Induction of immune tolerance to coagulation factor IX antigen by in vivo hepatic gene transfer

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Gene replacement therapy is an attractive approach for treatment of genetic disease, but may be complicated by the risk of a neutralizing immune response to the therapeutic gene product. There are examples of humoral and cellular immune responses against the transgene product as well as absence of such responses, depending on vector design and the underlying mutation in the dysfunctional gene. It has been unclear, however, whether transgene expression can induce tolerance to the therapeutic antigen. Here, we demonstrate induction of immune tolerance to a secreted human coagulation factor IX (hF.IX) antigen by adeno-associated viral gene transfer to the liver. Tolerized mice showed absence of anti-hF.IX and substantially reduced in vitro T cell responses after immunization with hF.IX in adjuvant. Tolerance induction was antigen specific, affected a broad range of Th cell subsets, and was favored by higher levels of transgene expression as determined by promoter strength, vector dose, and mouse strain. Hepatocyte-derived hF.IX expression induced regulatory $CD4^+$ T cells that can suppress anti-hF.IX formation after adoptive transfer. With a strain-dependent rate of success, tolerance to murine F.IX was induced in mice with a large F.IX gene deletion, supporting the relevance of these data for treatment of hemophilia B and other genetic diseases.

J. Clin. Invest. **111**:1347–1356 (2003). doi:10.1172/JCI200316887.

Introduction

Treatment of genetic disease by gene replacement therapy is complicated by the risk of an immune response against the therapeutic gene product. Depending on the underlying mutation in the defective gene and other genetic factors, the immune system of the recipient may not be tolerant to the functional protein antigen expressed by the donated gene. This issue is of particular concern in gene therapy for X-linked hemophilia, a bleeding disorder caused by absence of functional blood coagulation factor IX (F.IX, hemophilia B) or factor VIII (F.VIII, hemophilia A). Formation of inhibitory Ab's against the infused coagulation factor currently represents the most serious complication of treatment by conventional protein replacement therapy and occurs in 3–4% of hemophilia B patients (1). The F.IX genotype of individuals with inhibitor formation is typically characterized by extensive loss of coding information (2). Therefore, tolerance to wild-type F.IX antigen may not have been established due to lack of display of F.IX-derived T cell epitopes during development of the immune system.

Several laboratories have established gene therapy protocols based on muscle- or liver-directed F.IX gene transfer with adeno-associated viral (AAV) vectors (3–6). AAV serotype-2 vectors are derived from a replication-deficient, nonpathogenic DNA virus with a 4.7-kb single-stranded genome. The vector is produced in a helper virus-free system, does not contain viral coding sequences, and is capable of stable in vivo gene transfer (7). After successful scale-up to a large-animal model of the disease, hemophilia B dogs that lack circulating antigen because of a F.IX missense mutation, phase I clinical trials on the safety of intramuscular (IM) or hepatic artery infusion of AAV-F.IX vectors to adults with severe hemophilia B were initiated (4, 5, 8, 9).

IM administration of an AAV-2 vector resulted in sustained systemic expression of canine F.IX (cF.IX) in the missense mutation dogs (4). In contrast, identical vector doses injected into hemophilia B dogs with a F.IX null mutation (an early stop codon associated with unstable F.IX mRNA) caused induction of persistent, high-titer inhibitory anti-cF.IX (10). A similar immune response was observed in hemophilia B mice with a large F.IX gene deletion after IM injection of an AAV-murine F.IX (AAV-mF.IX) vector and in the missense mutation dogs at high vector doses per site of injection (11, 12). Consequently, enrollment in the muscle-

Received for publication September 11, 2002, and accepted in revised form February 26, 2003.

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Conflict of interest: The authors have declared that no conflict of interest exists.

Nonstandard abbreviations used: adeno-associated viral (AAV); intramuscular (IM); canine F.IX (cF.IX); murine F.IX (mF.IX); Freund's adjuvant (FA); incomplete FA (IFA); activated partial thromboplastin time (aPTT); Bethesda unit (BU); magnetic cell sorting (MACS); vector genome (vg); T regulatory 1 (Tr1); liver sinusoidal endothelial cells (LSECs).

directed gene transfer trial was limited to subjects with F.IX missense mutations (8). These experiments raise the question of whether gene therapy can be successful in a null mutation setting characterized by a lack of tolerance to the therapeutic antigen. While subjects currently enrolled in clinical trials are carefully selected based on their history with protein infusion, this question will need to be addressed before gene therapy can be extended (e.g., to previously untreated patients). Ideally, transgene expression itself would induce tolerance to the transgene product.

In previous experiments, sustained correction of murine hemophilia was documented for liver-directed gene transfer using vectors in mice bred on a C57BL/6 genetic background (5, 6, 13, 14). Implications from these experiments for inhibitor formation had been unclear, because results were likely influenced by the particular strain background (15). Other experiments showed that Ab formation against a secreted transgene product may be avoided by hepatic gene transfer or by restricting transgene expression to hepatocytes (16–18). Moreover, we have shown sustained therapeutic levels of cF.IX expression in two of three dogs with a F.IX null mutation that received hepatic gene transfer, while one animal (that additionally had auto-Ab's and liver pathology secondary to pyruvate kinase deficiency) developed inhibitory anti-F.IX by week 5 (19). In contrast, two of two dogs of this colony developed inhibitors within 2 weeks after IM injection of vector (10).

Here, we demonstrate that the greatly reduced risk of anti-F.IX formation following hepatic gene transfer with an AAV vector is the result of F.IX-specific induction of immune tolerance by this route of administration. This result has broad implications for gene therapy of systemic protein deficiencies and points out a role for in vivo gene transfer in tolerance induction.

Methods

Viral vectors. AAV-EF1α-hF.IX vector for expression of the hF.IX cDNA from the human elongation factor-1α enhancer/promoter (including the first intron of the human EF1α gene) was as described (20). To construct AAV-EF1α-mF.IX, the hF.IX cDNA in AAV-EF1α-hF.IX was replaced with a *Eco*RI-*Hind*III fragment containing the coding sequence of the mF.IX cDNA (11). Vector AAV-ApoE/hAAT-mF.IX was constructed by replacing the CMV promoter from the published AAV-CMV-mF.IX construct with the ApoE/hAAT enhancer promoter (as a 1.1-kb *Mlu*I-*Sac*II fragment) from pAAV-ApoE$_4$/hAAT-cF.IX plasmid (19). Therefore, AAV-ApoE/hAAT-mF.IX vector contains four copies of the apolipoprotein E gene enhancer upstream of the human α_1-antitrypsin promoter (21), a short chimeric intron, the mF.IX cDNA, and the human growth hormone polyadenylation signal. Vector AAV-ApoE/hAAT-hF.IX contains the expression cassette for hF.IX as designed by Kay and colleagues (22). This cassette includes the ApoE enhancer, hepatocyte control region, hAAT promoter, hF.IX cDNA (including a 1.4-kb portion of intron 1 of the hF.IX gene), and bovine growth hormone polyA signal. All expression cassettes are flanked by AAV-2 inverted terminal repeats. AAV vector (serotype 2) was produced by triple transfection of HEK-293 cells, purified by CsCl-gradient centrifugation, and quantitated by dot blot hybridization as described (23). AAV-GFP vector was kindly provided by Avigen Inc. (Alameda, California, USA).

