

Complete Short-Term Correction of Canine Hemophilia A by In Vivo Gene Therapy

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Hemophilia A is a severe bleeding disorder caused by a deficiency in clotting factor VIII (FVIII). A canine model that closely mimics the human disease was used to determine if an adenoviral vector expressing a human FVIII cDNA could be used to correct the hemophilia A phenotype. Within 48 hours after peripheral vein administration of the vector to FVIII-deficient dogs, the hemophilic phenotype was corrected, based on determination of the activated clotting time, the activated partial thromboplastin time, and the cuti-

cle bleeding time. Direct measurement of human FVIII in the dog plasma showed FVIII expression at amounts well above the human therapeutic level. FVIII expression in treated dogs was short-term, lasting 1 to 2 weeks, due to the development of a human FVIII-specific inhibitor antibody response. These data provide the first demonstration of in vivo gene therapy of hemophilia A.

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HEMOPHILIA A IS AN X-linked bleeding disorder caused by a deficiency of blood coagulation factor VIII (FVIII). Hemophilia A affects 1 in 5,000 to 10,000 men and, in the severe form, is a life-threatening, crippling disease.¹ Infusion of plasma-derived or recombinant FVIII in response to bleeding crises is currently the most widely accepted therapy.² Whereas prophylactic treatment of hemophilia A reduces the frequency and severity of bleeding episodes, such treatment is limited by the availability and high cost of purified FVIII, the short half-life of FVIII in vivo, and difficulties associated with repeated intravenous administration.²

Gene therapy for hemophilia A, which would furnish constant blood levels of FVIII, would be a significant treatment improvement. Hemophilia A has been widely discussed as a candidate disease for gene therapy for several reasons.³⁻⁵ (1) the molecular biology and biochemistry of FVIII and hemophilia A are well understood; (2) increasing plasma FVIII activity to 5% to 10% of normal would provide a significant clinical improvement; (3) current treatment is not curative; and (4) a canine model that closely mimics the human disease is available to evaluate new therapeutic products.

Although considerable progress has been made in the development of gene therapy strategies for hemophilia B (factor IX deficiency), FVIII gene therapy has been problematic.³⁻⁵ FVIII must be secreted directly into the plasma^{6,7} and must be complexed with von Willebrand factor (vWF) for protection from proteolytic degradation.⁸ Furthermore, expression of FVIII is inhibited by transcriptional silencers

present in the coding region⁹⁻¹³ and by inefficient protein processing and secretion.^{9,14,15} Most studies in gene therapy for hemophilia A have used an ex vivo retroviral transduction strategy for FVIII gene transfer.^{6,7,10,13,16-19} In general, expression of the FVIII protein in the genetically modified cells was low in vitro^{10,13,16-19} and undetectable in vivo.^{18,19} Recently, short-term expression of therapeutic levels of human FVIII in mice has been achieved via ex vivo gene transfer strategies.^{6,7} However, the necessity for ex vivo cell manipulation and reimplantation is a limitation to the application of gene therapy in large animal models and humans.

As an alternative approach to gene therapy of hemophilia A, we have developed adenoviral vectors for the in vivo delivery of a human FVIII cDNA.²⁰⁻²² Adenoviral vectors are attractive for in vivo FVIII gene delivery because a peripheral vein injection in rodents^{23,24} results in efficient transduction of hepatocytes that are cells capable of secreting FVIII directly into the circulation.⁸ Furthermore, high-level expression of human FVIII can be achieved in normal, adult mice treated with an adenoviral vector.²⁰⁻²² Human FVIII expression in the mice was sustained at levels fourfold greater than the human therapeutic range for at least 22 weeks.²² However, the efficacy of adenoviral-mediated delivery and expression of human FVIII in a clinically relevant large animal model had not been evaluated.

Canine hemophilia A, first reported approximately 50 years ago, has been thoroughly characterized as a model of hemophilia A in humans.^{25,26} Plasma-derived and recombinant human FVIII protein corrects the bleeding disorder in FVIII-deficient dogs.^{15,27-30} Furthermore, treated dogs frequently develop FVIII inhibitors,^{31,32} which is a common problem in human hemophilia A patients.³³ In this work, we have extended our investigations to a canine model of hemophilia A and show efficient adenoviral-mediated gene transfer and high-level human FVIII expression in the treated dogs. Hepatic expression of human FVIII resulted in phenotypic correction of the coagulation deficiency in the hemophilia A dogs.

