Eradication of neutralizing antibodies to factor VIII in canine hemophilia A after liver gene therapy

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Inhibitory antibodies to factor VIII (FVIII) are a major complication in the treatment of hemophilia A, affecting approximately 20% to 30% of patients. Current treatment for inhibitors is based on long-term, daily injections of large amounts of FVIII protein. Liver-directed gene therapy has been used to induce antigen-specific tolerance, but there are no data in hemophilic animals with pre-existing inhibitors. To determine whether sustained endogenous expression of FVIII could eradicate inhibitors, we injected adeno-associated viral vectors encoding canine FVIII (cFVIII) in 2 strains of inhibitor hemophilia A dogs. In 3 dogs, a transient increase in inhibitor titers (up to 7 Bethesda Units [BU]) at 2 weeks was followed by continuous decline to complete disappearance within 4-5 weeks. Subsequently, an increase in cFVIII levels (1.5%-8%), a shortening of clotting times, and a reduction (> 90%) of bleeding episodes were observed. Immune tolerance was confirmed by lack of antibody formation after repeated challenges with cFVIII protein and normal protein half-life. A fourth dog exhibited a strong early anamnestic response (216 BU), with slow decline to 0.8 BU and cFVIII antigen detection by 18 months after vector delivery. These data suggest that liver gene therapy has the potential to eradicate inhibitors and could improve the outcomes of hemophilia A patients. (*Blood*. 2010;116(26): 5842-5848)

Introduction

The development of neutralizing antibodies to replacement protein is a major complication of protein and enzyme replacement therapies for several genetic diseases. Hemophilia A is an X-linked bleeding disorder characterized by deficiency in the activity of factor VIII (FVIII), a key component of the coagulation cascade. The disease occurs in approximately 1 in 10 000 live births worldwide, and > 40% of these patients have severe disease, with FVIII activity < 1% of normal.[1] Infusion of plasma-derived or recombinant FVIII is the standard treatment. Alloantibodies (inhibitors) that neutralize the protein-replacement therapy develop in 20% to 30% of young patients with severe and moderate hemophilia A, resulting in high morbidity and mortality,[2,3] and this is a growing problem for adults as well.[4,5] Risk factors for inhibitor formation include both genetic and environmental factors. Underlying mutations in the FVIII gene, such as large gene deletions, nonsense mutations, and the most common mutation in severe hemophilia A patients, the inversion of intron 22, are all associated with inhibitor formation; however, it is not possible to predict with certainty which patients will develop inhibitors. For this reason, preventive strategies are not currently feasible.[6-8] Patients with high titers of inhibitors, defined as > 5 Bethesda units (BU), cannot usually be treated with FVIII replacement, necessitating the use of products that bypass the procoagulant effect of FVIII and are extremely expensive.[1] Thus, strategies for the eradication of inhibitors are of fundamental clinical relevance.

Currently, the only proven therapy for inhibitors is based on antigen-specific immune tolerance induction (ITI) protocols that stem from observations in the 1970s that continuous administration of large amounts of FVIII protein could lead to a reduction in inhibitor titers.[9] Current ITI involves daily infusions of FVIII protein for an average of 33 months to achieve complete eradication, which is commonly followed by long-term prophylaxis. This imposes enormous challenges for pediatric patients, who often require central venous catheters that are associated with a high risk of infection and thrombosis. In addition, the economic burden of this strategy is remarkable—approximately $1 million US—and thus it is prohibitive for many patients outside of the developed world.[2]

Adeno-associated viral (AAV) vectors are one of the most extensively studied and highly used vector platforms for gene-therapy applications. The safety profile of AAV vectors in clinical studies enrolling adult and pediatric populations has been excellent.[10-13] The first clinical studies using AAV to deliver the *F9* gene to the muscle or liver in subjects with hemophilia B found that this treatment was safe and without sustained toxicity.[10,14,15] The therapeutic doses defined in canine hemophilia B models were excellent predictors of the efficacy observed in clinical trials.[16,17] Thus, the use of large animal models has been essential for the successful translation of gene-therapy protocols "from the bench to the clinic."[18]

Liver-directed gene expression by AAV vectors has been associated with antigen-specific immune tolerance induction in naive, adult, large animals, including dog models of severe hemophilia A.[17-23] More difficult than preventing an immune response is the challenge of reversing an ongoing immune response

