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Lack of Germline Transmission of Vector Sequences Following Systemic Administration of Recombinant AAV-2 Vector in Males

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A potential consequence of systemic administration of viral vectors is the inadvertent introduction of foreign DNA into recipient germ cells. To evaluate the safety of *in vivo* recombinant adeno-associated virus (rAAV) mediated gene transfer approaches for hemophilia B, we explored the risk of germline transmission of vector sequences following intramuscular (IM) injection of rAAV in four species of male animals (mouse, rat, rabbit and dog). In vector biodistribution studies in mice and rats, there is a dose-dependent increase in the likelihood that vector sequences can be detected in gonadal DNA using a sensitive PCR technique. However, in dogs DNA extracted from semen is negative for vector sequences. To address this discrepancy, studies were done in rabbits, and both semen and testicular DNAs were analyzed for the presence of vector sequences. These studies showed that no AAV vector sequences were detected in DNA extracted from rabbit semen samples collected at time points ranging from 7 to 90 days following IM injection of 1×10^{13} vector genomes rAAV (vg) per kg. In contrast, DNA extracted from gonadal tissue was positive for vector sequences, but the positive signals diminished in number and strength with time. By FISH analysis, AAV signals were localized to the testis basement membrane and the interstitial space; no intracellular signal was observed. We observed similar findings following hepatic artery administration of rAAV in rats and dogs, suggesting that our findings are independent of the route of administration of vector. Attempts to transduce isolated murine spermatogonia directly with AAV-lacZ were unsuccessful. In clinical studies human subjects injected IM with an AAV vector at doses up to 2×10^{12} vg/kg have shown no evidence of vector sequences in semen. Together, these studies suggest that rAAV introduced into skeletal muscle or the hepatic artery does not transduce male germ cells efficiently. We conclude that the risk of inadvertent germline transmission of vector sequences following IM or hepatic artery injection of AAV-2 vectors is extremely low.

Key words: germline transmission, AAV vectors, gene therapy, hemophilia

INTRODUCTION

Reproductive toxicology in the setting of gene transfer is a relatively new area of investigation but is a central safety issue for the field [1,2]. The current position of the regulatory agencies is that gene transfer should be somatic only, that is it should not affect the germ cells of the recipient [3]. As *in vivo* gene transfer approaches are extended to larger numbers of patients, including those with reproductive potential, it is important to define the risks of inadvertent transmission of vector sequences into the germline of treated individuals.

The inadvertent integration of vector sequences into germ cells may vary as a function of vector type, route and dose of vector administration, and the identity of the germ

TABLE 1: Reproductive toxicology results in mice, rats, and dogs following IM injection of an AAV vector

Animal	Number of animals	Vector	Vector genome per kg	Time of sample collection	Samples	PCR positive signal/number of animals	Vector copy per μg DNA
Mice	10	AAV-CMV-murine F.IX	1.7×10^{11}	31 days 91 days	gonads gonads	1/5 2/5	10–100 1–10
Mice	8	AAV-CMV-murine F.IX	1.7×10^{12}	31 days 91 days	gonads gonads	3/3 3/5	10–100 10–100 (2/3) > 100 (1/3)
Rat	5	AAV-CMV-human F.IX	2.8×10^{11}	15 days	epididymal effluent	2/5	1–10
Rat	4	AAV-CMV-human F.IX	2.8×10^{13}	15 days	epididymal effluent	3/4	1–10
Dog	4	AAV-CMV-canine F.IX	1.3×10^{11} to 1.1×10^{13}	1.5, 4, 14, 16 months	semen	0/4	not detected

cell (that is, male or female) [4,5]. Spermatocytes can be accessed through the circulatory supply to the testis or by retrograde transport through the prostate gland [4]. Previous biodistribution studies of recombinant adeno-associated virus serotype-2 (rAAV-2) vectors have not specifically addressed biodistribution to gonadal tissues [6].

