6.

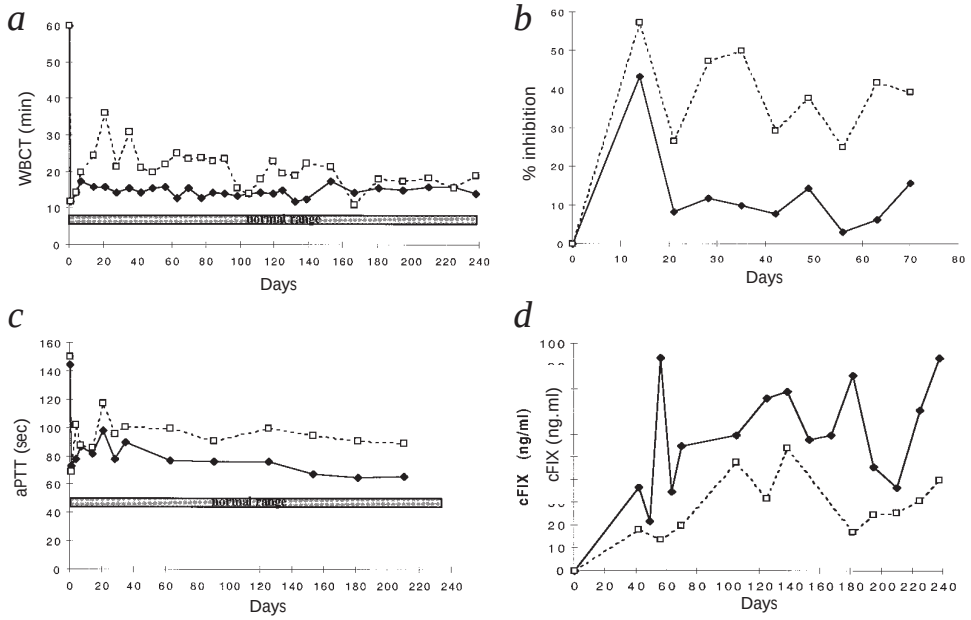


**Fig. 5** Gene therapy in hemophilia B dogs. Recombinant AAV-MFG-canine Factor IX was delivered to the liver of two hemophilia B dogs as a single intra-portal administration of $2.0 \times 10^{12}$ particles in a volume of 11 ml. ***a***, Whole blood clotting times (WBCT); normal WBCT is 6–8 min. ***b***, AAV neutralizing activity at a 1:1,000 dilution. ***c***, aPTT; normal aPTT is 42–50 s. ***d***, cFIX antigen levels. Dashed lines, dog B89; solid lines, dog B84.

neutralizing activity was detected in the young hemophilia B dogs, however, one of the donor dogs had substantial neutralizing activity and was eliminated from the donor pool.

After vector infusion, the whole-blood clotting time (WBCT), serum cFIX antigen, plasma biofunctional FIX protein (Fig. 5), serum liver enzymes, serum bilirubin and alkaline phosphatase concentrations (Table) were monitored. WBCT was reduced in both dogs (B84 and B89) immediately after surgery (Fig. 5*a*), because of the infusion of plasma from normal donors (the last infusion took place 2 days post-operatively). In hemophilia B dogs transfused with cFIX but not treated with rAAV, the WBCT did not return completely to baseline values until after approximately 20 days[26]. However, after this period, dogs B84 and B89 had sustained reductions in the WBCTs to about 13 and 20 minutes, respectively. Thus, the rise in WBCT in dog B89 over the first 10–21 days, before its stabilization, was probably due to the disappearance of the transfused cFIX (half-life, 20 hours). Dog B89 suffered a chest and shoulder bleed at day 95 (Table) that required 100 ml of normal dog plasma, and had a transient fall in WBCT between days 95 and119, but this returned to the steady-state level of about 20 minutes.

The more clinically relevant activated partial thromboplastin time (aPTT) was determined as a secondary means of monitoring the correction of the coagulation defect in the dogs. The pretreatment aPTT values were 145–150 seconds, whereas normal non-hemophiliac siblings had values ranging from 42 to 50 seconds. Both rAAV treated dogs had a reduction in aPTTs in a range of 65–110 seconds (Fig. 5*c*).