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Table 1. Summary of inhibitor eradication in hemophilia A dogs following cFVIII expression by AAV vector

			Inhibitors				Bleeds per month	
Dog	Age, y	Weight, kg	Duration before treatment, mo	Historical peak, BU	Time to eradication, wk	cFVIII plateau, activity	Pretreatment	Posttreatment
K01	1.7	20.1	8	12	5	1.5 %	3/20	1/23
K03	1	19.3	7	12	4	8 %	7/12	1/29
L44	0.7	16	4	4.5	4	1.5 %	5/8	0/16
Wembley	4.92	16.5	~ 24	3.6	80			
Total							15/40	2/68

to FVIII. We hypothesize that continuous expression of FVIII could mimic ITI protocols, with the additional advantage that after inhibitor eradication, the continuous expression of FVIII above 1% of normal would convert the disease phenotype from severe to moderate or mild.

Methods

AAV vector administration

Recombinant AAV vectors were produced by a triple-transfection protocol, as described previously,[10] using plasmids expressing canine FVIII (cFVIII) light chain (LC) or heavy chain (HC) in separate vectors under the control of a liver-specific promoter,[20] a second plasmid supplying adenovirus helper functions, and a third plasmid containing the AAV-2 *rep* gene and the AAV-8 *cap* gene. Vectors were purified by repeated cesium chloride density-gradient centrifugation.

Animal procedures

All animal experiments were approved by the institutional animal care and use committees at the Children's Hospital of Philadelphia, the University of North Carolina at Chapel Hill (UNC-Chapel Hill) , and Queen's University. Four adult male hemophilia A dogs were administered 2.5×10^{13} vg/kg of AAV8-cFVIII-LC and 2.5×10^{13} vg/kg AAV8-cFVIII-HC intravenously via the saphenous vein in a total volume of 10 mL/kg phosphate-buffered saline. Pooled normal plasma was concurrently given for cFVIII replacement to dog K03 to control an ongoing bleeding episode from a previous jugular vein puncture the day prior to vector delivery.

Systemic and local toxicity

Hematologic and comprehensive biochemical analyses of blood and serum samples for liver and kidney function tests were performed as described previously.[19,23]

cFVIII antigen, activity, and antibody assays

Whole-blood-clotting time was determined as described previously.[24] Pooled normal canine plasma was used as a standard for the quantitation of the activity of FVIII using a FVIII activity assay (Chromogenix Coatest SP4; Diapharma). cFVIII-LC antigen levels were analyzed by enzyme-linked immunosorbent serologic assay (ELISA) using a monoclonal antibody against cFVIII-LC (2C4.1C3) as a capture antibody, as described previously.[24] Anti-cFVIII antibodies were detected by Bethesda assay or as cFVIII-specific IgG antibodies by ELISA, as described previously.[24] It should be noted that the detection of inhibitor titers less than 1 BU is unreliable in the canine hemophilia A system.

Flow cytometry

Anticanine CD25 antibody (P4A10) was generously provided by V. K. Abrams (Seattle, WA).[25] P4A10 was conjugated to fluorescent dye using a commercially available kit (Alexa Fluor 488; Invitrogen). Peripheral blood mononuclear cells were surface stained for canine CD4-PE (AbD Serotech), CD25-AF-488 (P4A10), and intracellular stained with a cross-reactive mouse FoxP3 APC (eBioscience). Samples were run on a flow cytometer (FACSCanto; BD Biosciences), and data were analyzed using FlowJo Version 8.5.2 software (TreeStar).

Protein infusion for immunologic challenges and pharmacokinetic analysis

Recombinant, B domain–deleted cFVIII (rBDD-cFVIII) purified protein was infused intravenously (100 IU/kg) for pharmacokinetics assessment, and blood was collected at the time points indicated. Canine FVIII levels were determined by ELISA, and the half-life was calculated as described previously.[24] Immunologic challenges were carried out by infusion of 25 IU/kg/dose body weight of rBDD-cFVIII on a weekly basis (total 4 doses). Pooled normal dog plasma was infused at 25 mL/dose in a similar fashion.

