

Persistent Expression of hF.IX After Tolerance Induction by *In Utero* or Neonatal Administration of AAV-1-F.IX in Hemophilia B Mice

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The major complication associated with protein replacement therapy currently used in the treatment of hemophilia B (HB) is the development of antibodies to the infused human Factor IX (hF.IX). We hypothesized that vector-mediated expression of hF.IX, either at a prenatal stage or early in life may lead to tolerance to hF.IX and long-term transgene expression. Fetal, neonatal, and adult F.IX-deficient mice were injected with AAV-1-hF.IX, and the hF.IX levels as well as antibodies to hF.IX in the circulation were assayed. *In utero* injection followed by postnatal re-administration of adeno-associated virus 1 (AAV-1) vector achieved persistent expression of hF.IX in all animals, with no cellular or humoral immune response to F.IX. Similar results were seen after initial injection in neonatal mice followed by re-administration, whereas all mice injected at the adult stage developed antibodies to hF.IX. In contrast, after administration of AAV-2-hF.IX in the neonatal period, antibodies to hF.IX were formed in all the injected animals. We conclude that *in utero* or neonatal-stage injection of AAV-1-hF.IX can lead to long-term expression and absence of immune response. The differences in immune response between the AAV-1 and AAV-2 groups suggests that tolerance may be related to differences in bio-distribution, timing of expression, and/or the initial levels of hF.IX expression. This supports the concept of a narrow "window of opportunity" for tolerance induction.

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INTRODUCTION

Hemophilia B (HB) is an X-linked recessive disease caused by a deficiency in clotting Factor IX (F.IX), and characterized by repeated bleeding episodes in the joints and soft tissues. The current treatment for HB is based on protein replacement therapy with plasma-derived or recombinant human F.IX (hF.IX). However, some patients develop inhibitory antibodies that block the procoagulant activity of the F.IX protein, resulting in an

inability to achieve hemostasis. Gene therapy for this disease has focused on AAV transduction of muscle or liver cells to produce F.IX, and has been tested in Phase I clinical studies.¹⁻³ Although this may be a promising strategy, animal studies suggest that inhibitory antibody formation may pose a problem in a gene-based approach. Specifically, it has been observed that, in the absence of immune suppression, HB mice with a large gene deletion and HB dogs with a null mutation make antibodies to a species-specific F.IX transgene after intramuscular (IM) administration of AAV-2-canine-F.IX.⁴⁻⁸ Hemophilia is an excellent model for studying prenatal tolerance induction, given that levels of F.IX expression and antibody formation can be easily monitored over time.

In utero gene therapy is a promising strategy for treating a number of congenital diseases that can be diagnosed prenatally.⁹ A major advantage of fetal treatment is the potential to induce tolerance to the wild-type transgene product by introducing the gene product prior to the maturation of the immune system. Furthermore, expression of a transgene in the perinatal period can avert early-arising complications of genetic disorders, such as intracranial hemorrhage in hemophilic neonates.¹⁰ While hemophilia is not likely to be an early candidate for prenatal gene therapy (because alternative treatments are available) patients with this inherited disorder, like those with other such disorders, could certainly benefit from early intervention.

There is a rapidly-expanding body of literature dealing with the effect of *in utero* administration on induction of tolerance to a foreign transgene. A study that produced very convincing results used retroviral vectors in a sheep model; *in utero* intraperitoneal injection of pre-immune fetal lambs led to long-term expression of β -galactosidase in hematopoietic stem cells.¹¹ Postnatal challenge with purified protein failed to induce antibody response or lymphocyte proliferation to the transgene in these animals, in contrast to untreated age-matched controls in which these responses were routinely seen. Continuous expression of the transgene, as was achieved by hematopoietic stem cell transduction in that experiment, seems to be a requirement for maintenance of tolerance. *In utero* injection of adenovirus has not been shown to induce tolerance in the face of repeated

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exposure to a foreign protein.^{12,13} After *in utero* IM injection of AAV-2 and AAV-5 vectors, we¹⁴ and others¹⁵ have observed long-term expression of a foreign gene, thereby implying that some level of anergy or tolerance is achieved with this method.

Several groups of researchers have attempted *in utero* gene transfer of hFIX using adenoviral and AAV vectors. Although tolerance to hFIX has been reported following *in utero* injection of adenoviral vectors,¹⁶ the levels of expression were low, and not all of the animals were rendered tolerant to the transgene. It has been difficult to obtain high levels of expression with AAV-2 vectors.¹⁷ The effect of repeated exposure to the antigen was investigated in a study by Waddington *et al.*, in which *in utero* injections of adenovirus-hFIX were followed up with postnatal injections of hFIX protein in wild-type mice.¹⁶ In approximately half of the mice, this prevented the development of antibodies to hFIX. However, the existence of tolerance was not proven by cellular tests of immunity. A more recent report demonstrated sustained expression of hFIX after *in utero* injection of lentiviral vector, with expression mainly in the liver.¹⁸

Adeno-associated virus 1 (AAV-1) and AAV-2 are different serotypes of AAV. They are highly conserved but differ in terms of their ability to transduce skeletal muscle,² kinetics of gene expression, and tolerance induction.³ Membrane-associated heparin sulfate proteoglycan serves as the primary attachment site for AAV-2 on muscle fibers,¹⁹ while the receptor for AAV-1 has not been identified. AAV-1 is 10–20 times more efficient than AAV-2 as measured by circulating F.IX levels in HB mice and dogs following IM administration of similar doses of the two vectors.²⁰ When adult CD4 knockout mice are administered IM injections of AAV-2 vectors, the levels of hFIX in the circulation increase slowly and reach a plateau at 4 weeks.⁴ In contrast, the same dose of AAV-1 vector results in a rapid increase in hFIX expression to detectable levels within 1 week and reaches a plateau at a significantly higher level.²⁰ IM injections of AAV-1-hFIX induced tolerance to hFIX in HB mice in a dose-dependent manner, whereas AAV-2 failed to induce tolerance.^{20,21}

A comparison of results from AAV-1 and AAV-2 administered *in utero* has not been previously described. Differences in bio-distribution, timing of expression, and/or levels of transgene expression may be critically important to the outcome of an *in utero* gene transfer approach, particularly in terms of tolerance induction. In this study, we sought to examine a vector type and a route of administration, both clinically relevant, in a disease model of HB, focusing on investigating both humoral and cellular immunity to the transgene product. We hypothesized that AAV-1 and AAV-2 may exhibit differences in tolerance induction in mice in the fetal, neonatal, and adult stages, because of the differences in the transduction characteristics of the two vectors. We report here that *in utero* mice as well as neonatal mice can be rendered tolerant to the transgene by transduction with AAV-1, and that persistent levels of expression are achieved after a single re-administration, whereas neonatal animals that receive AAV-2 do not effectively achieve tolerance to the transgene.