Canine FIX serum antigen levels were 30–95ng/ml in dog B84, which correlated with a biological assay level of 1%, indicating that the cFIX has full biological activity (Fig. 5*d*). In dog B89, the cFIX antigen levels were 10–45ng/ml and the biological factor IX activity was about 0.5%. The difference in WBCTs and cFIX antigen levels between the two dogs may be attributed to their difference in weight at the time of infusion and over the course of the study (Table). There were no inhibitors detected against factor IX by Bethesda assay on days 0, 28 and 91.

Liver enzymes (ALT, AST and GGT), bilirubin and alkaline phosphatase were monitored for three weeks (Table). Levels were not significantly elevated except for a clinically insignificant rise in the ALT in dog B84 at day 1 after infusion. The alkaline phosphatase was slightly above the normal range in this same dog for several weeks, probably

**Table** rAAV-transduced hemophilia B dogs

| Dog | Day | AST[a] | Bilirubin[b] | GGT[c] | ALT[d] | Alk Phos[e] | Weight[f] | Event |
|---|---|---|---|---|---|---|---|---|
| B84, 3 mo old | 0 | 20 | 0.1 | 3 | 35 | 218 | 8.6 | Coverage/AAV |
| | 1 | 29 | 0.4 | 4 | 215 | 216 | | Coverage |
| | 4 | 25 | 0.3 | 3 | 69 | 206 | | |
| | 7 | 26 | 0.4 | 3 | 45 | 201 | | |
| | 14 | 34 | 0.4 | 2 | 32 | 189 | | |
| | 21 | 34 | 0.1 | ND[g] | 21 | 169 | | |
| | 47 | ND | ND | ND | ND | ND | 12.7 | |
| | 140 | ND | ND | ND | ND | ND | 15.9 | |
| B89, 3 mo old | 0 | 24 | 0.4 | 1 | 31 | 145 | 10.9 | Coverage/AAV |
| | 1 | 25 | 0.3 | 1 | 28 | 121 | | Coverage |
| | 4 | 20 | 0.4 | 2 | 26 | 133 | | |
| | 7 | 32 | 0.4 | 2 | 29 | 148 | | |
| | 14 | 32 | 0.4 | 2 | 34 | 129 | | |
| | 21 | 26 | 0.1 | ND | 21 | 127 | | |
| | 47 | ND | ND | ND | ND | ND | 18.1 | |
| | 95 | ND | ND | ND | ND | ND | Bleed/ Coverage | |
| | 140 | ND | ND | ND | ND | ND | 19.5 | |
| normal range | | 10–60 | 0.1–0.5 | 0–10 | 0–120 | 21–125 | | |

[a]AST, aspartate amino transferase levels in IU/L. [e]Alk Phos, alkaline phosphatase levels in IU/L [b]Bilirubin levels in mg/dL. [f]Weight in kg [c]GGT, gamma glutamyltransferase in IU/L. [g]ND, not determined [d]ALT, alanine amino transferase levels in IU/L.

due to bone growth.

Although there were no detectable anti-AAV antibodies before infusion, as expected, a humoral response to the AAV virions was observed after intra-portal rAAV therapy with maximal neutralization occurring at approximately 2 weeks after transduction. Dog B89 had a higher, more persistent level of neutralizing antibodies than did dog B84 (Fig. 5*b*).

In a separate experiment, a hemophilia B dog infused with $1 \times 10^{13}$ particles (five times more than the dogs studied here) of an irrelevant rAAV ($4.4 \times 10^{13}$ particles) vector had about 0.2–0.5 rAAV double-stranded rAAV DNA genomes per diploid dog genome more than 6 months after infusion (not shown). This establishes that, like the mouse, the dog has persistent double-stranded rAAV DNA genomes in the liver.