MATERIALS AND METHODS

Experimental animals and adenoviral vector. The experimental animals used in this study were mixed-breed dogs from the hemophilia A colony housed at Auburn University.³⁴ The adenoviral vector, Av1ALAPH81, contains a mouse albumin promoter, the first exon, first intron, and second exon to the translation initiation codon (ATG) of the human apolipoprotein A1 gene fused to the B-domain-deleted (BDD) FVIII cDNA in a viral backbone devoid of most E1 and E3 sequences.²¹ The vector was purified and titered by plaque

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assay on 293 cells, as described.²¹ A polymerase chain reaction (PCR) assay directed at E1a sequences³⁵ verified that the vector preparation contained less than 10 plaque forming units (pfu) of replication competent adenovirus per 10⁸ pfu. The adenoviral vector was diluted in 10 to 20 mL of Plasma-Lyte (Baxter Health Care Corp, Deerfield, IL) containing 1% heat-inactivated autologous serum and was administered through an in-dwelling cephalic vein catheter by slow infusion over 5 to 10 minutes. The number of hepatocytes per dog is estimated to be 2.0×10^{10} cells per 10 kg of body weight.³⁶ Dog B received cyclophosphamide (1 mg/kg; Mead, Johnson, Bristol Myers, Squib, Princeton, NJ) and prednisone (2 mg/kg; Roxane Laboratories, Inc, Columbus, OH) daily orally beginning 2 days before vector treatment and continuing for 24 days thereafter. Dog C received cyclosporin A (CsA; 10 mg/kg; Sandoz Laboratories, East Hanover, NJ) orally twice a day beginning 3 days before vector administration until day 60 after treatment. All animals were housed in facilities accredited by the American Association for the Accreditation of Laboratory Animal Care and experimental protocols were approved by the Institutional Animal Care and Concern Committee.

Coagulation and FVIII-specific assays. The activated clotting time (ACT) and the activated partial thromboplastin time (APTT) assays were performed in the Clinical Pathology Laboratory at the Auburn University School of Veterinary Medicine. The reference values for normal dogs in this laboratory are less than 2 minutes for the ACT and 10 to 14 seconds for the APTT.³⁴ The activated clotting time (ACT)³⁷ was measured as follows: 2 mL of blood was collected into a prewarmed (37°C) ACT tube containing 6 to 10 mg of siliceous earth (Becton Dickinson VACUTAINER Systems, Rutherford, NJ); the sample was mixed by inverting the tube five times. After 1 minute of incubation at 37°C, the tube was removed from the heating block at 15-second intervals to observe the appearance of the first visible clot. To measure the APTT,³⁸ plasma samples were obtained from blood collected with 0.109 mol/L trisodium citrate at a 9:1 ratio. One hundred microliters of actin cephaloplastin (Baxter) was added to 100 μ L of plasma and prewarmed to 37°C for 3 minutes. One hundred microliters of prewarmed 0.02 mol/L calcium chloride (Baxter) was added and the time to initial clot formation was determined with a fibrometer (Becton Dickinson and Co, Cockeysville, MD). The cuticle bleeding time (CBT), an *in vivo* test sensitive to discrete coagulation factor deficiencies,²⁶ was performed as follows. Dogs were lightly anaesthetized and placed in a supine position, and the fur around a nail was clipped. The nail was then severed directly proximal to the dorsal nail groove and the time in minutes until clot formation was recorded.

The FVIII biologic activity was quantitated using the Coatest chromogenic bio-assay (Chromogenic, Mölndal, Sweden). Coatest measures the FVIII-dependent generation of factor Xa from factor X, with 1 U defined as the amount of FVIII activity in 1 mL of pooled human plasma (100 to 200 ng/mL).^{39,40} Pooled human plasma (George King Bio-Medical, Inc, Overland Park, KS) was used as the FVIII activity standard. Human FVIII antigen levels were quantitated by enzyme-linked immunosorbent assay (ELISA).²⁰ Full-length recombinant FVIII protein, supplied by Genetics Institute (Cambridge, MA) was used to generate a standard curve ranging from 1 to 100 ng/mL. BDD FVIII protein (Genetics Institute) and full-length recombinant FVIII were similarly quantitated by this ELISA. Normal dog plasma samples did not interfere with the assay and the limit of sensitivity with dog plasma samples containing BDD FVIII was 3 ng/mL. Samples were diluted 1:5 to 1:10 for the ELISA. No human FVIII antigen was detected in plasma from untreated normal dogs.

The Bethesda assay was used to measure FVIII inhibitors by the one-stage method.⁴¹ Various dilutions of test plasmas were mixed 1:1 with a normal human plasma pool and incubated at 37°C for 2 hours and the clot time was determined with the one-stage APTT assay. The dilution with residual activity closest to 50% was used

to calculate the inhibitor titer, in which 50% residual FVIII activity equals 1 Bethesda unit (BU)/mL.