Results

We used 2 strains of severe hemophilia A dogs prone to inhibitor formation to test our hypothesis that continuous expression of FVIII could eradicate inhibitors. These dogs have circulating FVIII antigen and activity levels $< 1\%$ of normal, and thus accurately reproduce many of the symptoms and phenotype of severe hemophilia in humans. Moreover, the causative mutation in both canine models[26,27] mimics the intron 22 inversion observed in approximately 40% of severe disease in humans.[28] One strain was from a subset of the UNC-Chapel Hill colony that developed anti-cFVIII inhibitory antibodies upon exposure to normal canine plasma.[18] The second strain was from the Queen's University dog colony, which has a high risk of inhibitor formation upon cFVIII protein replacement.[29,30] This immunological phenotype makes these subsets of dogs more representative of the human hemophilia population, and dogs with inhibitors are ideal candidates for testing the safety and efficacy of AAV-mediated immune-tolerance induction protocols.

Due to the large size of the canine *F8* gene (even the fully functional rBDD-cFVIII[24]) and the limited packaging capacity of AAV vectors (4.7 kb), the cFVIII cDNA was divided into 2 different AAV8 vectors expressing either the cFVIII LC or HC under the control of a liver-specific promoter.[20]

Three hemophilia A dogs with inhibitors (K01, K03, and L44) from the UNC-Chapel Hill dog colony were administered 2.5×10^{13} vg/kg of AAV8 expressing LC and HC vector (5×10^{13} vg/kg total). The clinical characteristics of these dogs are shown in Table 1. The inhibitory antibodies identified in these dogs are restricted to the IgG2 subclass (equivalent to IgG4 in humans,[31] the most common inhibitor subclass).

To overcome the challenges of achieving hemostasis in these fragile animals, the vector was delivered by peripheral intravascular administration via the saphenous vein. Thus, no exogenous recombinant cFVIII or transfusion of normal plasma was required during the vector infusion (except in K03, see 2 paragraphs below).

K01 had a historical maximum inhibitor titer of 12 BU, and his inhibitor titer at the time of treatment was approximately 3 BU.

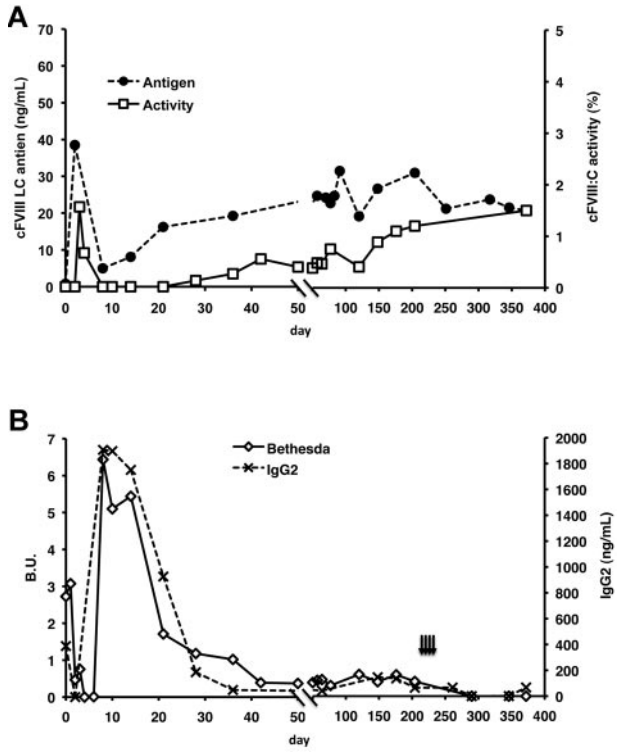

Figure 1. cFVIII expression and anti-cFVIII antibody responses in Chapel Hill hemophilia A dog K01 after liver delivery of AAV-cFVIII. One Chapel Hill dog (K01) with preexisting inhibitors to cFVIII was administered 2.5×10^{13} vg/kg of AAV8-TBG-cFVIII-HC and AAV8-TBG-cFVIII-LC by peripheral venous injection. (A) cFVIII antigen levels were assayed by a cFVIII-LC specific ELISA, and activity was monitored by FVIII assay. (B) Anti-cFVIII antibody responses were measured by anti-cFVIII IgG2 ELISA and Bethesda assays. Black arrows indicate 4 weekly challenges with 500 U of rBDD-cFVIII.