Mouse strains and experiments. Hemostatically normal C57BL/6, BALB/c, C3H, and CD-1 mice, as well as γδ-T cell receptor–deficient, CD8$^+$ T cell–deficient, IL-4–deficient, and Fas-deficient mice (all on C57BL/6 genetic background) were purchased from The Jackson Laboratory, Bar Harbor, Maine, USA. AAV vector (25 μl per injection) was delivered into the portal vein by splenic capsule injection with a Hamilton syringe following a ventral midline incision, as described (20, 24). Hemophilia B mice without endogenous F.IX expression (due to a targeted deletion of the promoter and the first three exons of the F.IX gene; ref. 25) received pooled normal mouse plasma (200 μl) by tail vein injection less than 30 min before and after surgery. These mice were generated by repeated breeding of F.IX knockout mice with BALB/c, C3H, or CD-1 mice (at least ten generations to obtain pure genetic backgrounds). Hemophilia B mice were identified by PCR-based genotyping as described (11). Immunizations were done by subcutaneous injection on the back using 2–5 μg human F.IX or F.X (or 10 μg of mF.IX in hemophilia B mice) protein formulated in CFA or incomplete Freund's adjuvant (IFA; Life Technologies Inc., Rockville, Massachusetts, USA). Normal mice were bled from the retro-orbital plexus using heparinized capillary tubes, while blood from hemophilia B mice was collected in 0.38% sodium citrate buffer during the bleed from the tail vein (14).

F.IX and Ab assays. Plasma levels of hF.IX antigen were measured by hF.IX-specific ELISA (14). Coagulation of plasma samples obtained from hemophilia B mice was determined by measurement of activated partial thromboplastin time (aPTT) using a fibrometer (11). Murine F.IX plasma concentrations were determined by ELISA using purified mF.IX protein as standard (11). Briefly, micotiter plates were coated with affinity-purified rabbit anti-mF.IX (1:50 dilution; Zymed Laboratories Inc., South San Francisco, California, USA), blocked with non-fat dry milk in 1× PBS/0.05% Tween-20, and mF.IX was detected with rabbit anti-mF.IX (1:2,000 dilution) chemically linked to HRP. Using these reagents, we determined the concentration of normal mouse plasma (C57BL/6-derived) to be 2,500 ng/ml, half the normal concentration of hF.IX in human plasma. Immuno-capture assay for determination and quantitation of anti-hF.IX or anti-mF.IX subclasses (IgG-1, IgG-2a, IgG-2b, IgG-3, IgA, and IgE) was performed as published using Ab's from Roche Molecular Biochemicals (Indianapolis, Indiana, USA) and immunoglobulin standards from Sigma-Aldrich (St. Louis, Missouri, USA) (11, 26). Bethesda assay for measurement of inhibitory anti-mF.IX was as described (11). One Bethesda unit (BU) is

equivalent to 50% of residual F.IX activity in this plasma-based coagulation assay.

T cell proliferation assay. Mice that had received two subcutaneous injections with 2 μg of hF.IX in Freund's adjuvant (FA) (1–1.5 months apart, with the first challenge in CFA, the second in IFA) were sacrificed 5 days after the second challenge, and lymphocytes extracted from spleens, and portal and inguinal lymph nodes were isolated as described (26) and pooled for in vitro T cell assays (three to five animals per experimental cohort). Lymphocytes were cultured in 2-MLC medium (DMEM, 2% heat-inactivated FCS, 1 mM sodium pyruvate, 10 mM HEPES, 0.1 mM nonessential amino acids, 10^{-6} M 2-mercaptoethanol, and antibiotics) for 5 days in the absence (mock) or presence of hF.IX antigen (10 μg/ml). Source of hF.IX protein for FA immunization and in vitro restimulation was high-purity plasma-derived hF.IX (Mononine; Armour Pharmaceutical Co., Kankakee, Illinois, USA). Lymphocyte proliferation was measured by scintillation count of ^{3}H-thymidine incorporation in hF.IX versus mock-stimulated cells (48 hours of in vitro restimulation followed by 8 hours of thymidine pulse) (12, 15). Proliferation in response to in vitro restimulation with hF.IX antigen was also determined for lymphocytes cultured in the presence of 10, 50, or 100 U/ml of murine IL-2 (PharMingen, San Diego, California, USA).

Adoptive T cell transfer. C57BL/6 mice (naive or 1.5 months after hepatic gene transfer with AAV vector; n = 4–5 animals per cohort) were sacrificed and splenocytes isolated (26). Total splenocytes were pooled and injected in the tail vein (5×10^7 cells in PBS/recipient naive C57BL/6 mouse, n = 4–5 per experimental group). Recipient mice were subcutaneously injected with hF.IX in CFA as described above 24 hours after adoptive splenocyte transfer. Anti-hF.IX formation was measured 14 days after immunization. Alternatively, CD4$^+$ T cells were purified from pooled splenocytes by magnetic cell sorting (MACS) using a column for positive selection with anti-murine CD4 (Miltenyi Biotech, Auburn, California, USA), according to manufacturer's instructions. FACS analysis showed more than 75% purity of CD4$^+$ T cells with less than 1% CD8$^+$ T cells and less than 3% B cells. Splenocytes depleted for CD4$^+$ T cells contained approximately 6% CD8$^+$ T cells and less than 1% CD4$^+$ T cells. CD4$^+$ T cells (10^7/recipient mouse) or CD4$^+$ T cell–depleted splenocytes (5×10^7/recipient) were adoptively transferred to naive C57BL/6 mice followed by hF.IX/cFA challenge 24 hours later as outlined above.

Results

Given the limited number of animals and reagents available to conduct immunological studies in large-animal models such as hemophilia B dogs, we designed experiments in mice that would allow us to investigate reduced incidence of inhibitor formation following hepatic gene transfer.