Southern analysis and RNase protection assay. DNA and RNA were isolated from liver and spleen biopsy samples as described.²⁰ Twenty micrograms of each DNA sample was digested with *Bam*HI and subjected to Southern analysis. The probe, prepared by random oligonucleotide priming, contained human FVIII cDNA sequences from +73 to +1345.^{42,43} Copy number control standards were prepared by adding 1.2 ng, 120 pg, or 12 pg of Av1ALAPH81 viral DNA, equivalent to 10, 1, and 0.1 vector copies per cell, respectively, to 20 μ g of normal dog liver genomic DNA and digesting with *Bam*HI. The band intensities were quantitated with a Molecular Dynamics PhosphorImager SF (Sunnydale, CA). RNase protection analysis was performed using the RNase Protection Kit II (Ambion, Austin, TX). For each sample, 50 μ g of total cellular RNA was hybridized with 50,000 cpm of a gel-purified RNA probe (see below), digested with the RNase A/T1 solution provided with the kit diluted 1:100, processed as directed, and analyzed on an 8% polyacrylamide-8 mol/L urea gel (SequaGel; National Diagnostics, Atlanta, GA). ³²P-labeled fragments from *Hpa* II-digested pBR322 were used as DNA size markers. The FVIII probe template, pGemSRpr, has been described.²¹ The glyceraldehyde 3-phosphate dehydrogenase (GAP) probe template was generated from the pTRI-GAPDH mouse plasmid (Ambion) digested with *Sry* I. Antisense RNA probes were synthesized with SP6 polymerase and α -³²P-CTP (3,000 Ci/mmol; Amersham, Arlington Heights, IL). To verify that the probe concentration in the reactions was in excess of the targeted RNA, an RNase protection assay was performed in which 50,000 cpm of the human FVIII-specific probe was hybridized to increasing concentrations (25, 50, 75, and 100 μ g) of mouse liver RNA isolated from a mouse that had received a high dose (4×10^9 pfu) of Av1ALAPH81²² (data not shown). The signal increased in proportion to the amount of RNA added, showing that the probe was in excess, and analysis of the band intensities with a Molecular Dynamics PhosphorImager SF demonstrated that the assay was quantitative.

RESULTS

Correction of the hemophilia A phenotype. The recombinant adenoviral vector, Av1ALAPH81, contains a human BDD FVIII cDNA expressed from a modified version of the mouse albumin promoter and incorporates the first intron of the human apolipoprotein A1 gene in the upstream untranslated region.²¹ Removal of the B-domain from the FVIII protein has no effect on function, activity, or immunogenicity of the protein.^{15,44,45} Av1ALAPH81 was administered to a 32-kg hemophilic dog, dog A, by cephalic vein infusion (Table 1). The ACT³⁷ and APTT³⁸ were determined before and at days 2, 4, and 7 after Av1ALAPH81 administration (Fig 1A and B). The ACT and APTT were both abnormal before treatment. Within 2 days after vector administration these clotting parameters normalized but returned to pretreatment values by day 7. The CBT,²⁶ which was greater than 20 minutes before Av1ALAPH81 administration, decreased to 2 minutes by day 2. On day 7, the CBT was 7.5 minutes, which is still within the normal range of 2 to 8 minutes.²⁶ Normalization of the coagulation parameters showed the complete correction of the canine hemophilic phenotype.

FVIII biologic activity was determined by analysis of plasma samples collected before vector administration and daily for 7 days after treatment using Coatest, a FVIII chromogenic bioassay (Fig 1C). FVIII biologic activity was not detected in plasma samples collected before Av1ALAPH81 treatment. However, at days 1 to 5 after treatment, plasma

Table 1. Vector Dose and Immune Response to AV1ALAPH81 Administration in Hemophilia A Dogs

Animal	Vector Dose (pfu/kg)	Immunosuppression	Peak FVIII Plasma Activity (mU/mL)	Bethesda Titer	
				Pretreatment	Posttreatment
Dog A	3.0×10^{10}	None	8,054	1	185
Dog B	1.5×10^{10}	CyP/prednisone	305	0	30
Dog C	3.6×10^{10}	CsA	0	0	0

Treatment summary of FVIII-deficient dogs. None of the dogs had been treated previously with human FVIII protein. The adenoviral vector, Av1ALAPH81,²¹ encoding human FVIII, was administered to each animal via cephalic vein infusion. Plasma samples were analyzed for FVIII biologic activity by the Coatest chromogenic bioassay. Peak FVIII plasma levels were obtained 2 and 5 days after vector treatment in dogs A and B, respectively. The immunosuppression regimes with cyclophosphamide (CyP) and prednisolone or CsA are described in the Materials and Methods. Plasma samples collected before and after treatment were analyzed for the presence of FVIII inhibitor antibodies using the Bethesda assay.⁴¹ Posttreatment Bethesda titers were performed on day-7 plasma samples collected from dogs A and C. Dog B posttreatment Bethesda titer was performed with day-14 plasma.

FVIII activity showed a dramatic increase. FVIII expression peaked 2 days after treatment at greater than 8,000 mU/mL. These levels are eightfold higher than the FVIII levels found in normal human plasma.^{39,40} By day 7, FVIII plasma levels had decreased below the level of detection.