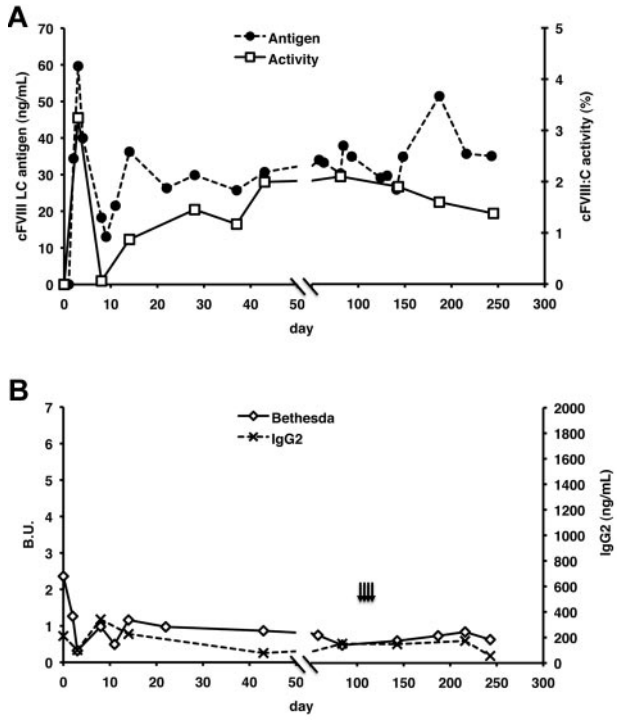

Figure 2. cFVIII expression and anti-cFVIII antibody responses in Chapel Hill hemophilia A dog L44 after liver delivery of AAV-cFVIII. One Chapel Hill dog (L44) with preexisting inhibitors to cFVIII was administered 2.5×10^{13} vg/kg of AAV8-TBG-cFVIII-HC and AAV8- TBG-cFVIII-LC by peripheral venous injection. (A) cFVIII antigen levels were assayed by a cFVIII-LC specific ELISA, and activity was monitored by FVIII assay. (B) Anti-cFVIII antibody responses were measured by anti-cFVIII IgG2 ELISA and Bethesda assays. Black arrows indicate 4 weekly challenges with 500 U of rBDD-cFVIII.

After vector administration, we observed a rapid increase in cFVIII expression (Figure 1A), peaking at day 3 (38 ng/mL LC antigen, 1.5% activity). This is consistent with the pattern of early expression of AAV-8 vectors. However, transgene expression levels decreased to near background levels for 3-4 weeks, and then slowly increased over time to reach cFVIII plateau levels of 30 ng/mL LC antigen and 1.5% activity. Inhibitor titers followed an inverse relationship with cFVIII antigen and activity levels. There was an initial decrease in inhibitor titer to undetectable levels, followed by a rapid increase, peaking at 7 BU on day 8 and then slowly decreasing over time, being no longer detectable by day 42 (Figure 1B). During this time, we documented an increase in inhibitor titers corresponding to a decrease in cFVIII expression, indicating an anamnestic inhibitor response followed by inhibitor eradication. This is a common observation during the early phase of ITI.[32]

The kinetics of cFVIII expression in dogs L44 (2.2 BU) and K03 (3 BU) were similar to those of K01. It should be noted that K03 was the only dog that received a transfusion of normal canine plasma at the time of vector injection to control bleeding from a jugular puncture wound suffered the previous day when collecting baseline samples. Thus, the cFVIII antigen and activity at early time points (days 2-3) from the transfusion confounds the quantification of the AAV-cFVIII-mediated expression. In both dogs, there was a rapid increase in the circulating cFVIII levels, followed by a decrease to undetectable levels from days 7-21 (Figures 2A, 3A). This transient decrease in transgene expression was not observed in noninhibitor hemophilia dogs administered AAV, and is an indication of an anamnestic immune response against cFVIII.[17,19,22,23] This expression profile was due to an increase in the inhibitor titers starting at 1 week after AAV treatment, followed by a slow decrease and complete eradication after 4-5 weeks (Figures 2B, 3B). We observed an expected inverse relationship between cFVIII expression and inhibitor titers, with cFVIII levels steadily increasing as inhibitor titers decreased to undetectable levels. Canine FVIII levels stabilized at 1.5% for dogs L44 and K01 and at 8% for K03. The reasons for this discrepancy in cFVIII expression levels are unclear; however, we previously showed that normal hemostasis at the time of AAV-2 vector delivery enhances transgene expression in murine models.[33] Thus, it is possible that correction of hemostasis by normal plasma infusion in K03 may have contributed to the higher efficiency of gene transfer.