RESULTS

Survival after *in utero* administration of AAV

Timed matings were set up between HB males and HB carrier females. Such matings would be expected to result in

approximately half of the offspring being HB and half being either wild-type or HB carriers. We have previously observed that HB as well as HB carrier or wild-type mice on a C57BL6/129 background develop a humoral immune response to hFIX.²² Therefore, results from all of the mice would be informative for tolerance to hFIX. On embryonic day 14, each fetus was administered vector IM in the left hind limb (**Figure 1a**). In the design of these experiments, our goal was to achieve comparable circulating levels of F.IX in recipient mice. This was based on previous observations suggesting that the strength of an immune response correlates with the amount of antigen delivered.^{20,23} Since the antigen of interest in this study is F.IX and not AAV capsid, this approach seemed more likely to yield useful information rather than an alternative design based on injection of equal numbers of vector particles.

The number of fetuses injected, with survival and genotype data, are shown in **Table 1**. In the *in utero* mouse model, survival depends on multiple factors, such as injection technique, vector preparation, maternal neglect, etc. The administration of AAV-1-CMV-hFIX resulted in survival to birth of 13 of the 17 fetuses injected (76%). Of the 17 fetuses that received AAV-2-CMV-hFIX *in utero*, 14 survived to birth (82%). The differences between AAV-1 and AAV-2 survival data are not statistically significant (Fisher exact test, $P = 1.0$) and are consistent with our experience with *in utero* injection of AAV vectors¹⁴ and lentiviral vectors²⁴ carrying lacZ in wild-type mice. Litters of mice that were not administered AAV (untreated) were comprised of wild-type, HB carrier, and HB mice in the expected ratios (**Table 1**), consistent with the predicted levels for an X-linked disease. The AAV-treated mice also have litters comprising wild-type, HB carrier, and HB mice in ratios that are not statistically different from those for untreated animals (Fisher exact test, $P = 1.0$).

Administration of AAV-2-CMV-h.FIX

The vector was administered *in utero* as described earlier. In neonatal mice, the vector was administered IM on day 2 of life, while adult mice were injected at 6–8 weeks of age (**Figure 1a**). All adult mice that received AAV-2-CMV-hFIX ($n = 5$) had very low-to-undetectable hFIX levels (0 to <9 ng/ml) and developed immunoglobulin G antibodies to hFIX within 3 weeks after vector administration (**Figure 1b**) as previously demonstrated.²² Ten neonatal mice were administered the vector on day 2 of life and five mice survived the procedure. All of the neonatal mice that survived were either wild-type or HB carriers (none of the HB mice survived), which is in contrast to the expected Mendelian ratios we observed with the *in utero*-treated mice (**Table 1**). It is assumed that maternal F.IX may cross the placental barrier and cover the fetuses during the injection at embryo day 14, whereas the neonates injected on day 2 of life face a substantial hemostatic challenge. The neonates initially produced very low levels of hFIX (0 to <9 ng/ml). By 8 weeks, hFIX was detectable in four of the five animals (0–25 ng/ml) and by 12 weeks all the animals had hFIX at the lower limit of detection. Antibody development to hFIX was first seen at 8 weeks, and by 12 weeks all five animals developed antibodies (**Figure 1c**). The neonates showed a significant delay in development of anti-hFIX antibodies (12 weeks after AAV-2-hFIX