Plasma samples were also assayed by an ELISA specific for human FVIII²⁰ to verify that the FVIII activity measured in the hemophilic dog plasma was vector-derived (Fig 1D). Human FVIII antigen was not detectable in the plasma before vector administration. At days 1 through 5, high levels of human FVIII were detected. The Coatest activity units and human FVIII antigen levels measured by ELISA were in good agreement, indicating that the FVIII was fully functional. Human FVIII was also detected in the dog plasma

with an ELISA in which the detection antibody was directed against canine vWF (data not shown). Consistent with the results of previous studies,^{28,29} these data show that the human FVIII protein complexed normally with canine vWF in the FVIII-deficient dog. Normalization of the clotting parameters and high-level human FVIII expression in the affected dog showed that the hemophilic phenotype can be completely corrected by adenoviral vector-mediated delivery of the BDD FVIII cDNA.

Vector persistence in transduced dogs. To assess vector DNA levels and FVIII gene expression, hepatic and splenic biopsies were performed 7 days after vector administration. DNA and RNA isolated from liver and spleen were assayed by Southern and RNase protection analyses (Fig 2). Vector

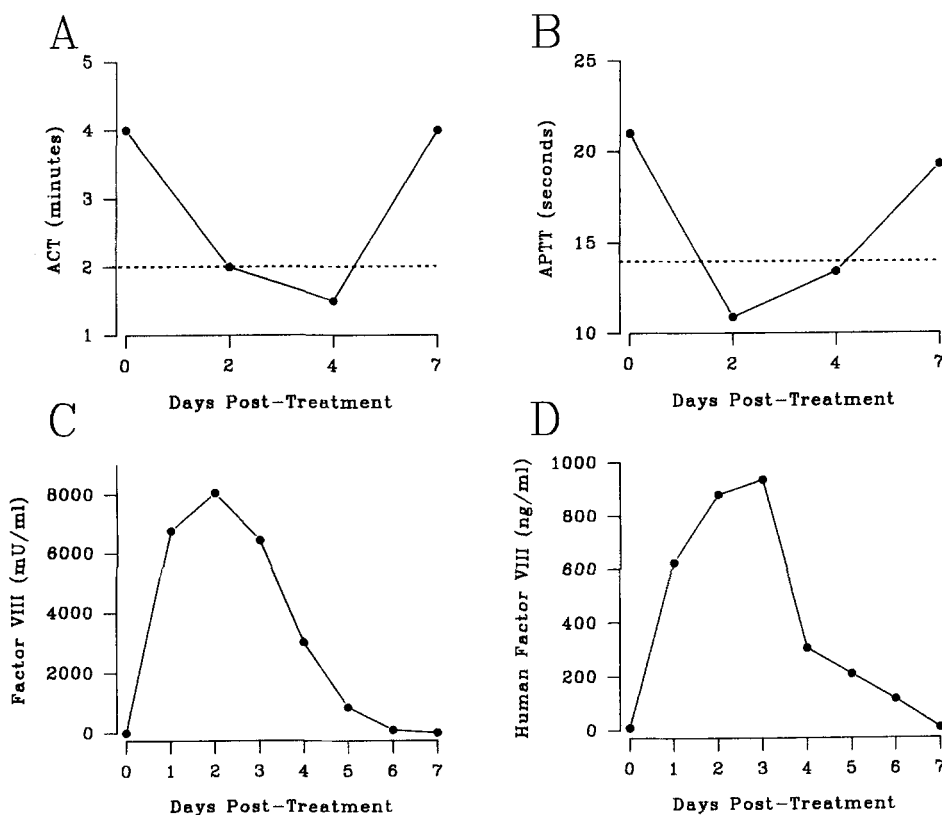


Fig 1. Correction of hemophilia A in FVIII-deficient dog A. Av1ALAPH81 (10^{12} pfu) was administered to 32-kg FVIII-deficient dog A. Plasma samples were collected before and at the indicated times after vector administration and assayed for the clotting parameters ACT³⁷ and APTT.³⁸ The upper limits of the canine normal range for ACT and APTT are indicated by the dotted lines. Plasma samples were analyzed for FVIII biologic activity by the Coatest chromogenic bioassay and for human FVIII antigen using a human FVIII-specific ELISA.²⁰ The Coatest assay using plasma from a normal dog yielded a value of 5,000 mU/mL. The potential human therapeutic range, the amount of FVIII required to convert a severe hemophilic to a mild or moderate hemophilic, is 50 mU/mL or 10 ng/mL.^{39,40} (A) ACT, (B) APTT, (C) FVIII biologic activity, (D) human FVIII antigen.