A consequence of using a dual-chain approach is that there is an imbalance in circulating cFVIII LC and HC antigen levels, with the LC antigen being secreted 10-25 times more efficiently than the HC antigen.[34,35] A large proportion of cells are transduced with only one of the vectors, and these cells will produce cFVIII antigen that is inactive without its complementary chain. Thus, cFVIII antigen levels will be higher than the cFVIII activity levels ($\sim$ 10-fold). We speculate that this excess of nonfunctional antigen is perhaps beneficial in inducing immune tolerance by increasing the overall amount of circulating antigen, as shown before in murine models.[36] The cFVIII activity observed in the dogs in this study reached therapeutic levels, as demonstrated by a sustained shortening of the whole blood clotting time (Figure 4) in all 3 dogs and a remarkable improvement of the disease phenotype, with a reduction of more than 90% of bleeding episodes (Table 1).

To determine whether these animals were tolerant to cFVIII, we performed immunological challenges with purified rBDD-cFVIII.[24] K01, K03, and L44 were challenged with 4 weekly

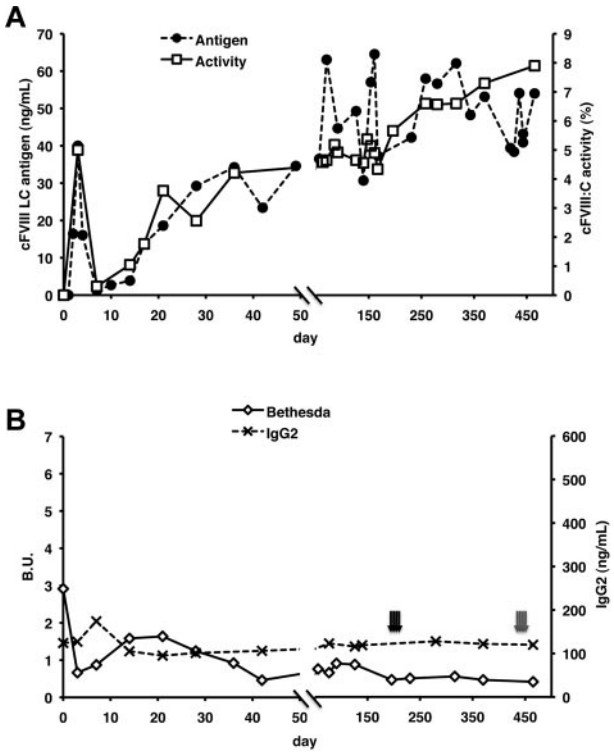

Figure 3. cFVIII expression and anti-cFVIII antibody responses in Chapel Hill hemophilia A dog K03 after liver delivery of AAV-cFVIII. One Chapel Hill dog (K03) with preexisting inhibitors to cFVIII was administered 2.5×10^{13} vg/kg of AAV8-TBG-cFVIII-HC and AAV8-TBG-cFVIII-LC by peripheral venous injection. (A) cFVIII antigen levels were assayed by a cFVIII-LC specific ELISA, and activity was monitored by FVIII assay. (B) Anti-cFVIII antibody responses were measured by anti-cFVIII IgG2 ELISA and Bethesda assays. Black arrows indicate 4 weekly challenges with 500 U of rBDD-cFVIII, and gray arrows indicate 4 weekly injections of 25 mL of pooled normal canine plasma per dose.

intravenous injections of 25 IU/kg body weight (2.5 μg/kg) of rBDD-cFVIII initiated on various days after vector administration (days 240, 125, and 113, respectively) and monitored for inhibitor formation. As can be seen in Figures 1 to 3, there was no change in cFVIII expression levels, nor was there any indication of either inhibitor formation or non-neutralizing antibodies after challenge in any dog. To confirm that tolerance induction is sustained upon exposure to the wild-type (full-length) cFVIII, we further challenged K03 starting on day 400 with 4 weekly injections of 25 mL of pooled normal canine plasma per dose, and no evidence of inhibitors or antibodies to cFVIII was observed.