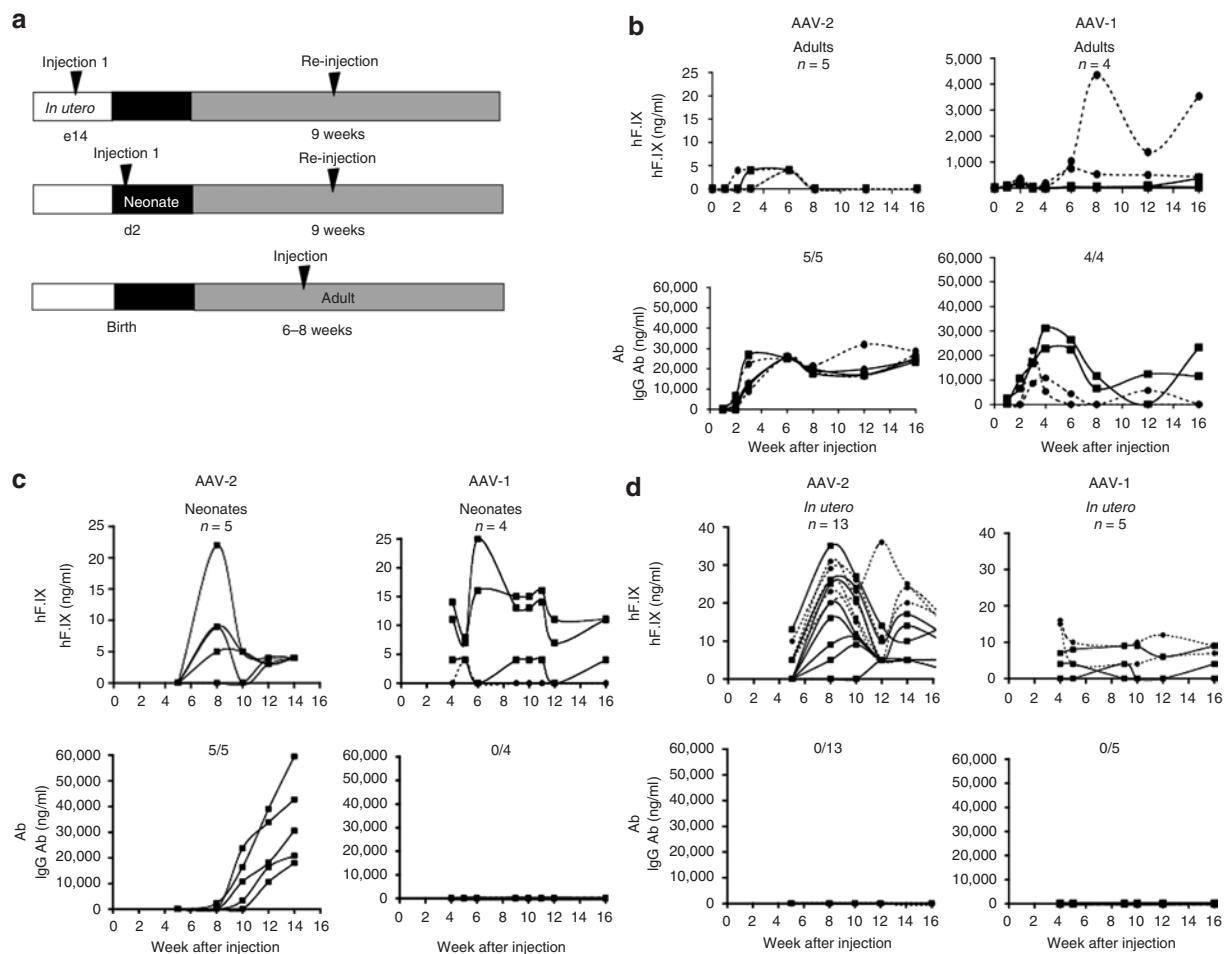


Figure 1 Comparison of adeno-associated virus 1 (AAV-1) and adeno-associated virus 2 (AAV-2) vector administration in adults, neonates, and fetuses *in utero*. **(a)** Experimental Design. Timed matings were set up between hemophilia B (HB) males and HB carrier females. On embryonic day 14 the pregnant females were prepared for surgery and each fetus was administered vector intramuscularly (IM) in the left hind limb. After birth, peripheral blood was collected beginning at week 5 after injection (4 weeks of age) for enzyme-linked immunosorbent assay to detect human Factor IX (hF.IX) and antibodies (Abs) to hF.IX. At a later time point (9 weeks) some of the mice were re-injected IM with the same serotype AAV vector in the right hind limb. Control animals were neonates injected on day 2 of life or adult animals injected at 6–8 weeks of age. **(b)** Administration of AAV-2-CMV-hF.IX (1.2×10^{13} vg/kg) ($n = 5$) and AAV-1-CMV-hF.IX (4×10^{12} vg/kg) ($n = 4$) to adult mice. The levels of hF.IX in adult animals were similar to levels previously observed in our laboratory for AAV-1 and AAV-2 vectors (top panels). Note the difference in scales for these graphs. HB mice are indicated by dotted lines while HB carriers or wild-type mice are indicated by solid lines. All the AAV-1 and AAV-2 injected adult mice developed Abs to hF.IX (lower panels). **(c)** Administration of AAV-2-CMV-hF.IX (1.8×10^{13} vg/kg) ($n = 5$) and AAV-1-CMV-hF.IX (4×10^{12} vg/kg) ($n = 4$) to neonatal mice. The levels of hF.IX for AAV-1- and AAV-2-injected neonates were measured beginning at 4 weeks of age, and were found to range between 0 and 25 ng/ml (top panels). None of the AAV-1-injected neonates developed Abs, whereas all the AAV-2 injected neonates developed anti-hF.IX Abs (lower panels). **(d)** *In utero* administration of AAV-2-CMV-hF.IX (2.7×10^{13} vg/kg) ($n = 13$) and AAV-1-CMV-hF.IX (5×10^{12} vg/kg) ($n = 5$). The levels of hF.IX after *in utero* vector administration resulted in low levels of hF.IX expression (0–35 ng/ml) (top panels). hF.IX was measured beginning at 4 weeks of age. None of the *in utero*-injected mice developed anti-hF.IX Abs up to 16 weeks after injection. CMV, cytomegalovirus; IgG, immunoglobulin G.

vector administration) as compared to the adult mice (3 weeks after vector administration).

Thirteen mice that were administered AAV-2-CMV-hF.IX *in utero* were analyzed long-term (13 of the 14 injected fetuses were live born). These mice expressed low levels of hF.IX (0–35 ng/ml) (Figure 1d). While adult and neonatal mice that received this vector developed antibodies within 3 and 12 weeks, respectively, mice in the *in utero* group did not develop antibodies through 16 weeks after the *in utero* vector administration. Antibodies to hF.IX were detected in 3 of the 13 animals at 20 weeks after injection (data not shown). Table 2 summarizes the findings after AAV-2 vector administration.

Administration of AAV-1-CMV-hF.IX

The levels of expression, the timing of expression, as well as the bio-distribution following injection may be crucial determinants of whether the fetus is rendered tolerant to the transgene. AAV-1 achieves 10-fold to 20-fold higher levels of expression of F.IX in muscle in adult mice than AAV-2 does, and also achieves these higher levels more quickly.²⁰ Given that administering AAV-2 prevented antibody formation to a substantial extent in *in utero* mice but not in neonates or in adult mice, we hypothesized that AAV-1 may be more effective at inducing tolerance during the early stages of development of the animals. In order to test this hypothesis, we performed another set of experiments with

AAV-1-CMV-hFIX, using the same experimental design as described earlier (Figure 1a).

Adult animals were administered AAV-1-CMV-hFIX ($n = 4$), and they expressed low levels of hFIX (36–81 ng/ml at 1 week; 181–366 ng/ml at 2 weeks), until they all developed anti-hFIX antibodies at 3 weeks (Figure 1b). One animal stopped making antibodies and began to express high levels (>500 ng/ml) of hFIX at 6 weeks. This pattern of transient antibody formation has been observed in our laboratory and is associated with high levels of hFIX expression at high vector doses.²⁰ Neonatal mice injected with AAV-1-CMV-hFIX ($n = 4$) on day 2 of life had low levels of hFIX expression (0–25 ng/ml) (Figure 1c). Antibodies to hFIX were not detected in any of the neonatal mice through 16 weeks after vector administration. This is in contrast to the results with AAV-2, which showed that all the neonates made anti-hFIX antibodies by 12 weeks. Despite the levels of expression of hFIX being similar with the use of either vector, the clear difference in neonatal immune response to hFIX suggests that the selection of the vector (AAV-1 and AAV-2) is important at the early stage of development of the mice. Mice that were administered AAV-1-hFIX *in utero* ($n = 5$) also expressed low levels of hFIX (0–16 ng/ml) (Figure 1d). None of these animals developed antibodies to hFIX through 16 weeks after vector administration.