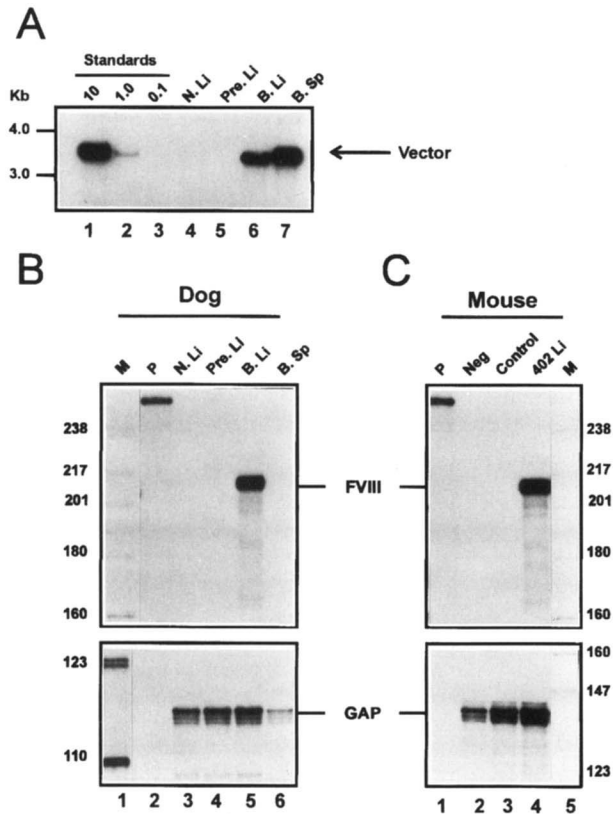


Fig 2. Dog A vector-derived DNA and RNA analyses. An open biopsy was performed 9 months before and 1 week after vector administration. DNA and RNA were isolated from liver and spleen biopsy samples.²⁰ (A) Southern analysis. A total of 20 μ g of DNA was used in each reaction. The copy number control standards (lanes 1 through 3) were generated by digesting purified Av1ALAPH81 viral DNA in amounts equivalent to 10, 1, and 0.1 vector copies per cell. Lanes 4 through 7 contain DNA isolated from a normal dog liver (N. Li), pretreatment liver biopsy DNA (Pre. Li), posttreatment liver biopsy DNA (B. Li), and posttreatment spleen biopsy DNA (B. Sp). DNA marker sizes are indicated in kilobases. (B) RNase protection analysis. A total of 50 μ g of RNA was used in each of the reactions. The upper panel displays an RNase protection analysis for human FVIII and the lower panel displays a separate RNase protection analysis using a mouse GAP-specific probe. Lane 1 contains ³²P-labeled DNA size markers. Sizes are indicated in base pairs. Lane 2 contains undigested full-length probe. Lanes 3 through 6 contain RNA isolated from a normal dog liver (N. Li), pretreatment liver biopsy RNA (Pre. Li), posttreatment liver biopsy RNA (B. Li), and posttreatment spleen biopsy RNA (B. Sp). Vector-derived human FVIII RNA protects a 212-bp probe fragment. The differences in the GAP RNA levels in the liver and spleen samples are due to organ to organ variation in GAP expression. The exposure shown is 5 days with intensifying screens. (C) Mouse liver RNase protection analysis. The upper panel displays an RNase protection analysis for human FVIII. Fifty micrograms of total cellular RNA isolated from mouse livers harvested 1 week after vector administration was used in each reaction. Lane 1 contains undigested full-length probe. Lanes 2 and 3 contain liver RNA isolated from an uninjected control mouse (Neg) and a mouse treated with 4×10^9 pfu of the control vector, Av1ALH9B²¹ (Control), respectively. Lane 4 represents liver RNA isolated from a mouse that received 4×10^9 pfu of Av1ALAPH81 (402 Li).²² Lane 5 contains DNA size markers. The exposure shown is 5 days with intensifying screens. The lower panel displays a separate RNase protection assay for mouse GAP using 30 μ g of total cellular mouse liver RNA. Exposure shown is 6 hours with intensifying screens. The dog and mouse FVIII-specific RNase protection assays displayed in (B) and (C) were performed simultaneously and analyzed on the same gel. The mouse data have been published previously.²²

DNA was present in both liver and spleen at an average of approximately 5 to 10 copies per cell, but was not detectable in liver before vector administration (Fig 2A). The RNA analysis showed the presence of vector-derived FVIII RNA only in the liver biopsy sample (Fig 2B). Comparison to the human FVIII-specific RNA detected in a mouse that was expressing human FVIII at levels greater than 1,000 ng/ml²² (10,000 to 20,000 mU/mL; Fig 2C) showed that the dog and mouse liver RNA samples contained similar amounts of human FVIII RNA. Human FVIII-specific RNA was not detected in the canine spleen biopsy sample, although the spleen contained more vector copies per cell than the liver. Thus, the albumin promoter remained transcriptionally active 7 days after vector administration and, consistent with previous observations in mice,²¹ the albumin promoter functioned in a liver-specific manner in the dog. As expected, human FVIII-specific RNA was not detected in the preinjection liver sample or in the normal dog liver RNA sample. Taken together, these results indicate that the decline in human FVIII expression was not due to a complete loss of vector DNA from the treated animal or to the transcriptional inactivation of the albumin promoter.