Sustained systemic expression of hF.IX in immunocompetent mice by hepatic gene transfer. Vectors AAV-EF1α-hF.IX and AAV-ApoE/hAAT-hF.IX were produced for expression of hF.IX from the ubiquitous EF1α promoter or a hepatocyte-specific ApoE enhancer/human α_1-anti–trypsin promoter combination. These vectors were infused into the portal circulation via the spleen for efficient gene transfer to the liver. Recipients of gene transfer were male immunocompetent mice of three different inbred strains with defined MHC haplotypes: C57BL/6 (H-2^b), BALB/c (H-2^d), and C3H (H-2^k). Following injection of AAV-EF1α-hF.IX (10^{11} vector genomes [vg's] per animal), C57BL/6 and BALB/c mice showed sustained systemic expression of hF.IX at therapeutic levels (Figure 1, a and b). C3H mice transiently expressed low levels of hF.IX (< 15 ng/ml, 6 weeks), but developed anti-hF.IX (mostly IgG-1) starting 9 weeks after injection (Figure 1, c and g). No anti-hF.IX was measured in BALB/c mice (Figure 1f).

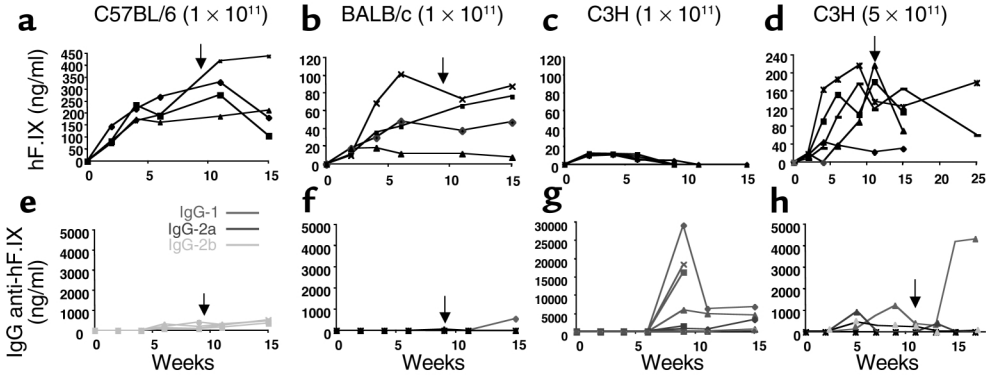

Figure 1

Plasma levels of hF.IX and anti-hF.IX (measured by ELISA or immuno-capture assay) in immune-competent mice as a function of time after liver-directed vector administration (AAV-EF1α-hF.IX vector). (**a**–**d**) hF.IX in ng/ml. (**e**–**h**) IgG anti-hF.IX in ng/ml. Red lines: IgG-1; blue lines IgG-2a; green lines: IgG-2b. (**a** and **e**) C57BL/6 mice (n = 4, 1×10^{11} vg/animal). (**b** and **f**) BALB/c mice (n = 4, 1×10^{11} vg/animal). (**c** and **g**) C3H mice (n = 4, 1×10^{11} vg/animal). (**d** and **h**) C3H mice (n = 5, 5×10^{11} vg/animal). Each line represents an individual animal. Symbols are identical for hF.IX and anti-hF.IX levels of the same animal (note that only one animal in **f** had an anti-hF.IX response). Vertical arrows indicate challenge by subcutaneous administration of 2 μg hF.IX formulated in CFA.

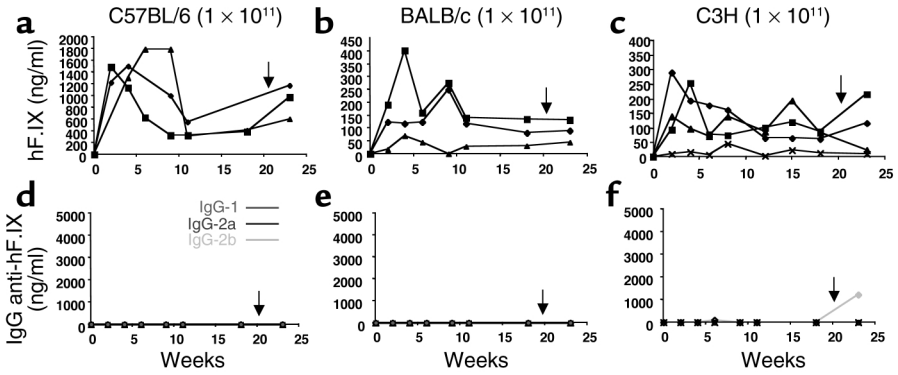

Figure 2
Plasma levels of hF.IX and anti-hF.IX (measured by ELISA or immuno-capture assay) in immunocompetent mice as a function of time after liver-directed vector administration (AAV-ApoE/hAAT-hF.IX vector, 10^{11} vg/animal). (**a**–**c**) hF.IX (ng/ml). (**d**–**f**) IgG anti-hF.IX (ng/ml). Red lines: IgG1; blue lines: IgG-2a; green lines: IgG-2b. (**a** and **d**) C57BL/6 mice (n = 3). (**b** and **e**) BALB/c mice (n = 3). (**c** and **f**) C3H mice (n = 4). Each line represents an individual animal. Symbols are identical for hF.IX and anti-hF.IX levels of the same animal. Vertical arrows indicate challenge by subcutaneous administration of 2 μg hF.IX formulated in CFA.

C57BL/6 developed low-titer, non-neutralizing IgG-2b at late time points. In other experiments with the AAV-EF1α-hF.IX vector, we have also observed a late IgG-2b response in BALB/c mice (data not shown). After injection of 5×10^{11} vg, C3H mice also showed sustained expression (Figure 1d), either without anti-hF.IX (four of five) or a mixed IgG response that was not neutralizing to transgene expression (one of five; Figure 1, e and h). Following gene transfer with the AAV-ApoE/hAAT-hF.IX vector, all mice displayed sustained expression without any detectable anti-hF.IX formation for at least 20 weeks after gene transfer (Figure 2). Levels of expression were substantially higher with this vector compared with the EF1α promoter. For both vectors, C57BL/6 gave the highest expression levels followed by BALB/c, then by C3H mice. None of the injected mice had circulating IgA, IgE, or IgG3 anti-hF.IX (data not shown).