Re-administration of AAV-1-hFIX after neonatal or *in utero* injections of AAV-1 vector

A comparison of the relative efficacies of AAV-1 and AAV-2 in neonates suggested that AAV-1 is superior at maintaining tolerance (Figure 1c). We therefore hypothesized that therapeutic levels of hFIX could be achieved by re-administration using AAV. We tested this hypothesis in the AAV-1-injected mice, since AAV-1 directs high levels of expression. Either AAV-1 or AAV-2 may be adequate to achieve tolerance if administered *in utero*, whereas AAV-1 is clearly better at inducing tolerance if administered in the neonatal period. The re-administration experiment was not possible in AAV-2 injected neonates, because all of these animals developed antibodies to F.IX. Therefore additional litters of mice were administered the vector *in utero* or as neonates and then, at a later time point (postnatally), the mice were re-injected IM with the same AAV serotype vector in the right hind limb (Figure 1).

Another litter of neonatal mice ($n = 8$) was injected with AAV-1-CMV-hFIX on day 2 of life and had low levels of hFIX expression initially, similar to those observed with the first litter ($n = 4$) previously described (0–25 ng/ml) (Figure 2). Antibodies to hFIX were not detected in any of these mice. They were re-injected at 9 weeks after the initial injection (9 weeks of age), and hFIX rose to therapeutic levels in all of the mice (382–6,855 ng/ml) at 20 weeks (10 weeks after re-injection). At 30 weeks hFIX had reached stable levels of 67–1,667 ng/ml (data not shown). None of the neonates produced antibodies to hFIX following the re-injection.

In an *in utero* group ($n = 7$), 6 mice out of the 7 displayed detectable but very low hFIX levels (<9–18 ng/ml) after the initial injection (Figure 2). None of these mice developed antibodies in the time period prior to re-injection. After re-injection at 9 weeks, the hFIX levels increased to 24–2,326 ng/ml in all the mice at the time point of 20 weeks after the initial injection, and were consistently at therapeutic levels (>1%) in 5 of the 7 mice. None of the mice developed antibodies to hFIX after the re-injection. An activated partial thromboplastin time assay demonstrated that the clotting activity correlated with the levels of hFIX detected by enzyme-linked immunosorbent assay (ELISA) (data not shown). Expression continued in both the *in utero* and neonatal groups for 11 months, the longest time point tested (data not shown). Table 2

Table 1 Survival after *in utero* AAV vector administration

Treatment	Expected based on Mendelian Inheritance	Untreated	AAV-1 ^b	AAV-2 ^b	Total AAV treated
Number injected	na	na	17	17	34
Number born	na	36	13 ^a	14 ^a	27
Genotype					
Wild type or HB carrier	50%	19 (53%)	7 (54%)	7 (50%)	14 (52%) ^c
HB	50%	17 (47%)	6 (46%)	7 (50%)	13 (48%) ^c

Abbreviations: AAV, adeno-associated virus; HB, hemophilia B; na, not applicable. ^aOne animal in each group died after 10 weeks and, therefore, was not included in the final analysis in Table 2. ^bNo statistical difference between AAV-1 and AAV-2 injected animals (Fisher exact test, $P = 1.0$). ^cNot statistically different from the untreated group (Fisher exact test, $P = 1.0$).

Table 2 Summary of AAV administration in *in utero*, neonatal, and adult mice

Vector	Age at time of injection	Total number analyzed in group	Number with detectable hFIX levels	Time to antibody development ^a	Re-administration ^b	Long-term hFIX levels	Long-term therapeutic levels (>1% hFIX)
AAV-1	<i>In utero</i> ^c	12 ^{d,e}	11	None detected	Yes ^b 7/12	7/7	5/7
	Neonate ^c	12 ^d	12	None detected	Yes ^b 8/12	8/8	8/8
	Adult ^c	4	0	1 wk	No	4/4	3/4
AAV-2	<i>In utero</i> ^c	13 ^e	13	None detected	No	13/13	Not re-injected
	Neonate ^c	5	5	8 wk	No	0/5	0/5
	Adult ^c	5	5	2 wk	No	0/5	0/5

Abbreviations: AAV, adeno-associated virus; CMV, cytomegalovirus; hFIX, human Factor IX; wk, week. ^aResults up to 16 weeks after injection. ^bData relating to the animals that were re-injected are shown in the graph in Figure 2. ^cAAV-1-CMV-hFIX doses as follows: *in utero* (5×10^{12} vg/kg), neonate (4×10^{12} vg/kg), adult (4×10^{12} vg/kg); AAV-2-CMV-hFIX doses as follows: *in utero* (2.7×10^{13} vg/kg), neonate (1.8×10^{13} vg/kg), adult (1.2×10^{13} vg/kg). ^dThis number represents total number of animals injected. Data relating to the neonatal animals that were not re-injected ($n = 4$) are shown in the graph in Figure 1c. Data relating to *in utero* mice that were not re-injected ($n = 5$) are shown in the graph in Figure 1d. ^eOne animal in each group died after 10 weeks. These are included in the survival study (Table 1) but not included in the final analysis.