Before initiating human trials of rAAV-2-mediated approaches for treating hemophilia B, an X-linked disorder, we analyzed risks of transmission of vector sequences to male germ cells. Biodistribution studies in four species and FISH analysis in rabbits suggest that although vector may spread to the gonads by hematogenous dissemination, it does not transduce germ cells. Thus, the risk of germline transmission is very low.

Results and Discussion

We first analyzed risk in the setting of delivery of vector to skeletal muscle following intramuscular (IM) injection of a rAAV vector at doses ranging from 1.7×10^{11} to 2.8×10^{13} vector genomes (vg)/kg. Biodistribution studies in mice and rats showed the presence of AAV vector sequences by PCR in genomic DNA from gonadal tissue; however, no PCR signal was identified in genomic DNA extracted from semen from four hemophilic dogs injected with similar doses of vector (Table 1).

To resolve this discrepancy between data in dog semen and rodent gonadal tissue, we carried out a series of experiments in which both gonadal tissue and semen were analyzed. In these experiments, we injected 12 adult male rabbits with 1×10^{13} vg/kg of a rAAV null vector and 4 animals with saline as negative controls. Injections were carried out under ultrasound visualization to avoid introduction of vector into large vessels in muscle. After semen collection, the animals were sacrificed at 7, 30, 60, and 90 days postinjection and DNA was extracted from semen and from gonadal tissue samples. These time points were chosen based on the duration of spermatogenesis in the rabbit, 46 days [7].

We devised a PCR assay sensitive enough to detect as few as 1–10 copies of AAV vector plasmid per 3×10^5 haploid genomes (Fig. 1A), and carried out a vector

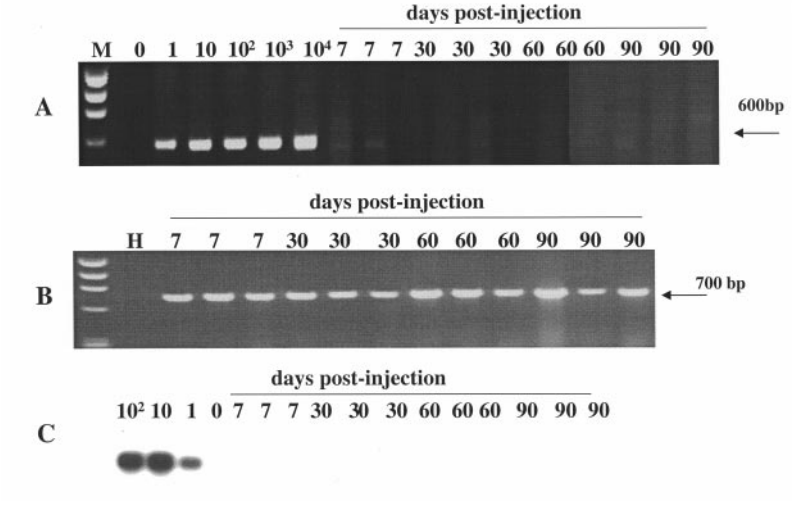

FIG. 1. PCR amplification of rAAV null vector sequences from rabbit semen DNA. (A) PCR analysis for AAV null vector sequences in genomic rabbit DNA isolated from semen collected on days 7, 30, 60, or 90 following IM injection of 1×10^{13} vector genome per kg. Each lane represents a sample from an individual animal. Lane M, size marker. Standard controls consisted of 0 to 1×10^4 copies of AAV null vector mixed with 1 μg genomic rabbit DNA. (B) PCR analysis for a 700bp of rabbit growth hormone using genomic DNA extracted from rabbit semen following IM injection of 1×10^{13} vg/kg of AAV. A PCR product was obtained in all samples, and a "no added DNA" control (H) was negative. (C) Southern blot of PCR products amplified from rabbit semen as outlined in (A). The probe was a 3-kb fragment containing sequences from the β-galactosidase and *neo* genes in the null vector. Positive controls contained 0 to 1×10^2 copies of AAV null vector plasmid mixed with 1 μg genomic rabbit DNA.