Sustained expression is associated with induction of immune tolerance. Since mice of different strains showed sustained expression of the hF.IX antigen and failed to mount a neutralizing anti-hF.IX response following hepatic gene transfer, we sought to investigate the nature of this immunological unresponsiveness to the transgene product. Immune-competent mice are not tolerant to the non–species-specific hF.IX antigen (despite approximately 80% homology with mF.IX) (18, 26). Unresponsiveness of the immune system may be the result of ignorance (e.g., due to lack of efficient antigen-derived peptide presentation following this route of administration). If the murine immune system was simply ignoring the hF.IX antigen, an immune response should occur given the proper immunological challenge. Alternatively, transgene expression may have induced immune tolerance. After challenge by subcutaneous injection of hF.IX (2 μg) in CFA, mice treated previously with hepatic gene transfer continued to express hF.IX without anti-hF.IX formation (Figures 1–3), while naive mice or mice injected with an AAV-GFP vector developed anti-hF.IX within 14 days after immunization. These mice developed IgG-1 anti-hF.IX, with some animals additionally synthesizing IgG-2a

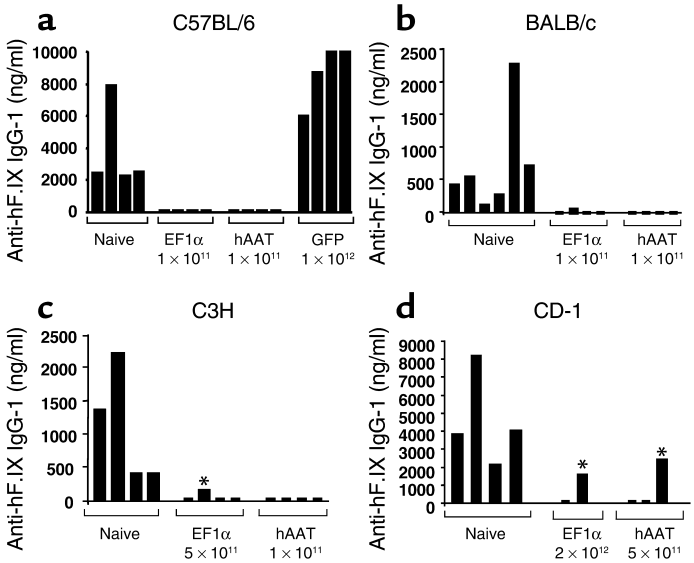

Figure 3
Plasma levels of IgG1 anti-hF.IX on day 14 after immunological challenge by subcutaneous administration of 2 μg hF.IX formulated in CFA. Mice were naive or had received hepatic gene transfer with AAV-EF1α-hF.IX (EF1α), AAV-ApoE/hAAT-hF.IX (hAAT), or AAV-GFP (GFP) vector 2–3 months earlier at the indicated vector doses. Each bar represents an individual animal. (**a**) C57BL/6, (**b**) BALB/c, (**c**) C3H, (**d**) CD-1 mice. Note that (in contrast to naive mice) none of the vector-treated animals had detectable IgGa after challenge (not shown). *IgG-1 anti-hF.IX levels are shown as increase over levels before CFA challenge for those mice that had IgG-1 anti-hF.IX after gene transfer.

Table 1
Outcome of liver-directed hF.IX gene transfer in CD-1 mice as a function of promoter and vector dose

		Before hF.IX/cFA challenge		After hF.IX/cFA challenge	
Vector	Dose (vg/animal)	Sustained systemic hF.IX expression	Anti-hF.IX formation	Sustained systemic hF.IX expression	Anti-hF.IX formation
EF1α	1×10^{11}	0 ng/ml (4/4)	Predominantly IgG-2a (1–27 μg/ml)	ND	ND
	4×10^{11}	0 ng/ml (3/4) or 30 ng/ml (1/4)	Predominantly IgG-2a (1/4), or IgG-1 (2/4), or none (1/4)	ND	ND
	2×10^{12}	30–200 ng/ml (4/4)	None (3/4) or IgG-1 (1/4; 1 μg/ml)	4/4	None (3/4) or IgG-1 (1/4; 2 μg/ml)
hAAT	2×10^{10}	0 ng/ml (4/4)	IgG-1 (4/4; 1–28 μg/ml)	ND	ND
	1×10^{11}	10–20 ng/ml (4/4)	None (4/4)	10–20 ng/ml (4/4)	IgG-1 and IgG-2a (3/4; 0.5–3 μg/ml)
	5×10^{11}	50–500 ng/ml (4/4)	None (3/4) or IgG-1 (1/4; 2 μg/ml)	4/4	None (3/4) or IgG-1 (1/4; 5 μg/ml)

Vectors were AAV-EF1α-hF.IX (EF1α) or AAV-ApoE/hAAT-hF.IX (hAAT). Animals with sustained hF.IX expression received immunological challenge by subcutaneous injection of hF.IX in CFA 1.5 months after vector administration. Shown are range of expression levels and anti-hF.IX levels, immunoglobulin subclass of anti-hF.IX, and number of animals for each cohort showing expression or anti-hF.IX formation before and after challenge. ND, not done (challenge was not administered).

and IgG-2b (Figure 3 and data not shown). A limited number of mice that failed to produce anti-F.IX after the first adjuvant challenge (n = 2 per strain, n = 6 for C57BL/6 mice) were followed for several weeks after the second adjuvant boost and also did not show anti-hF.IX at these later time points (data not shown). Since mice lacked immune responses after stringent immunological challenge, unresponsiveness cannot be explained by ignorance. Thus, hepatic gene transfer does not simply avoid immune responses, but induces tolerance to the hF.IX transgene product.

Higher levels of transgene expression favor tolerance. When we performed hepatic gene transfer in the outbred CD-1 strain, three of four mice did not have detectable hF.IX plasma levels 2 weeks after injection of 10^{11} AAV-EF1α-hF.IX, and four of the mice developed anti-hF.IX by week 4 (Table 1). Ab subclass analyses revealed primarily IgG-2a production (indicating a primarily Th1-driven response) and, additionally, IgG-1 and IgG-2b anti-hF.IX. Based on results obtained with C3H mice (see above), we hypothesized that Ab formation could be avoided by an increase in vector dose. Subsequent injection of CD-1 mice with increasing vector doses (4×10^{11} vg/animal or 2×10^{12} vg/animal) confirmed this hypothesis. The mid-dose cohort showed mixed results with mice showing IgG-1 or IgG-2a anti-hF.IX or sustained expression without anti-hF.IX (Table 1). In the high-dose cohort, sustained expression was achieved in four of four mice (three of four animals without anti-hF.IX, one of four animals with non-neutralizing IgG-1; Table 1). Injection of the more powerful AAV-ApoE/hAAT-hF.IX vector gave sustained subtherapeutic levels of expression (10–20 ng/ml; lower than in the three strains described above) at a dose of 10^{11} vg/animal, while anti-hF.IX formation without detectable expression was observed at lower vector doses (Table 1). Therapeutic levels of expression were measured in the high-dose cohort (5×10^{11} vg/animal, again three to four animals without anti-F.IX, one of four animals with non-neutralizing IgG-1; Table 1). Therefore, levels of expression as determined by vector dose and promoter strength mainly determined incidence of Ab formation. Mice treated with 10^{11} AAV-ApoE/hAAT-hF.IX produced low-titer anti-hF.IX after immunological challenge with hF.IX in CFA, whereas mice in the high-dose cohorts of either vector generally did not produce anti-hF.IX after challenge (see Figure 3d and Table 1). Those mice with IgG-1 anti-hF.IX before CFA injection, one in four in each high-dose cohort, continued to express hF.IX, but showed an increase in Ab titer (Figure 3d).