## Discussion

Recombinant AAV vectors can integrate into the genome of mice after intrahepatic delivery. After transduction, up to 5% of hepatocytes each have an average of 60–70 integrated rAAV genomes configured in head-to-tail concatamers[24]. Moreover, gene expression as detected by RT–PCR and RNA *in situ* hybridization showed hepatocyte directed gene expression. This results in constitutive and, in some cases, curative concentrations of human factor IX for the lifetime of the treated mouse. The rise in factor IX expression over a period of about 5 weeks is consistent with the slow conversion of the single-stranded genomes into double-stranded DNA (ref. 24). To determine if these factor IX levels would actually be therapeutic in animal models of hemophilia, we designed preclinical studies in hemophilia B mice and dogs. To gain access to the portal vein for vector infusion, the hemophilic animals were treated with normal plasma to prevent bleeding during surgery. There were no complications observed with this or subsequent procedures.

The studies here demonstrate that rAAV-mediated gene transfer of the hFIX cDNA can correct the bleeding diathesis in hemophilia B mice. The untreated animals rarely survive longer than 2–3 months because of spontaneous hemorrhage[1,20], and until now all the rAAV treated animals have survived for at least 1 year. Whether the success obtained in mice would translate into successful results in non-rodent or larger animal models could not be predicted. Thus, confirmation in another species was important to help predict the eventual success and the dose required in humans. In the mouse studies here and in our previous studies, the concentration of hFIX ranged between 5 and 40% of the normal concentration depending on the dose of vector. In the dog studies, the total dose of the vector given was 40-fold greater than that given to mice, yet the starting dose of the dogs were 500-fold greater than the weights of the mice. Thus, the effective dose in the dogs was about 8% of that given to the mice. If the response was linear, then the dogs would produce between 0.4% and 3% of the normal level of factor IX. One dog had 0.5% the normal concentration of factor IX, whereas the other dog had about 1%. This resulted in a 50 and 75% reduction in the whole blood clotting time in the two dogs. In addition, the hemophilia B dogs have on average five spontaneous bleeding episodes per year. We have followed two treated dogs for 8 months each, and during that time (16 months), we would expect six to seven 'bleeds' and only observed one. Thus a reduction in spontaneous bleeding episodes was observed in the two dogs treated with rAAV-MFG-cFIX over the course of this study. Further studies are required to determine the statistical significance of the diminished number of bleeding episodes in canine hemophilia B dogs treated with rAAV vectors. Eventually, higher doses of rAAV will be required to achieve higher levels of biofunctional cFIX and a possible cure.

Although antibodies were detected in hemophilic mice receiving rAAV-MFG-hFIX, the serum concentrations of hFIX were steady. Steady-state concentrations of hFIX were slightly higher in C57Bl/6-scid mice than in C57Bl/6 immunocompetent mice[23], indicating that the antibodies may have resulted in a slight increase in hFIX clearance. Antibodies in normal mice did not affect *in vitro* clotting activity[23]. However, the hemophilia B mice had never been exposed to any source of mouse or human factor IX before the start of the study. Thus, the absence of factor IX in these mice did not seem to be the reason for antibody formation, because anti-hFIX antibodies were also detected in normal C57Bl/6 mice and no antibodies were detected in control hemophilia mice given normal mouse plasma injections. The antibodies detected here did not prevent the correction of hemophilia. The data indicate the species differences between the protein and host were responsible for antibody generation. The hemophilia B dogs did not form inhibitors to cFIX by Bethesda detection assays. Moreover, after the administration of plasma from normal donors to B89 at day 95, no interference with the gene therapy was observed at day 119 (when the coverage had 'worn off'). This also indicates that the AAV-expressed cFIX is immunologically indistinguishable from the normal cFIX that was administered. Finally, this indicates that standard treatment can be administered in combination with gene therapy without adverse effects.

Pre-existing immunity to the vector is also potentially important. Plasma from normal donor dogs can contain neutralizing antibodies against AAV. Further studies will be required to determine if prescreening both donor and recipient animals before vector administration will be necessary to avoid anti-vector antibodies that may reduce or inhibit gene transfer, either by adoptive transfer or by pre-existing immunity. Vector mobilization from the liver with concomitant wild-type adenovirus, wild-type AAV and rAAV will need to be addressed also, for safety considerations.