Expression is limited by antibodies to human FVIII. FVIII-deficient dogs have previously been shown to rapidly develop inhibitory antibodies to human FVIII within 1 to 2 weeks after exposure to the human protein.³¹ This observation provides a likely explanation for the decrease in FVIII plasma levels between 5 to 7 days after treatment (Fig 1). The Bethesda assay, a standardized test to quantitate inhibitors in hemophilic patients,⁴¹ was used to measure FVIII inhibitors in pretreatment and day-7 posttreatment plasma samples. A value of 1 BU was measured in the pretreatment plasma sample (Table 1). At day 7, the antibody titer had increased to 185 BU, showing the development of FVIII inhibitors in the treated dog. Therefore, the limited duration of expression of human FVIII in the hemophilic dog was due, at least in part, to the development of antibodies to the human protein. Dog A did not survive a surgical biopsy and died from bleeding complications.

To minimize the humoral immune response to human FVIII, a second 19-kg FVIII-deficient dog, dog B, was immunosuppressed with cyclophosphamide and prednisone before and after vector administration (Table 1). At 2 to 10 days after treatment, the ACT and APTT approached the normal range, thus showing phenotypic correction in the second dog (Fig 3A and B). Evaluation of FVIII activity by Coatest showed FVIII expression at amounts well above human therapeutic levels that were sustained for 10 days (Fig 3C). However, plasma levels were lower than those achieved in dog A. The Bethesda assay,⁴¹ performed using plasma samples collected from dog B, measured 0 BU before vector administration (Table 1). However, by days 7 and 14, the antibody titer had increased to 14 BU and 30 BU, respectively. Therefore, the immunosuppressive treatment did not prevent the development of an antibody response to human FVIII and, consequently, did not substantially prolong FVIII expression in this animal. A biopsy was not performed on dog B.

Based on previous studies with adenoviral vectors, the persistence of FVIII expression in the treated dogs may also

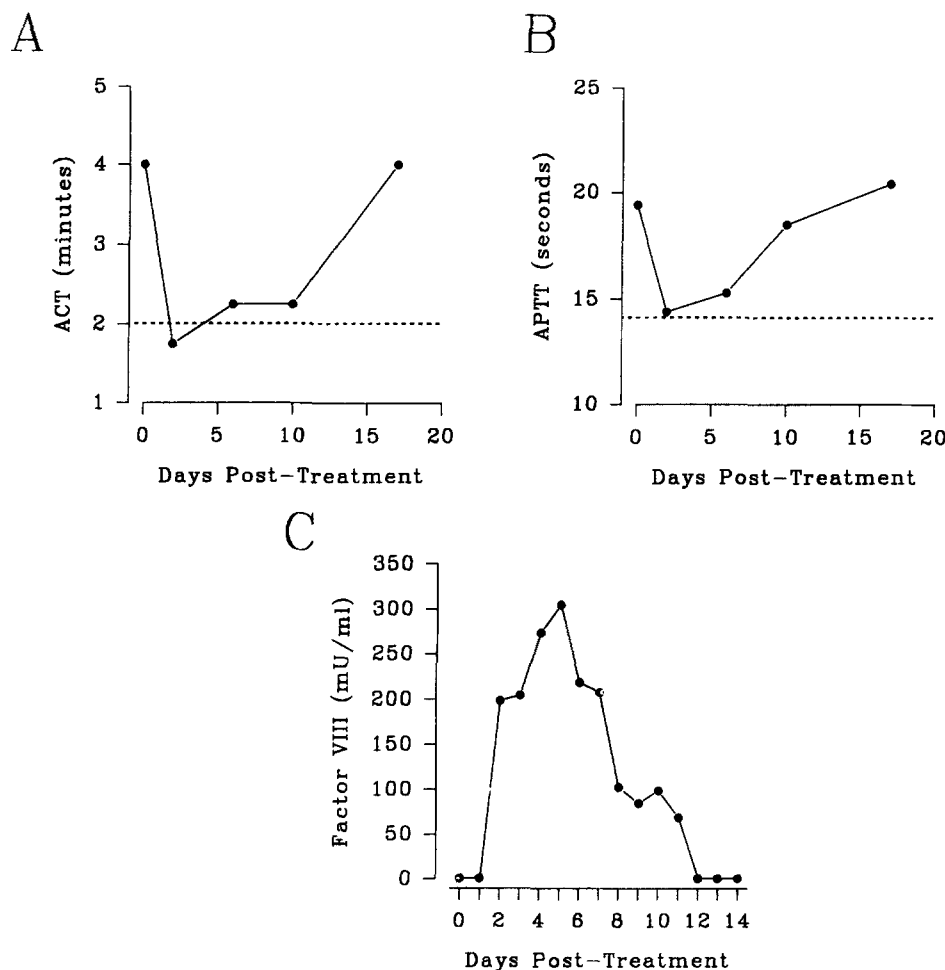


Fig 3. Correction of hemophilia A in FVIII-deficient dog B. Av1ALAPH81 (2.8×10^{11} pfu) was administered to 19-kg, FVIII-deficient dog B. The dog was treated daily with cyclophosphamide (1 mg/kg) and prednisone (2 mg/kg) beginning 2 days before vector administration. Plasma samples were collected at the indicated times and analyzed as described. The upper limits of the canine normal range for ACT and APTT are indicated by the dotted lines. (A) ACT, (B) APTT, (C) FVIII biologic activity.

have been limited by a cellular immune response against expressed adenoviral genes or the FVIII transgene.⁴⁶⁻⁴⁹ Therefore, a third, 22-kg, FVIII-deficient dog, dog C, was immunosuppressed with CsA before and after Av1ALAPH81 vector administration (Table 1). No significant improvement of clotting parameters were observed in this dog, and FVIII activity was undetectable (data not shown). To assay for the presence of human FVIII antibodies, a Bethesda assay⁴¹ was performed using pretreatment and day-7 plasma samples. No Bethesda titer was detected at either time point, indicating the lack of inhibitors to human FVIII (Table 1).