Tolerance induction is antigen specific. To test antigen specificity of tolerance induction, we challenged C57BL/6 mice that had received AAV-hF.IX vector by subcutaneous injection of the closely related serine protease hF.X formulated in CFA. These mice formed anti-

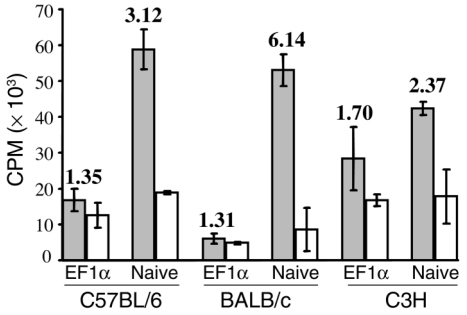

Figure 4
Lymphocyte proliferation following in vitro restimulation with hF.IX protein. Naive or AAV-EF1α-hF.IX–treated mice (portal infusion of 10^{11} vg/animal for C57BL/6 and BALB/c mice and 5×10^{11} vg/animal for C3H mice) were boosted twice with hF.IX formulated in adjuvant (1.5 months after gene transfer for vector-treated mice for the first challenge with hF.IX/cFA and 1 month later with hF.IX/iFA) and sacrificed on day 5 after the second boost (animals were identical to those used in Figure 1). Total pooled splenocytes and inguinal lymph node cells (n = 3/strain) were cultured in the presence or absence (mock, white bars) of hF.IX antigen (10 μg/ml media, gray bars) for 5 days prior to pulse with ^{3}T-thymidine. ^{3}T-thymidine incorporation was measured by scintillation counting. All lymphocyte cultures were set up in quadruplicate. Average counts per minute ± 90% confidence interval are shown. Numbers above bars are stimulation indexes (ratio of counts per minute for antigen versus mock-stimulated cells).

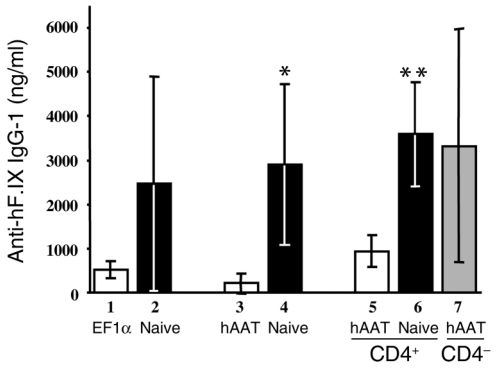

Figure 5
Plasma levels of IgG-1 anti-hF.IX on day 14 after immunological challenge by subcutaneous administration of 2 μg hF.IX formulated in CFA in C57BL/6 mice that had received adoptive transfer of splenocytes from naive or vector-treated C57BL/6 mice. Each bar is average Ab titer for four to five animals ± 90% confidence interval. Adoptive transfer was by tail vein injection 24 h before challenge. Vector-treated mice had received hepatic gene transfer with 10^{11} vg/animal of AAV-EF1α-hF.IX (EF1α) or AAV-ApoE/hAAT-hF.IX (hAAT) vector 1.5 months before the experiment. Total splenocytes (5×10^7, bars 1–4), $CD4^+$ T cell–depleted splenocytes (1.5×10^7, bar 7), or MACS-purified $CD4^+$ T cells (bars 5 and 6) were transferred. *Difference in anti-hF.IX titer between mice that received splenocytes from naive versus AAV-ApoE/hAAT-hF.IX–treated animals was statistically different ($P < 0.01$). **Difference in anti-hF.IX titer between mice that received $CD4^+$ cells from naive versus AAV-ApoE/hAAT-hF.IX–treated animals was statistically different ($P < 0.05$).

hF.X at titers (8.5 ± 3.5 μg IgG-1/ml at day 14 after immunization) similar to naive control mice (10.7 ± 3.3 μg IgG-1/ml, data not shown). Next, we performed subcutaneous injections of a mix of hF.IX and hF.X (5 μg each per mouse) in CFA in C57BL/6 mice that had received hepatic AAV-hF.IX gene transfer. Naive control mice formed high-titer anti-hF.IX (21.5 ± 10.3 μg IgG-1/ml) and anti-hF.X (8.6 ± 4 μg IgG-1/ml) by day 14, as expected, while mice with hepatocyte-derived hF.IX expression formed only high-titer anti-hF.X (6 ± 2.2 μg IgG-1/ml anti-hF.X versus 0.5 ± 0.4 μg IgG-1/ml anti-hF.IX; data not shown), illustrating antigen specificity of tolerance induction.

Unresponsiveness to hF.IX on the T cell level. Anti-F.IX formation in protein therapy, as well as in gene therapy, is a Th cell–dependent process (26, 27). To investigate whether induction of immune tolerance is reflected in $CD4^+$ Th cell responses, we challenged tolerized mice a second time with hF.IX in IFA and sacrificed the animals 5 days later for in vitro restimulation of lymphocytes with hF.IX antigen. As compared with naive mice challenged in parallel, lymphocytes from AAV-EF1α-hF.IX–transduced mice showed no (C57BL/6 and BALB/c) or reduced (C3H) proliferation following in vivo challenge with hF.IX/iFA and in vitro restimulation with hF.IX antigen, whereas the identical experiment resulted in a proliferative response to hF.IX after immunization of naive mice of these strains (Figure 4).

Evidence for $CD4^+$ regulatory T cells. If tolerance induction involves regulatory or suppressor lymphocytes, we should be able to transfer unresponsiveness to the hF.IX antigen from tolerized animals to naive mice of the same strain. To address this question, we adoptively transferred pooled splenocytes from C57BL/6 mice that had received hepatic gene transfer or from naive C57BL/6 mice (controls) to naive C57BL/6 mice (5×10^7 of total splenocytes were injected into the tail vein). Mice were challenged by subcutaneous injection of hF.IX in CFA 24 hours after receiving splenocytes and plasma samples analyzed for anti-hF.IX 2 weeks after the challenge. As compared with controls, mice that had received cells from vector-treated animals produced, on average, four- to eightfold lower IgG-1 anti-hF.IX levels (Figure 5). This result was similar for splenocyte transfer from AAV-EF1α-hF.IX– and AAV-ApoE/hAAT-hF.IX–treated mice. When purified $CD4^+$ T cells were transferred (10^7 cells/animal), an identical result was obtained, whereas $CD4^+$ T cell–depleted splenocytes (5×10^7 cells/animal) failed to transfer unresponsiveness (Figure 5).