The delivery of rAAV into the liver for treatment of hemophilia is a reasonable approach because the portal circulation can be accessed by interventional radiologic techniques that do not require surgery. The ability to treat hemophilic animals is a step forward towards human clinical trials.

## Methods

**Recombinant AAV vector construction**. Standard cloning methods were used for rAAV vector construction. The vector plasmid SSV9-MFG-S-K9F9 was derived from pXCJL-K9FIX (ref. 12) and SSV9. The SSV9 plasmid (also called psub201; ref. 27) was digested with *Xba*I to remove almost the entire AAV genome, leaving only the terminal repeats, and was filled in by Klenow. The blunt-ended backbone was ligated to a 3.24-kb *Nhe*1–*Sal*1 fragment (filled in by Klenow fragment) containing the Moloney murine leukemia virus (MLV) 5' long terminal repeat, adjacent splice donor/acceptor sequences, and the canine factor IX (K9FIX) cDNA sequence precisely connected to the MLV *env* ATG from plasmid MFG-S-K9FIX and the poly(A) site of bovine growth hormone from pRc/CMV (Invitrogen, Carlsbad, California). MFG-S has been described[28]. The vectors rAAV-MFG-hFIX, rAAV-MFG-hTH, and pTR-UF5 have been described[23,29]. The accuracy of the vector plasmids was verified by DNA sequencing.

**Recombinant AAV vector preparation**. Recombinant AAV (rAAV) vectors were prepared according to a described method[23] with modifications. Subconfluent 293 cells were co-transfected with the vector plasmids, and

the AAV helper plasmid pUC19.ACG was used to supply AAV rep and cap functions. The pUC19.ACG construct contains the *Xba*I fragment of pACG2-1 described[30], which was isolated by PCR to change the 5' *Xba*I site to *Hin*dIII and the 3' *Xba*I site to *Bam*HI. This fragment was inserted into the *Hin*dIII and *Bam*HI sites in pUC-19, and does not contain the adenoviral terminal repeats. rAAV virions were purified on two sequential isopycnic CsCl gradients formed in a Beckman NVT65 rotor at 291,000 *g* for 6 h minimum each. Fractions were collected using a Beckman Fraction Recovery System (catalog number 343890; Beckman Instruments, Palo Alto, California). After CsCl banding, the fractions containing rAAV were dialyzed against PBS (catalog number 59300-78P; JRH Biosciences, Lenexa, Kansas). The vector preparations had particle titers of $1 \times 10^{11}$–$1 \times 10^{12}$ per ml. Wild-type AAV contamination was less than 200 particles per $1 \times 10^{10}$ particle injection. After purification of rAAV virions, there was no contaminating infectious adenovirus. Endotoxin was less than 1 endotoxin unit/ml by gel clot limulus amoebacyte lysate and kinetic turbidimetric assays.

**Animal Studies.** C57Bl/6 mice were obtained from Jackson Laboratories (Bar Harbor, Maine), and hemophilia B mice[1] were mated into a C57Bl/6 background. Animals were treated according to the NIH Guidelines for Animal Care and the Guidelines of the University of Washington, Stanford University and the University of North Carolina, and housed in specific pathogen-free conditions. The AAV vectors were administered by intraportal or by intravenous tail vein infusion[21]. The 100- to 300-μl volume was infused over 30 seconds. Mice were periodically bled using the retroorbital technique. Hemophilic mice were treated with 0.5 ml of 'pooled', normal mouse plasma 30 min before abdominal surgery and with 0.2 ml of plasma by IP infusion about 12 h after a portal vein infusion of the rAAV vector.

Inbred hemophilia B dogs 3 months old, maintained at the Francis Owen Blood Research Laboratory (University of North Carolina at Chapel Hill), were pre-screened for anti-AAV neutralizing antibodies. These dogs had been previously immunized for canine parvoviruses. Dogs were given plasma 'coverage' from normal dogs (also prescreened for anti-AAV antibodies) on the day of surgery (200 ml) and one day after surgery (100 ml) to prevent bleeding. The AAV vectors were injected directly into the portal vein, which had been isolated during laparotomy of animals sedated by general anesthesia[21]. The 11-ml volume was infused over approximately 30 min.