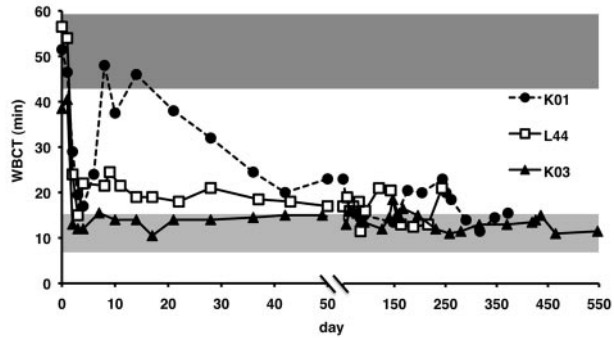

Figure 4. Whole-blood clotting time of Chapel Hill inhibitor dogs after vector administration. Whole blood clotting time (WBCT) for all 3 hemophilia A Chapel Hill dogs is shown. The WBCT range for a normal dog is shaded in light gray (8-12 minutes), and the range for a hemophilia A dog is shaded in dark gray (> 45 minutes).

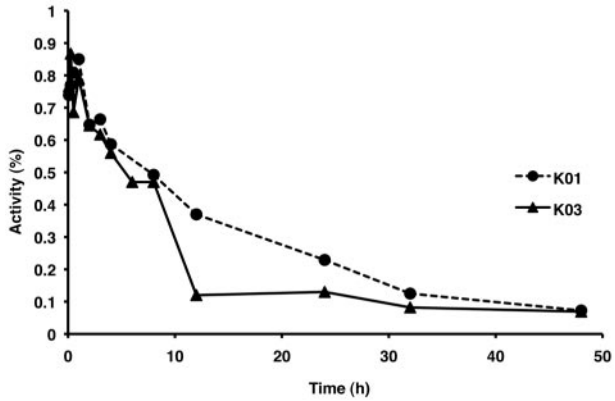

Figure 5. Recovery of cFVIII after intravenous administration. rBDD-cFVIII was administered (100 IU/kg) to Chapel Hill dogs K01 and K03 by intravenous injection, and cFVIII activity was monitored over time by FVIII assay.

To further confirm the eradication of inhibitors and to exclude the presence of non-neutralizing antibodies that might increase the clearance of cFVIII, we determined the recovery and half-life of the cFVIII protein in K01 and K03. Both dogs were infused with 100 IU/kg of rBDD-cFVIII, and plasma was collected between 5 minutes and 48 hours after infusion. As can be seen in Figure 5, there was an excellent recovery of more than 80% of the infused protein measured at 5-10 minutes after infusion. We determined similar cFVIII falloff curves with a terminal half-life of ~ 14 hours in both dogs. These findings are comparable to our previously reported data on pharmacokinetic parameters obtained in naive hemophilia A dogs.[24]

Because previous data using liver-directed gene transfer to prevent immune responses in animal models have shown the involvement of regulatory T cells,[37-40] we used flow cytometry to determine the frequency of $CD4^+$, $CD25^+$, and $FoxP3^+$ T cells at baseline, week 1, and week 12. Interestingly, we observed an increase in $CD4^+$, $CD25^+$, and $FoxP3^+$ T cells at week 1, with a return to baseline levels by week 12 (Table 2). While further studies are required to determine the exact mechanism of tolerance induction in this model, our data are consistent with the hypothesis that regulatory T cells might be, at least in part, involved in this phenomenon.

Next we treated a dog (Wembley) from the colony at Queen's University.[41] Wembley developed inhibitors after primary exposure to human FVIII, and these inhibitory antibodies were found to cross-react with canine FVIII. He received further infusions of cryoprecipitate containing large amounts of cFVIII. At the time of vector injection, the inhibitor titers against cFVIII were 3.5 BU. After administration of AAV8-cFVIII vector, we observed a rapid increase in cFVIII antigen and activity, which reached levels of 74 ng/mL and 10%, respectively. These levels quickly decreased to pretreatment levels that coincided with a remarkable increase in

Table 2. Flow cytometric analysis of total peripheral blood mononuclear cells from Chapel Hill inhibitor dogs

	% $CD25^+FoxP3^+$ of total $CD4^+$ (SD)		
Dog	Baseline (d 0)	Peak (wk 1)	Plateau (wk 12)
K01	1.05 (0.09)	2.61 (0.30)	0.94 (0.06)
K03	2.45 (0.05)	4.60 (0.26)	2.58 (0.03)
L44	1.01 (0.10)	1.50 (0.11)	1.16 (0.10)

SD indicates standard deviation of triplicate analysis of sample.