Requirements for tolerance induction. To test requirements for tolerance induction by hepatic F.IX gene transfer, we performed injections of AAV-EF1α-hF.IX vector in several knockout strains (C57BL/6 genetic background) deficient in molecules that affect immune function. $CD4^+$ and $CD8^+$ T cells express a T cell receptor composed of α and β subunits. Previous work has shown that in mice deficient in T cells expressing the rarer γδ-T cell receptor it is more difficult to induce oral tolerance to antigens (28). Literature on oral tolerance also describes induction of regulatory $CD8^+$ T cells secreting TGF-β cytokine (29). Both γδ-T cell receptor–deficient mice and $CD8^+$ T cell–deficient mice, however, showed sustained expression of hF.IX without anti-hF.IX formation following hepatic gene transfer (Table 2). Immunological unresponsiveness was upheld after challenge with hF.IX/cFA (Table 2).

Table 2
Liver-directed gene transfer with AAV-EF1α-hF.IX vector in knockout mice deficient in γδ-T cell receptor, $CD8^+$ T cells, IL-4 cytokine expression, or Fas

	Before hF.IX/cFA challenge		After hF.IX/cFA challenge	
Strain	Sustained systemic hF.IX expression	Anti-hF.IX formation	Sustained systemic hF.IX expression	Anti-hF.IX formation
γδ-TCR deficient	100–300 ng/ml	0/4	100–300 ng/ml	0/4
$CD8^+$ T cell deficient	100–300 ng/ml	0/4	100–300 ng/ml	0/4
IL-4 deficient	100–300 ng/ml	0/4	100–300 ng/ml	0/4
Fas deficient	50–200 ng/ml	0/6	0 ng/ml (2/6) or 100–500 ng/ml (4/6)	IgG-1 (0.5–16 μg/ml; 6/6)

Animals received immunological challenge by subcutaneous injection of hF.IX in cFA 1–2 months after vector (2×10^{11} vg/animal) administration. Shown are range of expression and number of animals for each cohort showing expression or anti-hF.IX formation before and after challenge. TCR, T cell receptor.

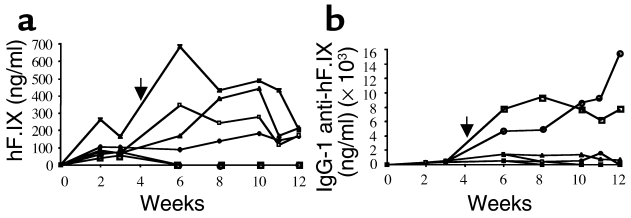

Figure 6
Liver-directed gene transfer with AAV-EF1α-hF.IX vector (2×10^{11} vg/animal) in C57BL/6 mice deficient in cell death receptor Fas ($n = 6$). Shown are plasma levels of hF.IX and anti-hF.IX (measured by ELISA or immuno-capture assay) as a function of time after vector administration. (**a**) Plasma levels of hF.IX (ng/ml). (**b**) IgG1 anti-hF.IX. Vertical arrows indicate challenge by subcutaneous administration of 2 μg hF.IX formulated in CFA. Symbols are identical for hF.IX and anti-hF.IX levels of the same animal.

In previous studies on muscle-directed gene transfer with AAV-F.IX vector, we found a predominantly Th2-driven anti-F.IX response. Since results documented above show a predominant Th1 response in the context of low levels of hF.IX expression in liver-directed gene transfer, one could hypothesize that transduced liver is prone to produce a Th1 response, but at higher expression levels this Th1 response is suppressed by regulatory Th2 cells. To test this interpretation, we transduced IL-4–deficient mice, which cannot produce Th2-dependent Ab's, but form IgG-2a anti-hF.IX after IM injection of vector (26). These mice also showed sustained expression without evidence for anti-hF.IX (Table 2). In particular, no IgG-2a was detected, indicating that tolerance induction cannot be explained by suppression of an imminent Th1 response by Th2 cells.

To evaluate a potential requirement for apoptotic cell death mediated by the Fas-Fas ligand pathway, we performed hepatic gene transfer in Fas-deficient C57BL/6 mice. These mice also did not develop anti-hF.IX during the first month after vector administration (Figure 6b). At this time point, mice were challenged with hF.IX/cFA. Subsequently, Fas-deficient mice produced IgG1 anti-hF.IX (six of six) mice within 2 weeks after challenge. This immune response neutralized expression in only two of six mice, however, while four of six Fas-deficient animals continued to show circulating hF.IX levels (Figure 6a). Mice with a neutralizing response had high-titer anti-hF.IX, while the other four animals developed only low-titer anti-hF.IX (Figure 6b). As described above for other experiments, normal C57BL/6 controls ($n = 4$) continued to express hF.IX without Ab formation when challenged 1 month after vector administration, and Fas-deficient mice not challenged with hF.IX/cFA continued to express hF.IX without anti-hF.IX formation ($n = 6$; data not shown).

Treatment of hemophilia B mice with large F.IX gene deletion. To test tolerance induction to F.IX by hepatic gene transfer in animal models of hemophilia B, we bred F.IX knockout mice onto three different genetic backgrounds, BALB/c, C3H, and CD-1 (sustained expression of F.IX transgenes from different viral vectors following intravenous or portal infusion is already well documented in the literature for C57BL/6 mice) (5, 6, 14). As summarized in Table 3, sustained expression of mF.IX was obtained after hepatic gene transfer in three of five BALB/c mice treated with the AAV-EF1α-mF.IX and in four of four BALB/c mice treated with the AAV-ApoE/hAAT-mF.IX vector (3×10^{11} vg of either vector per mouse for all hemophilic mice injected). In CD-1 mice, expression was achieved in three of five mice injected with AAV-ApoE/hAAT-mF.IX vector, while none of the AAV-EF1α-mF.IX–injected mice showed mF.IX expression in the circulation (zero of five). Those mice that did not express mF.IX had developed inhibitory anti-mF.IX (Table 3). Inhibitory anti-mF.IX included IgG-1 and IgG-2a subclasses (data not shown).