To measure vector transduction in dog C, liver and spleen biopsies were performed 14 days after vector treatment. Southern analysis of DNA isolated from the biopsy samples showed a significant, although lower level of liver and spleen transduction in dog C compared with the day-7 biopsy samples obtained from dog A (Fig 4A). A second biopsy at 60 days showed that vector DNA remained in the liver, although at fivefold lower levels than detected at 14 days (data not shown). However, FVIII RNA could not be detected by RNase protection analysis (Fig 4B), indicating that the vector was not expressing the FVIII cDNA in this animal.

DISCUSSION

We have shown complete correction of canine hemophilia A by *in vivo* gene therapy. A noninvasive, peripheral vein

injection of an FVIII adenoviral vector to hemophilia A-affected dogs resulted in normalization of clinical clotting parameters and expression of human FVIII in the plasma at amounts well above human therapeutic levels. Previous studies performed in normal adult mice showed that the FVIII adenoviral vector could achieve high plasma levels of human FVIII.^{21,22} However, the effectiveness and feasibility of this approach in a clinically relevant large animal model had not been established.

FVIII-deficient dogs represent an excellent model system to test the efficacy of adenoviral vector-mediated gene therapy for hemophilia A. The pathophysiology of the canine disease is analogous to human hemophilia A^{25,26} and FVIII-deficient dogs have been used previously to support the development of FVIII pharmaceutical products.^{15,27-29} The data presented here show that hepatic expression of human FVIII resulted in phenotypic correction of the coagulation defect in an animal with hemophilia A, therefore verifying the efficacy of *in vivo* gene therapy for hemophilia A.

Although phenotypic correction was achieved, its duration was limited by a humoral immune response to the human FVIII protein. The rapid development of anti-FVIII antibodies was not unexpected because purified human factor VIII is highly immunogenic in dogs when delivered intravenously.³¹ An attempt to block the antibody response with cyclophosphamide and prednisone in the second dog did not