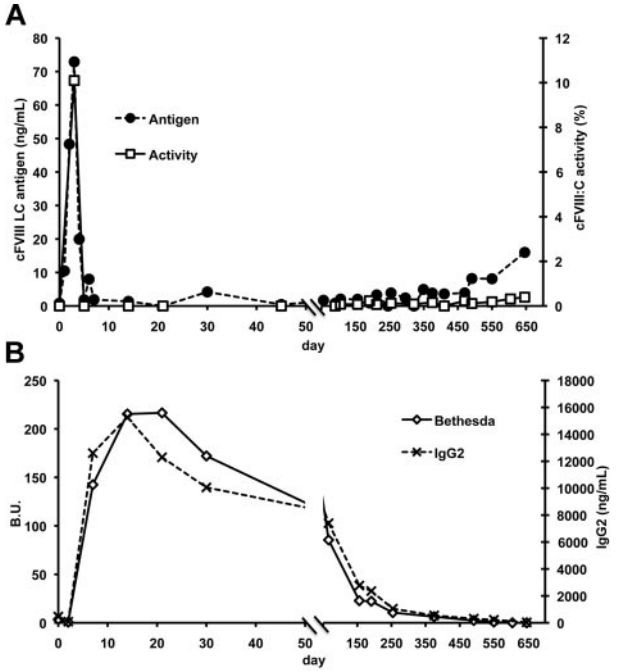

Figure 6. cFVIII expression and anti-cFVIII antibody response in a high-responding hemophilia A dog after AAV-mediated liver expression of cFVIII. A high-responding hemophilia A dog from the Queen's University colony (Wembley) was administered 2.5×10^{13} vg/kg of AAV8-TBG-cFVIII-HC and AAV8-TBG-cFVIII-LC by peripheral venous injection. (A) cFVIII antigen levels were assayed by a cFVIII-LC-specific ELISA, and activity was monitored by FVIII assay. (B) Anti-cFVIII antibody responses were measured by anti-cFVIII IgG2 ELISA and Bethesda assays.

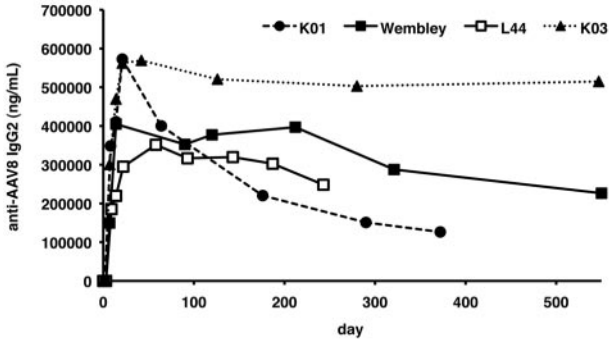

Figure 7. Anti-AAV8 capsid humoral responses. Anti-AAV8 capsid IgG2 responses were assayed in all 4 dogs. Plasma samples were assayed for capsid-specific IgG2 by ELISA with plates coated with empty AAV8 capsid.

inhibitor titers and anti-cFVIII IgG2 (Figure 6A). A similar finding was observed for anti-cFVIII IgG1 (data not shown). Wembley's inhibitors showed a strong anamnestic response, with titers peaking at 216 BU on day 21 (Figure 6B). We continued to monitor the levels of cFVIII and anti-cFVIII antibodies, and over the following 80 weeks, the inhibitor titers gradually decreased to current levels of 0.8-1.0 BU/mL at day 550 (ongoing observation). As the inhibitor titers decreased, we began to observe a rise in cFVIII LC antigen (12 ng/mL); however, cFVIII activity levels remained below the limit of detection ($< 1\%$). We are continuing to follow this animal, and hypothesize that when the inhibitor titers are completely eradicated, the cFVIII activity levels will increase and eventually reach a detectable level. This dog resembles to a certain extent the kinetics of high-responder patients, showing a substantial increase in inhibitor levels upon exposure to FVIII protein.[32] In high-responder patients, ITI failure rates increase to more than double that of nonhigh-responders.[2] Interestingly, Wembley's anti–human FVIII inhibitors rose from a baseline value of 7.4 BU to a peak of 271 BU at day 14, and have stabilized at 2.2 BU. A similar pattern was observed for the anti-hFVIII IgG2 levels, with current levels of ~ 20 μg/mL (data not shown). We speculate that this indicates that Wembley was tolerized to specific epitopes shared between human and canine FVIII; however, there continue to be inhibitors specific for epitopes unique to hFVIII.