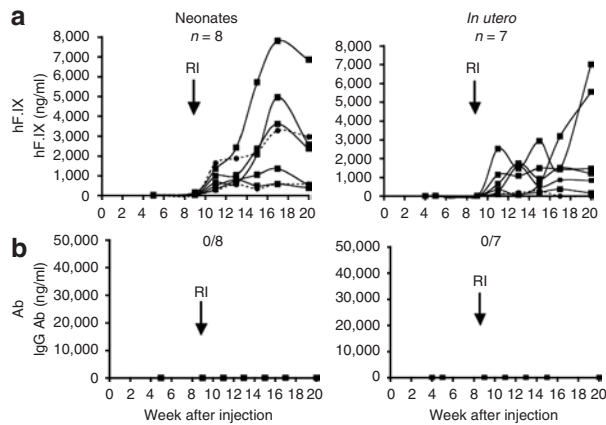


Figure 2 Therapeutic levels of human factor IX (hF.IX) expression and absence of antibody formation after intra-muscular re-administration of AAV-1-CMV-hF.IX. **(a)** hF.IX expression after *in utero* administration of adeno-associated virus 1 (AAV-1) vector and re-injection (RI) at 9 weeks after initial administration in neonates and *in utero* mice. All the mice injected as neonates ($n = 8$) (left panel) and *in utero* ($n = 7$) (right panel) expressed low levels of hF.IX prior to re-administration (0–25 ng/ml), which increased to therapeutic levels after re-administration. This is a different group of animals from the mice presented in **Figure 1c** and **d** (right panels). **(b)** Anti-hF.IX antibodies (Abs) were not detected either before or after vector re-administration at 9 weeks. Hemophilia B (HB) mice are indicated by dotted lines and HB carriers or wild-type mice are indicated by solid lines. CMV, cytomegalovirus; IgG, immunoglobulin G.

summarizes the findings after AAV-1 vector administration and re-injection.

Antibody response to AAV capsid after *in utero* injection and following re-administration

Anti-AAV antibody titers were determined after IM administration of AAV-hF.IX to *in utero*, neonatal, and adult mice. Total anti-AAV immunoglobulin G could not be detected after *in utero* or neonatal administration of AAV-1-hF.IX; however, anti-AAV antibodies were detected after re-administration (data not shown), as previously observed.¹⁴ In adult mice, anti-AAV-1 antibodies (2,000 ng/ml) were generated within 3 weeks after vector administration. Similar observations were made with AAV-2, *i.e.*, no anti-AAV antibodies were detected after *in utero* or neonatal administration, but were observed in adult mice after injection. More antibodies (7,000 ng/ml) were found in the adult control mice in the case of AAV-2 than in AAV-1. These data suggest that there was no difference between AAV-1 and AAV-2 as regards antibody production after *in utero* or neonatal injection. That is, when the vector is administered *in utero* or in neonatal mice, anti-AAV antibody production plays no role in the initial levels of expression or in the efficacy of subsequent vector re-administration.

Cellular immunity studies in mice injected with AAV-1-hF.IX

In order to perform a more rigorous test of tolerance to hF.IX, mice in the *in utero* and neonatal groups that had received re-injection of AAV-1 vector were challenged with hF.IX in complete Freund's adjuvant (cFA), a stringent immunological challenge. After 1 month, the mice were boosted with hF.IX in incomplete

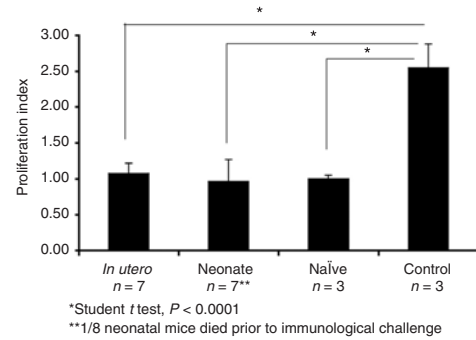


Figure 3 Cellular tests of immunity to challenge mice that were administered AAV-1-hF.IX. Mice that had received high titer AAV-1-CMV-hF.IX were challenged with human factor IX (hF.IX) in complete Freund's adjuvant, and T cell proliferation assays were performed. The proliferation index (mean counts per minute (cpm) hF.IX/mean cpm) was calculated based on the cpm of ^3H detected with splenocytes incubated with hF.IX protein and the cpm detected in the absence of protein (media alone or mock). *In utero* mice ($n = 7$), neonates ($n = 7$) (1 of the 8 neonates receiving re-administered vector died prior to challenge), and control C57Bl/6 mice ($n = 3$) were challenged, while naïve C57Bl/6 mice ($n = 3$) were not challenged. AAV, adeno-associated virus; CMV, cytomegalovirus.

Freund's adjuvant for a T cell proliferation assay (**Figure 3**). All the *in utero*-injected mice ($n = 7$) had T cell proliferation indices less than 1.3. Similarly, all the neonatal mice ($n = 7$) (1 of the 8 re-injected neonates died prior to challenge) had proliferation indices less than 1.2, similar to naïve mice that were not challenged with cFA. In contrast, control mice ($n = 3$) that had not been administered AAV-hF.IX and were immunized with hF.IX in cFA had high antibody titers to hF.IX after challenge, and proliferation indices of 2.5 ± 0.33 (Student *t*-test, $P < 0.0001$). Cytokine release assay performed with cells from the same mice demonstrated that only the control mice immunized with Freund's adjuvant produced high levels of interferon- γ (mean 2305 ± 277); it was undetectable in the challenged *in utero* and neonatal AAV-injected mice (data not shown). No differences were seen in the interleukin-2 and interleukin-10 levels (data not shown). The cytokine profile was consistent with the T cell proliferation results as well as with previous studies in our laboratory, in which a Th2 response to the secreted transgene product was observed in C57Bl/6 mice.^{22,25}

Bio-distribution of AAV vector after *in utero* administration of AAV-hF.IX

In order to examine whether differences in bio-distribution contribute to differences in immune response to the transgene product, we harvested tissues from *in utero* (embryo day 14)-injected mice at 1 week after vector administration (day 2 of life) ($n = 4$) and at 5 weeks after vector administration (4 weeks of age) ($n = 4$) (**Figure 4**). DNA was isolated and real time polymerase chain reaction analysis was performed using primers and probe specific to the hF.IX transgene. At 1 week after vector administration, both AAV-1 and AAV-2 vectors were widely distributed in all tissues that were analyzed (**Figure 4**), thereby suggesting that the vector is systemically distributed after *in utero* IM injection. This is not surprising, given that there are documented data of serum samples that tested positive for vector DNA after IM injection of