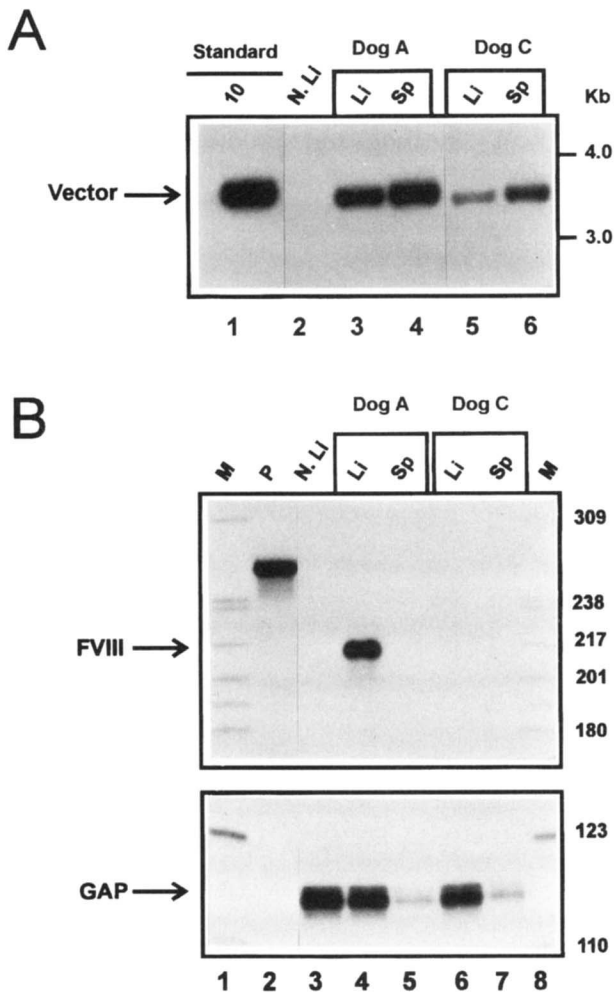


Fig 4. Dog C vector-derived DNA and RNA analyses. Dog C (22 kg) received 8×10^{11} pfu of Av1ALAPH81. The dog was treated twice daily with CsA (10 mg/kg) beginning 3 days before vector administration. A surgical biopsy was performed 2 weeks after vector administration. DNA and RNA were isolated from liver and spleen biopsy samples. (A) Southern analysis. A total of 20 μ g of DNA was used in each reaction. The copy number control standard (lane 1) was generated by digesting purified Av1ALAPH81 viral DNA in an amount equivalent to 10 vector copies per cell. Lanes 2 through 6 contain DNA isolated from a normal dog liver (N. Li), dog A liver (Li), dog A spleen (Sp), dog C liver (Li), and dog C spleen (Sp) DNAs. DNA marker sizes are indicated in kilobases. (B) RNAse protection analysis. A total of 50 μ g of RNA was used in each reaction. The upper panel displays an RNAse protection analysis for human FVIII and the lower panel displays a separate RNAse protection analysis using a mouse GAP-specific probe. Lanes 1 and 8 contain DNA size markers. Lane 2 contains undigested full-length probe. Lanes 3 through 7 contain RNA isolated from a normal dog liver (N. Li), dog A liver (Li), dog A spleen (Sp), dog C liver (Li), and dog C spleen (Sp) RNAs. The exposure shown is 5 days with intensifying screens. The differences in the GAP RNA levels in the liver and spleen samples are due to organ to organ variation in GAP expression.

substantially improve the duration of phenotypic correction. A similar immunosuppressive regimen also showed limited success when used to treat human patients with high FVIII inhibitor titers.³³ In contrast to human FVIII, the canine FVIII protein is less immunogenic in hemophilic dogs and some

dogs can be repeatedly treated with canine plasma without developing inhibitory antibodies.³² The establishment of sustained phenotypic correction in hemophilic dogs may require the development of adenoviral vectors that encode the canine cDNA. This strategy would be comparable to treating human hemophiliacs with vectors encoding the human cDNA. Indeed, retroviral and adenoviral vectors encoding the canine factor IX cDNA have been used to treat hemophilia B dogs and expression persisted at low levels (0.1% of human physiologic levels) for several months.⁴⁹⁻⁵¹ No evidence of a humoral immune response to the canine factor IX protein in the treated animals was reported.⁴⁹⁻⁵¹ Whereas the inhibitory antibody response to human FVIII was sufficient to explain the loss of FVIII expression in dogs A and B, even in the absence of a humoral immune response to the human protein, sustained FVIII expression also could be limited by a cellular immune response directed against hepatocytes expressing adenoviral backbone gene products.⁴⁶⁻⁴⁹

It remains unclear why vector treatment of dog C did not result in human FVIII expression. Inhibitory antibodies to human FVIII were not detected before or after vector administration, confirming that human FVIII protein was not expressed in this animal (Table 1). DNA and RNA analyses of liver and spleen biopsy samples showed that, although dog C was transduced with the vector, no human FVIII-specific RNA was detected in the liver (Fig 4), indicating that the vector was not functional. Comparison of the transduction efficiencies of dog A and dog C showed that, although both dogs received a similar dose of vector (Table 1), dog C had significantly less vector DNA in the liver and spleen than did dog A. Therefore, it is possible that a threshold of vector transduction may exist below which the DNA is not transcribed. Alternatively, lack of vector function in dog C may have been caused by the administration of CsA. Preliminary studies in mice have suggested that CsA may inhibit expression from the FVIII vector by reducing the FVIII RNA levels (data not shown). However, the manner in which CsA may suppress transcription from the vector remains unclear but may be related to interactions with cellular transcription factors.⁵² The variation in vector function described in this work underscores the importance of evaluating gene therapy in large animal models, such as hemophilic dogs.

One notable finding in this study is that peripheral vein administration of an adenoviral vector to dogs resulted in efficient liver transduction. This observation is consistent with a previous study in which dogs received an adenoviral vector encoding the β -galactosidase or human FIX cDNA (Mauser et al, manuscript submitted). Preferential accumulation of adenoviral vectors in liver has been described in mice,^{21,24} and the extension of this phenomenon to large animals lends credence to the intravenous use of these vectors in humans. Interestingly, the tissue distribution of vector in the dog model differed from that observed in mice; after intravenous vector administration, mouse spleens were transduced at only 1% to 3% of the level seen in liver,^{21,24} whereas the dog spleen was transduced with more vector copies per cell than the liver (Figs 2 and 4). The explanation for this disparity is not clear and may represent differences in the vector tropism in the two models or simply more efficient

phagocytosis in the canine spleen. Expression patterns, on the other hand, were the same in the two animal models in that vector-derived human FVIII RNA was detected only in the liver.²¹ The absence of expression in a highly transduced organ such as the spleen may provide a margin of safety for liver-based gene therapy protocols, because the consequences of ectopic expression of FVIII are unknown.

In this report, effective treatment of canine hemophilia A was achieved with a noninvasive, peripheral vein administration of a human FVIII adenoviral vector. Although further advances in vector design will be needed to enable sustained expression, correction of the coagulation defect in a clinically relevant large animal model is a crucial step in verifying the feasibility of gene therapy for hemophilia A.

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