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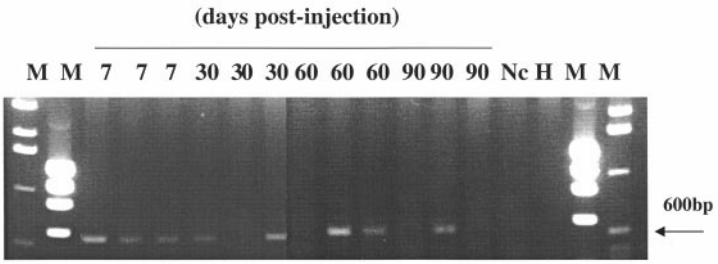

FIG. 2. PCR amplification of rAAV null vector sequences from rabbit gonadal tissue. AAV null vector sequences are detected in genomic rabbit DNA from gonadal tissue. Lane M, size markers; NC, genomic DNA from noninjected rabbit; H, water. Each lane represents a sample from an individual animal; positive signals were detected in 3/3 at day 7, 2/3 at day 30, 2/3 at day 60, and 1/3 at day 90.

biodistribution study. Vector was disseminated into the circulation as detected by positive PCR signals on DNA extracted from serum samples obtained at 15 minutes, 24 hours, and 48 hours postinjection in all animals (data not shown). At 7 days following injection, vector was no longer detectable in any serum samples. The presence of vector in rabbit serum at time points immediately following injection is in clear agreement with data previously obtained in hemophilic dogs and subsequently obtained in a clinical trial using identical ultrasound-guided IM injection of rAAV in human subjects with severe hemophilia B [8]. Vector sequences were also detected in all skeletal muscle samples from rAAV-injected animals, but in none of those injected with saline (data not shown).

A total of 3 μg of DNA extracted from semen samples was analyzed for each animal. The assay detected as little as one copy of vector sequence per microgram of DNA (Fig. 1A). None of the test samples displayed this level of positivity, but faint bands that seemed to migrate to approximately the same position can be seen, for example, in lanes from 7-day time points (Fig. 1A). To further analyze the faint signals, we carried out Southern blot analysis of PCR products. Hybridization to a radiolabeled 3-kb probe confirmed the absence of vector-derived sequences from semen DNA (Fig. 1C). As an additional control, to establish that the extracted DNA was intact, a 700-bp fragment of the rabbit growth hormone gene was amplified from DNA isolated from semen using appropriate primers and the same reagents (Fig. 1B). The results demonstrate the predicted PCR fragment in all semen samples. To exclude the possibility of a specific inhibitory effect of the DNA sample on the vector PCR assay, 1 μg of genomic DNA from each sample was mixed with one copy of null vector plasmid (data not shown). Amplification of the 600-bp fragment was successful for 14 of 16 samples, whereas 2 samples yielded a PCR product when spiked with 10 copies of null vector plasmid.

In DNA obtained from gonadal tissue, vector sequence was detected in 8 of 12 injected animals as follows: 3 of 3 at 7 days, 2 of 3 at 30 days, 2 of 3 at 60 days, and 1 of 3 at 90 days following injection (Fig. 2). In all animals the positive signal strength was equal to or lower than 10