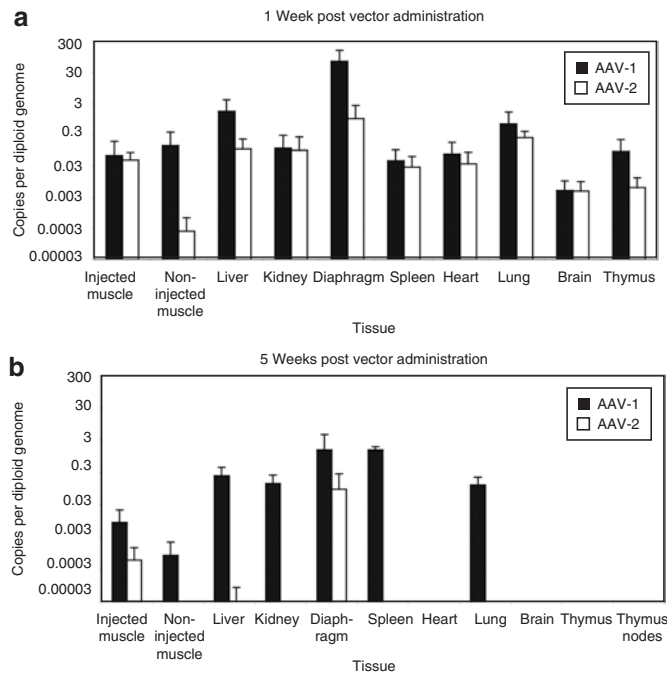


Figure 4 Tissue bio-distribution analysis of mice injected with adeno-associated virus (AAV) *in utero*. Mice were injected on embryo day 14 as previously described (Figure 1a) with doses of 5×10^{12} vg/kg for AAV-1-CMV-hF.IX and 2.7×10^{13} vg/kg for AAV-2-CMV-hF.IX. Tissues were harvested from *in utero*-injected mice at (a) 1 week after vector administration (day 2 of life) ($n = 4$) or (b) 5 weeks after vector administration (4 weeks of age) ($n = 4$). Real time polymerase chain reaction was used for detecting the number of copies in 200 ng of genomic DNA, and the data is displayed as the number of copies per diploid genome. The negative control tissue samples from untreated mice had no detectable human factor IX (hF.IX) genomes (data not shown). CMV, cytomegalovirus.

AAV in human subjects.² The numbers of vector copies in injected muscle is similar for both groups at 1 week after injection, thereby suggesting that the fivefold higher particle dose given to the AAV-2 injected mice was appropriate in terms of achieving comparable levels of gene transfer. However, marked differences appear at a later time point. At 5 weeks after vector administration, AAV-1 injected mice had vector genomes in liver (all 4 mice), kidney (all 4 mice), spleen (all 4 mice), lung (3 of the 4 mice), diaphragm (1 of the 4 mice), injected muscle (all 4 mice), and uninjected muscle (non-injected limb) (2 of the 4 mice), whereas AAV-2-injected mice had only ≥ 1 vector genome/diploid genome in the injected muscle (2 of the 4 mice) and diaphragm (2 of the 4 mice). The relatively high numbers observed in diaphragm are in keeping with our previous observations after AAV2-lacZ injection *in utero*.¹⁴ The mechanism(s) of this marked difference in persistence of vector genomes over time between the two serotypes is unknown; it may involve differential uptake by reticuloendothelial cells in liver, spleen, and lung. However, attempts to transduce murine splenic dendritic cells *in vitro* with AAV-1 and AAV-2 vectors, as evaluated by F.IX expression were unsuccessful (data not shown). Interestingly, this difference between AAV-1 and AAV-2 *in utero*-injected mice with regard to gene copy number did not result in marked differences in postnatal circulating F.IX levels or in the presence or absence of antibodies to F.IX (Figure 1d). Nevertheless, the differences in bio-distribution between the two

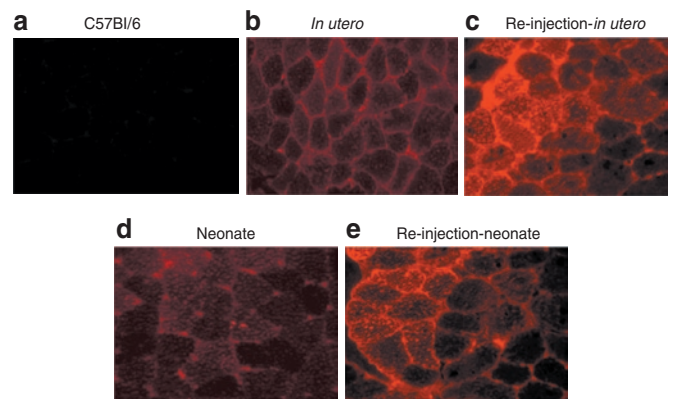


Figure 5 Comparison of immunofluorescence detection of human factor IX (hF.IX) in *in utero*- and neonatal-injected mice after AAV-1-CMV-hF.IX administration. In the *in utero* group as well as in the neonatal group, the initial injection was in the left leg while the booster re-administration was in the right leg. Muscles from each leg were harvested when the animals were at least 8 months old. Original magnification $\times 200$. (a) control, un-injected adult mouse (C57Bl/6); (b) *in utero* group, left hindlimb; (c) *in utero* group, after re-administration, right hindlimb; (d) neonatal group, left hindlimb; (e) neonatal group, after re-administration, right hindlimb. AAV, adeno-associated virus, CMV, cytomegalovirus.

serotypes may have affected the development of antibodies to F.IX in the mice injected at the neonatal stage.

Immunofluorescence staining of hF.IX in muscle after administration of AAV-1-hF.IX

Immunofluorescence staining for hF.IX was performed in muscle harvested from the *in utero* and neonatal groups when the animals were at least 8 months old (Figure 5). In all the animals, the initial injection was in the left leg while the re-injection was in the right leg. This was done in order to distinguish intensities of expression from injections administered at different stages of life. The animals that were injected in the left hind limb with vector *in utero* had very low but detectable staining for hF.IX in the injected muscle groups when compared with untreated muscle in control mice. The right limb (injected during adult life) had a greater number of patches of more intense staining for hF.IX, consistent with the higher levels of expression that were detected in the circulation after the second injection. Similarly, in the neonatal groups the left leg muscle, injected on day 2 of life, revealed only low-level positive staining for hF.IX, whereas the right leg muscle (injected during adult life) showed more intense staining. These analyses support the findings that the postnatal injections were successful in increasing the levels of expression. That is, immunofluorescence staining for hF.IX is consistent with the circulating levels of F.IX.