To confirm that the immune tolerance to cFVIII was specific, we measured anti-AAV8 IgG2 antibody levels. As seen in Figure 7, all animals developed a robust and sustained anti-capsid immune response, indicating that they were fully capable of generating and maintaining humoral immune responses to other antigens after vector administration.

Discussion

The contrast in the immunological profile and response after AAV-mediated expression of cFVIII between the 3 Chapel Hill dogs and the dog from the Queen's colony was remarkable. Although these dogs had a similar underlying causative mutation, all 3 Chapel Hill dogs (K01, K03, L44) showed mild anamnestic responses and rapid eradication of inhibitors in 4-5 weeks, while Wembley had a very strong immune response that has taken over a year and a half for inhibitor titers to decrease to background levels. There are several factors that may explain these distinct outcomes. First, previous exposure to xenoantigen (human FVIII) that could hamper the ability to induce antigen-specific immune tolerance. Second, there are the differences in strains of dogs that may reflect inherited factors similar to ethnicity as a genetic risk factor in humans.[7,8] Lastly, the long duration (~ 2 years) between inhibitor development and AAV administration may also influence the rates of success, as has been observed in humans on ITI.[2]

The data presented here demonstrate for the first time in an adult large animal model of disease the potential of liver-directed, AAV-mediated gene expression to induce tolerance to the transgene in the setting of preexisting inhibitory antibodies. The sustained expression of cFVIII from the transgene after inhibitor eradication recapitulates the secondary prophylactic replacement protocols required to maintain the immune tolerance after successful inhibitor eradication.[2] In this model, we observed both inhibitor eradication and complete normalization of pharmacokinetics of FVIII protein infusion and improved disease phenotype. Overall, vector administration was well tolerated, with no abnormalities on serial determinations of hematologic and biochemical analyses of blood and serum samples for liver and kidney function tests.

The underlying mechanism of the success of ITI in humans is still unclear, but this has been investigated in preclinical studies and has been shown to depend on both B- and T-cell response.[42,43] The exact mechanism of the immune-tolerance induction in this hemophilia A dog study is currently unknown. Previous work in murine and nonhuman primate models has shown that sustained AAV-mediated expression of transgenes can induce tolerance, and that this sustained expression is dependent on regulatory T cells.[37-39] In addition, recent work using microRNA to restrict transgene expression from a lentiviral vector to hepatocytes has also shown sustained transgene expression and the induction of antigen-specific T-regulatory cells.[40] While our observation that $CD4^+CD25^+FoxP3^+$ T cells are transiently up-regulated after gene transfer is consistent with the hypothesis that regulatory T cells are involved, much work still needs to be done, including testing the

function and antigen specificity of these cells, to fully investigate the mechanism of immune tolerance induction in this model. It is also likely that this immune-tolerance induction involves multiple mechanisms, including anergy and/or deletion, and this is currently under investigation.

Our group and others have previously demonstrated that using viral vectors to direct gene transfer to the liver of adult[17,19,21,23] or neonatal[44,45] large animal models can induce tolerance to the expressed transgene and prevent immune responses. Data on tolerance induction by gene therapy and/or immune modulatory strategies in preexisting immune responses has been limited to murine models.[46-48] Considering the limited numbers of hemophilia A dogs in this study and the modest inhibitor titers at the time of vector administration, these findings have to be considered a proof-of-principle that immune tolerance induction is feasible in the setting of preexisting immunity in a large animal model for an unmet medical need. These data may have relevance not only for hemophilia, but also for a variety of diseases in which antibody formation to the therapeutic protein or enzymes could prevent optimal clinical responses.[49,50]

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Authorship

Contribution: J.D.F. directed the design and execution of the experiments and drafted the paper; M.C.O., H.W.G.F., E.P.M., J.M.C., D.L., and T.C.N. performed the vector administration, provided care to the animals, collected laboratory samples, and assisted with experimental design and interpretation; S.Z. provided the AAV vectors used in this study; D.E.S. and H.H.K. provided insights on protocol design, interpretation of data, and manuscript preparation; and V.R.A. directed experimental design, conducted data analysis and interpretation, and drafted the manuscript.

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