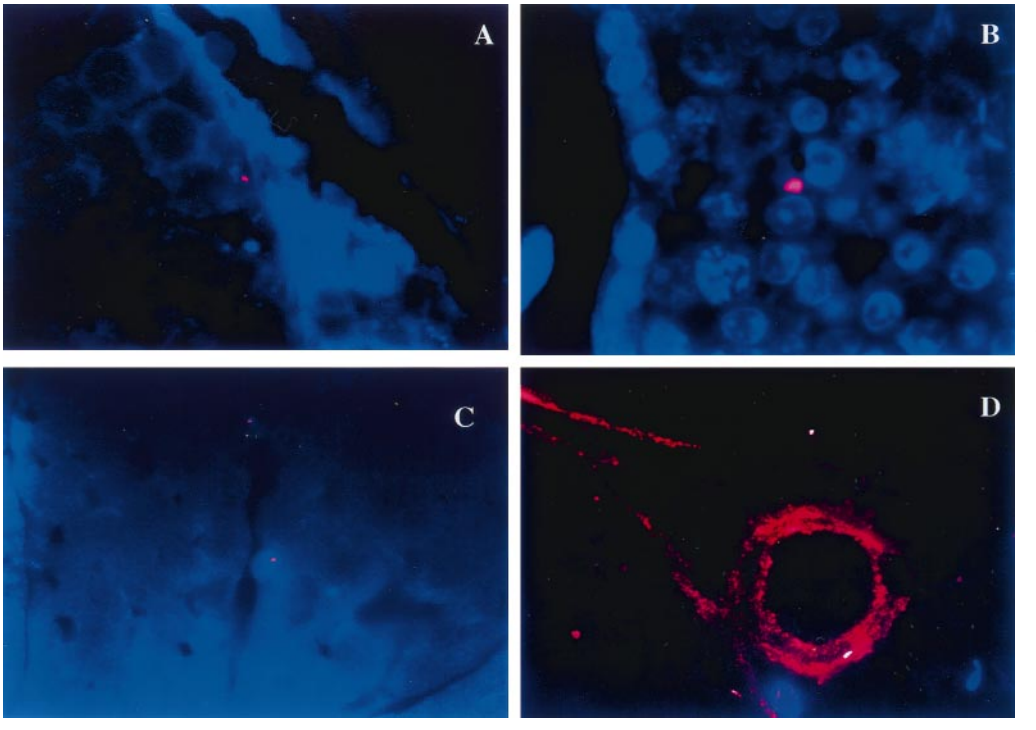

FIG. 3. Fluorescent *in situ* hybridization of AAV-null vector sequences. Testis sections from a rabbit injected with AAV-null vector (1×10^{13} vg/kg) and harvested on day 60 were cryosectioned (5 μm thickness (A) or 3 μm thickness (B)) and processed for FISH. Fluorescent images of sample stained with DAPI (blue) to delineate the nucleus and the hybridization signal (red) showed that the rAAV genome is located near the seminiferous tubule basement membrane (A) or intercellular space (B). (C) Muscle sections from an injected rabbit were harvested on day 30; the hybridization signal is located within the nucleus. (D) FISH signal is demonstrated on the vessel wall of a testis harvested on day 7 following AAV injection. Magnification, ×400.