DISCUSSION

Numerous animal studies indicate that gene therapy is a promising approach to the treatment of HB. However, there is ample evidence that immune response to the donated gene product may be a serious obstacle to overcome, especially when skeletal muscle is the target tissue.^{5,7,21} *In utero* or neonatal gene transfer is a potential method of overcoming this problem, because it

enables expression of the transgene product before the immune system is completely established. Hemophilia is an excellent model for studying tolerance induction in the pre-immune period. The current studies demonstrate that it is possible to establish tolerance to hFIX by *in utero* or neonatal injections of AAV vectors and to obtain long-term therapeutic levels of hFIX in immunocompetent mice. Furthermore, we show that the AAV serotype may have an impact on the success of re-administration of the viral vector and induction of immune tolerance to the transgene.

In immunocompetent adult mice, IM administration of either AAV-1-hFIX or AAV-2-hFIX invariably results in the formation of antibodies to human F.IX (Figure 1b), as previously observed.^{4,20} However, the administration of AAV-1-hFIX in either newborn or fetal mice results in low-level F.IX expression (Figure 1c and d) and an absence of antibodies to F.IX (Figure 1c and d). Moreover, mice injected with AAV-1 in the fetal or neonatal period do not develop antibodies to the AAV capsid, and this makes re-administration of the vector possible. When we re-administered AAV-1-hFIX to mice 9 weeks after the initial injection (either neonatal or fetal), we found expression of F.IX at therapeutic levels (~380–6800 ng/ml) in all the mice (Figure 2). Moreover, these mice were tolerant to hFIX as measured by T cell proliferation assays (Figure 3). It follows that administration of AAV-1-hFIX early in life can promote tolerance to a foreign F.IX protein. Also, because antibodies to the capsid do not form after this first injection, it is possible to administer a second booster injection to raise the levels of F.IX in the circulation to within the normal range. The contrast between this result and the result seen in adult mice shows that the stage of development at which the injection is administered is of central importance in determining the level of immune response to vector and transgene product. Adult mice injected with AAV-1-hFIX develop antibodies to both capsid and transgene product, while mice injected as neonates or fetuses do not develop either type of antibody after the first injection. These findings have implications for human therapy as well. The ability to promote tolerance to a foreign protein, *e.g.*, F.IX or F.VIII, in an individual with hemophilia would be extremely useful in preventing the formation of inhibitory antibodies to these proteins, thereby overcoming the major complication of protein infusion therapy for this condition. Notably, such an approach would effectively induce tolerance for either a protein infusion or gene transfer approach in postnatal life. Moreover, the problem identified in a recent trial of AAV-mediated gene transfer to liver in human adults with hemophilia, namely, destruction of the transduced hepatocytes by host T cells,³ would be unlikely to occur. This is because such an immune response would be possible only if the individual has pre-existing T-cell immunity to wild-type AAV and this situation would not exist in the fetus or neonate.

The response in neonatal mice injected with AAV-2-hFIX was quite different from the response seen in a similar group that received AAV-1-hFIX. Despite similar low levels of transgene expression in both the groups, all of the AAV-2 injected mice went on to develop antibodies to human F.IX, while none of the AAV-1 injected mice did (Figure 1c). The difference in kinetics of expression may be a factor influencing this difference in immune response; AAV-1 expression begins earlier (compare values at 4–5

weeks in Figure 1c, measurable in AAV-1 mice, still undetectable in AAV-2-injected mice). Although the AAV-2-injected mice did receive a fivefold higher number of vector particles, it is difficult to postulate that the vector particle number accounts for this difference in immune response, because there is very little evidence in any species, including humans, of a robust innate immune response to AAV. The bio-distribution analysis results look quite similar for the two serotypes 1 week after vector injection, but there is much better persistence of AAV-1 genomes than of AAV-2 at 5 weeks after injection. It is possible that the higher gene copy number at the later time point (5 weeks) reflects better persistence and/or stabilization of the AAV-1 genome following entry into the cell. Since both vectors have the same inverted terminal repeats, the difference cannot be attributed to differences in conversion to double-stranded DNA. Rather, it likely has to do with differences in the kinetics or efficiency of uncoating, with vector DNA that uncoats in the nucleus or in the perinuclear region probably persisting longer than DNA that uncoats in the cytoplasm. The precise etiology of the difference in immune response to human F.IX in this setting is still unknown.

The difference in the host immune response to the two major antigens of the vector (AAV capsid and human F.IX) is intriguing. For instance, in mice first injected with AAV-1-hFIX as fetuses or neonates, antibodies to human F.IX do not appear either after the first injection or after re-administration, while antibodies to capsid clearly develop after vector re-administration. The factors that may be involved in this difference include the persistence of antigen (in the case of F.IX, antigen is continuously expressed, whereas capsid is present at high levels initially, but gradually disappears), and the fact that tolerance to a eukaryotic protein with high homology to other circulating proteins (F.VII, F.X) develops more readily than tolerance to a viral capsid protein. Studies of hepatic gene transfer have demonstrated that antigen levels as well as persistence of antigen are critically important for the establishment and maintenance of tolerance.²⁵ Our studies demonstrate that the very low level of hFIX in the circulation of mice injected during fetal life (2×10^{-10} mol/l = 10 ng/ml) ensured that tolerance was maintained, so that the hFIX could be increased to therapeutic levels with re-administration.

The fact that therapeutic levels of hFIX can be established by postnatal AAV vector administration in animals that had previously undergone *in utero* vector injection is an exciting finding. It suggests that even low levels of hFIX are sufficient to induce tolerance to the transgene product, as long as expression is continuous. Furthermore, these studies demonstrate that multiple factors may influence the outcome of gene therapy at early stages of development. Each serotype has characteristics that will impact the success of fetal or neonatal gene transfer; for example, a more detailed characterization of the distribution of the receptors for these viral vectors during early development is needed. The kinetics of transgene expression may also play a role in the ability to maintain tolerance *i.e.*, a minimal level of transgene expression may be required at a defined period of time in early development. Gene transfer approaches during these early stages of development have the potential to circumvent the obstacle of pre-existing immunity to viral vectors, and to induce tolerance to the transgene.