**Blood analysis**. Mouse serum was analyzed for total human factor IX antigen by an ELISA assay using polyclonal antisera to human factor IX. Mouse plasma samples for factor IX clotting activities were collected by periorbital capillary pipette and analyzed as described[23]. The specific activity of hFIX in a mouse plasma background may be slightly varied because of unknown interactions of the human protein with mouse plasma proteins. The bleeding time in mice was calculated by clipping a section of tail 2–3 mm in length and collecting blood in a tube and recording the time when bleeding stopped. Hemophilia B mice do not stop bleeding, and the tails were cauterized by heat at 30 min or when 300 μl of blood was lost. Whole-blood clotting time (WBCT) and bioactivity of canine factor IX were measured as described[22]. Canine blood chemistries (GGT, ALT, AST, alkaline phosphatase and bilirubin) were analyzed in an automated clinical laboratory.

**Antibody analysis**. Western blot analysis was done by separating 500 pg of purified factor IX (Sigma) by 10% SDS–PAGE and transferring it to nitrocellulose. After transfer, the blot was cut into strips and blocked for 2 h at room temperature in TBST: 8% milk (Carnation Instant Milk, Carnation, Nestle Food Company, Glendale, California) and 1% BSA (Fisher) in TBS containing 0.1% Tween-20 (BioRad, Richmond, California). The strips were then incubated overnight at 4 °C with the diluted serum samples. The dilution buffer was 3% milk and 0.5% BSA in TBST. Next, the strips were washed twice for 5 min each, then twice for 10 min each in TBST. After being washed, the strips were incubated in HRP-labeled goat anti-mouse IgG (Jackson Immunoresearch, West Grove, Pennsylvania) at a 1:5,000 dilution for 1 h at room temperature and then washed. The blot was then developed using the ECL kit (Amersham). Factor IX antibodies were detected by the Bethesda inhibitor assay[31]. For the assay, a subject plasma with a residual FIX activity of 50% of the normal control is defined as one Besthda units of inhibitor per ml.

**Detection of neutralizing anti-AAV antibodies**. 293 cells were seeded in a 24-well plate at a density of $1 \times 10^5$ cells/well, in 1 ml of IMDM media (JRH Biosciences, Lenexa, Kansas). The cells were allowed to adhere for 2 h at 37 °C. The media was then removed by aspiration before $6 \times 10^6$ particles of adenovirus dl309 were added in a final volume of 200 μl per well. The cells were further incubated at 37 °C for 1 h and then washed twice in the same media before the following mix was added. Antibody binding was done by incubating rAAV-CMV-GFP (1 μl = $5 \times 10^8$ total particles or $9 \times 10^6$ transducing units) virus with serum diluted in PBS at 4 °C for 2 h in a total volume of 25 μl. The final dilution of the test serum from hemophilia B mice and dogs, normal mice and dog plasma donors, and transduced animals taken over the course of the experiment was 1:100 or 1:1,000. This mix was added to the washed cells in a final volume of 200 μl and incubated for 1 h at 37 °C. Media (400 μl) were then added to each well and cells were incubated overnight. Cells were collected, washed in PBS/BSA (1%), and analyzed by fluorescence activated cell sorting. The percent inhibition was calculated using a 'no antibody' control sample as a reference. Another control was anti-AAV guinea-pig sera, which showed maximal inhibition.

*Acknowledgments*

*We thank S. Powell, V. Vimal, B. Winther, B. Kaspar, T. Dull, D. Colvin, R. Lazar, M. Moskalenko, Y.-L. Li, R. Raymer and P. McElveen for technical assistance, and L. Naldini for comments. This work was supported by NIH grants, HL53682 (M.A.K.) and HL01648 (U.N.C.).*

RECEIVED 16 SEPTEMBER; ACCEPTED 30 NOVEMBER 1998

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