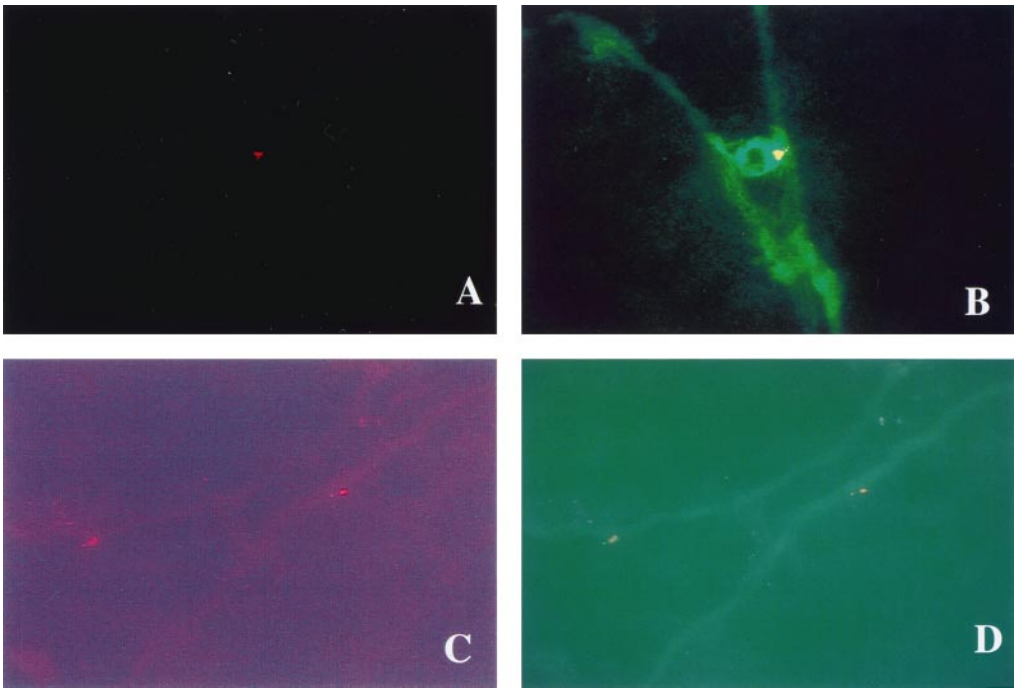

FIG. 4. IF staining for AAV capsid and for heparan sulfate proteoglycan in testis. Testes sections from rabbit injected with AAV-null vector harvested on day 7 were stained with an antibody specific for the intact AAV capsid (red). AAV particles are localized on the vessel wall (A) or basement membrane (C). Images obtained by confocal microscopy from cryosections staining with both anti-heparan sulfate proteoglycan antibody (green) and anti-AAV capsid (red) revealed that AAV colocalized with surface HSPG on the testis vessel wall (B) or basement membrane (D).

copies of AAV vector plasmid per 3×10^5 haploid genomes. No PCR product was detected in noninjected animals. These data suggest that vector may be present in the testis, but it is not present in cells, including germ cells, found in semen.

To localize the anatomic structures giving rise to the positive PCR signals from testicular DNA, we carried out FISH analysis on rabbit testicular tissue. In four of seven sections analyzed by this method, AAV sequences were present in testis basement membrane and in the interstitial space. No intracytoplasmic or intranuclear signals were detected (Figs. 3A and 3B). No hybridization was detected in three gonadal sections or skeletal muscle tissues from uninjected animals. In contrast, intranuclear fluorescent signals were readily demonstrated in sections taken from injected skeletal muscle (Fig. 3C).

We were surprised by the extent and intensity of the positive signal shown in Fig. 3D. We hypothesized that this signal arises from vector particles adhering to the vessel wall and the basement membrane of the testis, which are both rich in heparan sulfate proteoglycan. However, because it is usually difficult to visualize a target of < 10 kb using FISH, we were puzzled as to the origin of the signal. A possible explanation is that, if there are a large number of particles adherent to these structures, the density of smaller targets may have the same capacity to enhance the signal from the FISH probe as if the sequences were actually concatemerized. It should be noted that the bright signal along basement membranes was seen in 7-day samples but never at later time points, nor in saline-injected control animals. Because of this bright signal (Fig. 3D), we attempted to identify intact AAV particles by immunofluorescence (IF) staining. The staining demonstrated that occasional particles can be identified (Figs. 4A and 4C) along the vessel wall or basement membrane, and that they colocalize with staining for heparan sulfate proteoglycan (Figs. 4B and 4D). Again, these findings were present in gonadal tissue harvested at day 7 after injection, but not in sections harvested at later time points or in samples from saline-injected control animals.

Together with the positive serum PCR results, these data suggest that hematogenous dissemination of the vector does occur. Through this route, rAAV binds to HSPG on the testis blood vessel as evidenced by IF and FISH signal obtained from tissue harvested at 7 days following injection. However, these findings were not seen in gonadal tissue harvested at later time points, suggesting that vector is eventually cleared.