Interestingly, there was one hemophilic BALB/c mouse with transient expression at 1 month after AAV-EF1α-mF.IX gene transfer, followed by inhibitor formation at later time points. This animal synthesized IgA and IgG-2b anti-mF.IX at 1 month, which was not neutralizing to mF.IX expression or partial correction of coagulation (data not shown). Immune deviation toward this Th3-type, TGF-β–dependent response shifted to a Th1 response with neutralizing IgG-2a by

Table 3
Immunity versus tolerance in hemophilia B mice of different strain backgrounds receiving hepatic gene transfer with AAV-EF1α-mF.IX or AAV-ApoE/hAAT-mF.IX vector[A]

		Before challenge			After challenge with mF.IX/cFA		
Strain	Vector	Sustained mF.IX expression	Sustained correction of aPTT	Inhibitor formation	Sustained mF.IX expression	Sustained correction of aPTT	Inhibitor formation
BALB/c	EF1α	3/5 (50–250 ng/ml)	3/5 (42–50 s)	2/5 (2–5 BU)	1/1 (50 ng/ml)	1/1 (56 s)	0/1
	hAAT	4/4 (300–1,000 ng/ml)	4/4 (35–42 s)	0/4	4/4 (400–1,000 ng/ml)	4/4 (30–45 s)	0/4
CD-1	EF1α	0/5	0/5	5/5 (6–10 BU)	–	–	–
	hAAT	3/5 (30–250 ng/ml)	1/5 (45–50 s)	2/5 (2–6 BU)	1/2 (200 ng/ml)	1/2 (50 s)	1/2 (7 BU)
C3H	hAAT	1/5 (500 ng/ml)	1/5 (40 s)	4/5 (5–11 BU)	1/1 (500 ng/ml)	1/1 (40 s)	0/1

Number of mice expressing mF.IX, range of expression (nanograms mF.IX per milliliter plasma), and correction of aPTT coagulation time (in seconds) is given for each cohort of mice. For those mice that had developed inhibitory anti-mF.IX, the range of Ab titer is given in BUs. A total of eight mice with sustained mF.IX expression was chosen for subcutaneous administration of mF.IX (10 μg) in CFA 2–4 months after vector administration. Mouse plasma samples were assayed up to 8 months after vector administration. Range of aPTT for normal mouse plasma was 25–35 s, and > 60 s for untreated hemophilia B mice. [A]3×10^{11} vg/mouse. AAV-EF1α-mF.IX (EF1α); AAV-ApoE/hAAT-mF.IX (hAAT).

month 4 (data not shown), however. In contrast, expression was sustained in all other mice that had mF.IX levels at a 1-month time point.

Some mice with sustained mF.IX expression were challenged by subcutaneous administration of mF.IX in CFA (2–4 months after vector administration) and were assayed 1.5 months later for transgene expression and inhibitor formation. Of eight mice challenged, seven continued to express mF.IX (without evidence for inhibitor formation) at a level identical to that prior to challenge, while the hemophilia B CD-1 mouse developed an inhibitor after challenge (Table 3). This mouse had the lowest level of transgene expression (approximately 30 ng/ml), whereas all mice expressing more than 50 ng/ml did not form inhibitors after challenge. While the success rate of tolerance induction for these vector/strain combinations was as predicted from experiments with hF.IX in hemostatically normal mice (see above; i.e., higher levels of expression such as in BALB/c mice versus CD-1 mice or with the ApoE/hAAT versus the EF1α promoter gave a higher success rate), C3H mice gave a much lower rate of success than predicted (only one in five mice injected with AAV-ApoE/hAAT-mF.IX; Table 3).

Discussion

Several publications provide examples of absence of an immune response to the transgene product by liver-directed or hepatocyte-restricted expression from viral vectors (5, 6, 13, 17–19). If an immune response to the transgene-derived antigen was simply avoided by hepatic gene transfer through ignorance of the immune system (e.g., due to insufficient peptide presentation to T cells), this could have dangerous consequences at later time points given an adequate immunological stimulus, such as tissue damage during an injury or during surgery, infectious disease, etc. Our study provides strong evidence for tolerance induction to the F.IX antigen by AAV-mediated hepatic gene transfer.

Absence of Th cell–dependent responses in tolerant mice. Inhibitor formation is a $CD4^+$ Th cell–dependent process, and our data, specifically the lack of Th cell–dependent immunoglobulin (IgG-1 and IgG-2a, reflecting absence of Th2- and Th1-dependent responses, respectively) and of F.IX-specific T cell proliferation, support the hypothesis that hepatic gene transfer can induce $CD4^+$ T cell tolerance. Theoretically, tolerance may be induced by clonal deletion of F.IX-specific T cells, induction of T cell unresponsiveness (anergy), by induction of regulatory or suppressor cells, or by a combination of these mechanisms. Several sets of data rule out an immune deviation mechanism, that is, induction of an immune response that is inefficient in clearance of the F.IX antigen (e.g., by synthesis of a different immunoglobulin subclass). First, all AAV-ApoE/hAAT-F.IX– and a number of AAV-EF1a-F.IX–transduced animals with sustained F.IX expression had no evidence for circulating anti-F.IX of any immunoglobulin subclass. Second, immune deviation toward IgA/IgG-2b response (as commonly observed in the eye or gut) (30, 31) was seen in only one mouse and was transient. Furthermore, naive CD-1 mice, following a second challenge with hF.IX in adjuvant, produced IgA anti-hF.IX in addition to IgG subclasses (three of four mice, data not shown). CD-1 mice treated with the highest vector doses shown in Table 1 did not produce this immunoglobulin subclass, suggesting that there was also a decrease in the potential for Th3-mediated Ab formation (data not shown). In summary, tolerant mice displayed a broad and sustained reduction in the potential for Th cell responses to hF.IX.