MATERIALS AND METHODS

Animal breeding and procedures. HB male mice (C57Bl6/129) with a large gene deletion²⁶ were allowed to mate with HB carrier females (C57Bl6/129) in order to decrease the risk of bleeding during surgery. Approximately half of the offspring from this mating would be expected to have HB, one quarter would be HB carriers, and one quarter would be wild-type. At 14–15 days gestation, surgery was carried out on the pregnant dams and each fetus was injected with AAV-hFIX as previously described.¹⁴ All experimental protocols were approved by the Institutional Animal Care and Use Committee at the Children's Hospital of Philadelphia and followed guidelines set forth in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Each fetus was injected with 5 μ l of viral suspension (5×10^9 vector genomes (vg)/fetus or 5×10^{12} vg/kg of AAV-1-CMV-hFIX; 2.7×10^{10} vg/fetus or 2.7×10^{13} vg/kg AAV-2-CMV-hFIX). Neonatal controls were injected in the left hind limb at day 2 using 10 μ l of vector (1×10^{10} vg/neonate or 4×10^{12} vg/kg AAV-1-CMV-hFIX; 2.7×10^{10} vg/neonate or 1.8×10^{13} vg/kg AAV-2-CMV-hFIX) and adult controls were injected in the left hind limb at age 8 weeks using 75 μ l of vector in 5% sorbitol (dose of 1×10^{11} vg/mouse or 4×10^{12} vg/kg AAV-1-CMV-hFIX; 3×10^{11} vg/mouse or 1.2×10^{13} vg/kg AAV-2-CMV-hFIX). Animals were re-injected with the same serotype of AAV-hFIX in the right leg at 9 weeks (AAV-1-human skeletal actin/C512-hFIX) (2×10^{10} vg/mouse or 8×10^{11} vg/kg).²⁷ Each injection or re-injection was at a single site. Previous studies have demonstrated that, in adult mice, the levels of hFIX after IM administration of AAV-1-HSA/C512-hFIX are similar to the levels after administration of AAV-1-CMV-hFIX.²⁸ The experimental design is illustrated in **Figure 1**. Statistical analysis of survival was performed using the Fisher exact test.

AAV vector construction and production. The vectors were produced by triple transfection of HEK-293 cells in a helper-virus-free system and purified by CsCl gradient centrifugations as previously described.^{4,20,29}

Analysis of hFIX protein and anti-hFIX and anti-AAV antibodies. Blood was obtained by retro-orbital puncture beginning at 4 weeks after injection in all animals to determine hFIX levels and hFIX antibodies by ELISA. Levels of hFIX in the plasma were measured by ELISA as previously described.³⁰ In some animals a detectable signal was observed, which was below the standard curve; these animals were recorded as <9 ng/ml and a distinction was made between this low level and zero. We chose to distinguish between 0 and <9 ng/ml since this allows analysis of data to account for even very low levels of antigen. In order to detect mouse immunoglobulin specific to hFIX, ELISA was performed as previously described using mouse immunoglobulin standard (Sigma-Aldrich, St. Louis, MO) and an antibody specific for mouse immunoglobulin G (Chemicon, Temecula, CA).^{7,22} Activated partial thromboplastin time was used in order to determine the clotting activity of hFIX, as previously described.³⁰ Anti-AAV capsid immunoglobulin G antibodies were detected with the same ELISA assay described for the anti-hFIX antibodies; however, the ELISA plate was coated with 1×10^9 capsid particles/well of AAV-2 empty capsid or AAV-1 empty capsid.

Biodistribution analysis. Mice were administered vector *in utero* as described in "Animal Breeding and Procedures" and the mice were sacrificed at day 2 of life (1 week after vector injection) and at 4 weeks of age (5 weeks after vector injection) for tissue harvesting. Tissues were snap frozen in liquid nitrogen. DNA was isolated from all tissues using the QIAmp DNA Mini Kit (Qiagen, Valencia, CA), except for muscle and heart tissues that were isolated using the Puregene DNA Isolation Kit (Gentra Systems, Minneapolis, MN). Real time polymerase chain reaction was performed with 200 ng of genomic DNA with primers and probe specific to the F.IX (F9) gene. The primers were as follows: forward primer, 5' TTCGATCTCAAAAGTTCACCATCTATAAC 3'; reverse primer, 5' AAACCTGGTCCCTTCCACTTCAG 3'; and probe 6FAM-5' AATCTC TACCTCCTCATGGAAGCCAGCA 3'-TAMRA (Applied Biosystems,

Foster City, CA). The real time polymerase chain reaction assay was performed in triplicate and the standard was AAV-h.FIX linearized plasmid. The gene copy number was determined as follows: since 200 ng of DNA was used in each reaction and one diploid genome is equivalent to 6 pg of DNA, we estimated that there were 33,333 genomes in the DNA in each reaction. The number of copies detected in the real time polymerase chain reaction was divided by 33,333 to estimate the number of copies per diploid genome.

T cell proliferation and cytokine release assay. Mice were challenged with plasma-derived human F.IX (Mononine; Aventis Behring, Kankakee, IL) in cFA (5 μ g per mouse) (Sigma-Aldrich, St. Louis, MO) (200 μ l volume of 1:2 cFA and phosphate-buffered saline; subcutaneous injections). After 1 month the mice received a booster with 2 μ g hFIX (Mononine) in incomplete Freund's adjuvant (Sigma-Aldrich, St. Louis, MO). Three days later the splenocytes were harvested and placed in culture for a T cell proliferation assay and a cytokine release assay as previously described.^{22,25} Statistics were performed using the Student *t*-test.

Immunofluorescence staining. At the time of sacrificing the mice, muscle tissue was frozen in 2-methylbutane and transferred to liquid nitrogen. Cryosections (5 μ m) were prepared and stained for hFIX as previously described.⁴ Stained sections were viewed with an Eclipse E800 microscope (Nikon, Tokyo, Japan) using a Plan Apo x 20/0.75 objective and epifluorescent light (TRITC HYQ filter). Images were captured with a Cool Snap-Pro camera and analyzed with Image Pro-Plus software (Media Cybernetics, Silver Spring, MD).

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