In a different approach, we carried out *in vitro* experiments to determine whether murine spermatogonia exposed directly to AAV vector can be transduced. Using a vector expressing LacZ under the control of a cytomegalovirus (CMV) promoter, murine spermatogonia cells were transduced at a multiplicity of infection (MOI) of 5000. Efficient expression from the CMV promoter has been documented in murine spermatogonia [9]. Spermatogonia were identified and distinguished from testicular somatic cells or Sertoli cells by morphological criteria and immunostaining using a monoclonal antibody to the germ cell nuclear antigen (GCNA) [10]. The results show that, even up to 7 days in culture, no spermatogonia were transduced. In control experiments, detection of

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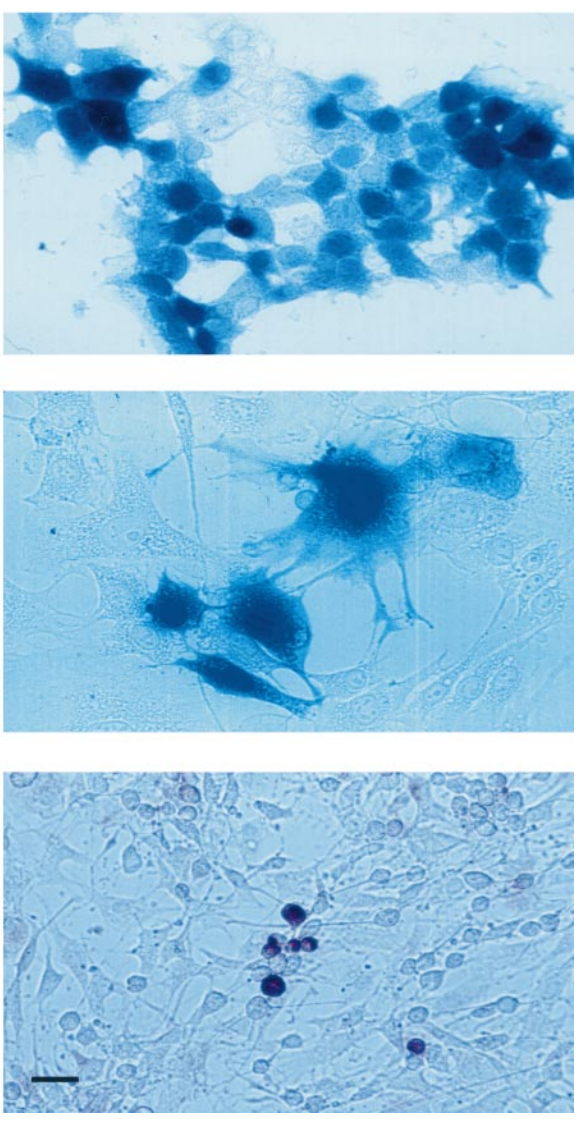

FIG. 5. AAV does not transduce murine germ cells. Human 293T cells (A), mouse fibroblast cell lines (STO cells; B), and mouse spermatogonia and Sertoli cell cocultures (C) are shown. All cultures were transduced with AAV-LacZ, and gene expression is shown by X-gal histochemistry in both human and mouse control cultures (blue cells in A and B). Neither Sertoli cells nor spermatogonia were found positive after 3, 5, or 7 d in culture. Spermatogonia are identified by anti-GCNA immunohistochemistry (red cells in C). Bar, 40 μm.

cytoplasmic expression of LacZ revealed that rAAV efficiently transduced control murine fibroblasts and human embryonic kidney cells (Figs. 5A and 5B). These results suggest that rAAV cannot efficiently transduce murine male germ cells following direct *in vitro* exposure to the vector.

In additional studies we examined DNA extracted from testes and semen of animals following administration of vector into the hepatic artery. In rats, administration of doses of vector up to 1×10^{13} vg/kg also showed a dose-dependent increase in likelihood of detecting AAV sequences in gonadal DNA (Table 2). In dogs, however, when vector was administered via the hepatic artery, vector sequences were not detected in either semen DNA or gonadal tissue at any time point up to 90 days.