Induction of regulatory $CD4^+$ T cells by liver-directed F.IX gene transfer. While studies in knockout mice and adoptive T cell transfer do not support involvement of regulatory $CD8^+$ or γδ T cells, we have demonstrated that hepatic gene transfer induces regulatory $CD4^+$ T cells that can suppress anti-F.IX formation after adoptive transfer to non–vector-treated mice. Candidates for such regulatory cells include T regulatory 1 (Tr1) cells (which produce high levels of IL-10, but no IL-4), regulatory Th2 cells, and regulatory Th3 cells (secreting high levels of TGF-β) (31, 32). Our previous results on adenoviral F.IX gene transfer (indicating that tolerance induction was independent of IL-10 expression) may argue against involvement of Tr1 cells. (15). Increased potential for induction of Th2 responses should raise the risk of an anti-F.IX response. TGF-β–mediated immune suppression is likely involved in tolerance induction given the synthesis of IgG-2b anti-F.IX and detection of this cytokine in hF.IX-stimulated lymphocyte cultures for certain vector/strain combinations (Figure 1 and unpublished results). TGF-β is known to suppress lymphocyte proliferation as well as activation of dendritic cells and thus may prevent T cell activation or clonal expansion (31, 33). We do not know at this point whether TGF-β in hepatic tolerance is derived from regulatory Th3 cells or a different cell type. Furthermore, suppression may not only be mediated through cytokine secretion, but also other mechanisms. Knolle and colleagues have shown that $CD4^+$ T cells primed by liver sinusoidal endothelial cells (LSECs), in contrast to priming by dendritic cells, fail to become Th cells and display a phenotype similar to Th0 cells (34). The authors speculate that such cells by default become regulatory T cells. Further studies will be required to identify the regulatory subset of $CD4^+$ T cells in hepatic gene transfer. As illustrated in Figure 5, adoptive lymphocyte transfer did not provide complete protection from Th2-mediated anti-F.IX formation. This result may reflect that recipients of T cell transfer did not have circulating hF.IX antigen, which may be required for full suppressor cell activity or may indicate that T cell–mediated suppression is not the only mechanism involved in tolerance.

Potential role for apoptotic cell death in tolerance. To assess the possibility that unresponsiveness of Th cells in hepatic tolerized mice to hF.IX was due to T cell anergy, we attempted to induce cytokine release and prolif-

eration by addition of murine IL-2 to lymphocyte media (see Methods, experiment performed with C57BL/6 and BALB/c mice). Unresponsiveness could not be reverted by in vitro incubation with IL-2 (data not shown), however. While our study showed no evidence for T cell anergy, experiments in Fas-deficient mice suggest a role for Fas-FasL–mediated cell death in establishment of a level of tolerance that cannot be broken subsequently by a strong immunogenic challenge, such as in the presence of adjuvant. Deletion of antigen-specific T cells through the Fas-FasL pathway may be the result of interaction with professional APCs or may be induced by activity of regulatory cells.

Role of expression levels and immunogenicity of the F.IX antigen on success rate for tolerance induction. Experiments in CD-1 mice, in which we controlled levels of transgene expression by adjustment of vector doses to compensate for differences in promoter strength in our two expression cassettes, demonstrate that higher levels of F.IX expression promote a shift from Th1-driven Ab formation (IgG-2a production) to Th2 responses (IgG-1 production) or tolerance, with tolerance being the most likely outcome at high expression levels. This is in contrast to muscle-directed gene transfer, where increased vector doses per injection site promoted Ab formation (12). Moreover, therapeutic levels of expression (>50 ng/ml, 1% of normal F.IX levels in humans) were sufficient for tolerance induction. A comparison between data from hF.IX gene transfer in the four mouse strains tested indicates that success of tolerance induction can be correlated with levels of transgene expression, which are higher in C57BL/6 and BALB/c mice than in C3H and CD-1 mice and higher with the ApoE/hAAT than with the EF1α promoter. Higher levels of expression may favor induction of regulatory cells, while a F.IX antigen containing a strong T cell epitope may increase the risk of T cell activation that may promote an immune response. As expected from experiments in normal mice, success rates of tolerance induction to mF.IX antigen in hemophilia B mice correlated with levels of transgene expression, as determined by promoter strength and strain background (compare results in BALB/c and CD-1 mice and published results with C57BL/6 mice). Success of tolerance induction, however, was generally lower than in normal mice, which can be explained by higher levels of F.IX expression being required in gene deletion mice for tolerance induction or by a tolerogenic effect of endogenous F.IX expression. Furthermore, it is likely that the threshold level of expression required for tolerance induction varies for different antigen and strain/genotype combinations. Experiments in C3H hemophilia B mice show that, although the hepatic route is generally tolerogenic compared with other routes, strain/antigen combinations can be identified that are more likely to result in an immune response.

Implications for gene therapy. It has long been the hope of the gene therapy community that gene transfer of a therapeutic protein could induce tolerance to the antigen by sustained transgene expression (35). Besides reports documenting avoidance of immune responses, there are examples of transient neutralizing Ab responses that were downregulated over a period of months (4, 36). There are, furthermore, attempts to induce tolerance by ex vivo gene transfer to hematopoietic stem cells combined with ablation of bone marrow and T cells, by in utero gene transfer, or by a combination of gene transfer and administration of immunomodulatory drugs (11, 37–39). This study provides, to our knowledge, the first clear and detailed evidence that transgene expression by itself can induce tolerance to the therapeutic protein antigen. Tolerance was achieved by in vivo gene transfer to adult animals of several strains of mice, which represents a clinically feasible and relevant treatment strategy. Relevance of these data is further supported by results in the null mutation dog model (19). Successful tolerance induction was dependent on proper selection of vector and target tissue. The liver has been implicated in oral and in portal venous tolerance (40, 41). Liver transplants are often not rejected even across MHC barriers, and LSECs have been shown to represent specialized APCs that can induce T cell tolerance (40, 42). Results with the ApoE/hAAT construct show that hepatocyte-restricted expression can induce tolerance. Even with a ubiquitous promoter, hepatocytes are the major site of F.IX synthesis following portal infusion of AAV vector, which has a strong tropism for liver and does not appear to transduce other cell types within the liver or APCs (24, 43, 44). It is likely that the microenvironment of the liver (APCs, cytokine milieu, etc.) promotes tolerance rather than immunity to the transgene product (40).

Our results on the effect of expression levels on tolerance induction would explain earlier data on sustained expression of hF.VIII in C57BL/6 mice from an adenoviral vector (conferring superphysiological expression levels) as opposed to neutralizing anti-hF.VIII formation with a low-expressing AAV vector after hepatic gene transfer (45, 46). Because of the potential of adenoviral vectors for innate and adaptive immunity and inflammation, it is uncertain whether this vector system can be adapted as a clinical tool for tolerance induction to a therapeutic protein (15, 47). Induction of tolerance to a therapeutic systemic protein has broad implications for design of clinical trials and gene-based treatment strategies for genetic diseases and is of particular importance in treatment of patients with mutations in the coagulation factor gene that may predispose to increased risk of inhibitor formation.

Acknowledgments

This work was supported by NIH grants R01 AI/HL51390-01 to R.W. Herzog and R01 HL61921 to K.A. High and by a research grant from Hemophilia of Georgia to K.A. High. R.W. Herzog was additionally supported by a Career Development Award from the National Hemophilia Foundation. The authors thank Avigen Inc. for providing pAAV-ApoE/hAAT-hF.IX and pAAV-GFP plasmids.

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