These data suggest that AAV does not transduce male germ cells present in the testis. Such findings are consistent with a body of work that has documented the difficulty of obtaining stable and efficient DNA transfer to spermatogenic cells in rodent models, by either viral or nonviral gene transfer techniques, a factor that has been an important limiting step in the development of transgenic animals [4,11,12]. Moreover, anatomical and statistical considerations also support the validity of our experimental findings. To efficiently transduce the spermatogonia, the vector must traverse the basement membranes of two structures, the vascular wall and the seminiferous tubule wall. If efficient transfer occurs across the vessel wall, the cells first encountered would be interstitial cells; to reach spermatogonia, it would be necessary to traverse the basement membrane of the seminiferous tubule as well [4]. Even if spermatogonia are transduced, the event would result in only transient positivity unless early precursors (A_0 spermatogonia) were transduced. Ideally the number of insertions into the genome of gametes from exogenous DNA vector transfer should be zero. However, it should be noted that the rate of endogenous insertion by L1 retrotransposons is 1 in every 50–100 human sperm [13]. In our analysis it remains a formal possibility that the semen samples are positive just below the level of detection of the assay. One microgram of genomic DNA represents approximately 300,000 haploid mammalian genomes; the PCR assay developed for this study is sensitive and can detect as little as 1–100 vector copies per microgram of DNA. Thus, these data indicate a probability of germline transmission of vector sequence of less than 1 in 3000, which is significantly lower than the rate of endogenous insertional mutation.

These data suggest that AAV-2 introduced via IM or intravascular injection at doses up to 1×10^{13} vg/kg does not transduce male germ cells at a detectable level. Additional studies in humans will be required to establish the safety of this procedure, and female germ cells have not yet been analyzed. Two early disease targets for AAV-mediated gene transfer, hemophilia and muscular dystrophy, affect males almost exclusively, so these results support the safety of planned and ongoing clinical investigations. The data thus far suggest that the risk of germline transmission of vector sequences in the setting of AAV-mediated gene transfer is extremely low.

TABLE 2: Reproductive toxicology results in rats and dogs following injection into the hepatic artery of an AAV vector

Animal	Number of animals	Vector	Vector genome per kg	Day of sample collection[a]	PCR positive signal/number of animals	Vector copy per μg DNA
Rat	12	AAV-ApoEhAAT human F.IX	1×10^{11}	50 92	1/6 0/6	2 not detected
Rat	11	AAV-ApoEhAAT human F.IX	1×10^{12}	50 92	4/6 0/5	1–3 not detected
Rat	12	AAV-ApoEhAAT human F.IX	1×10^{13}	50 92	6/6 4/6	4–16 2–7
Dog	3	AAV-null vector	3.7×10^{12} to 7×10^{12}	90[b]	0/3	not detected

[a]Results from gonadal tissue harvested from all animals at the time of sacrifice.

[b]Semen samples collected for all three dogs at 7, 30, 60, and 90 d following injection were persistently negative.

MATERIALS AND METHODS

Biodistribution studies in mice, rats, and dogs following IM administration of AAV vectors. All animal experiments were performed in accordance with IACUC-approved protocols. We injected mice at IM [14] sites with an AAV vector encoding murine F.IX; a 2.7-kb murine F.IX cDNA (provided by D. Stafford, UNC) was inserted as a *Bam*HI fragment into a AAV vector described previously [14], and tissue harvested 30 and 90 d postinjection. Semen samples were not isolated from mice. As part of an acute toxicity study of the vector to be used in clinical trials, we injected rats with an AAV vector encoding human F.IX under the control of a CMV promoter [14]. Rats were sacrificed at 15 d postinjection, and epididymal effluents collected as follows. Immediately after sacrifice, animals were bilaterally castrated and testis and epididymis were isolated and placed in phosphate buffered saline to elute epididymal contents (gametes and other cell types). The buffer containing effluent material from epididymis was centrifuged and DNA extracted from the pellet. PCR assays were carried out using the upstream primer located in the CMV enhancer region and the reverse primer in the human factor IX coding region, as described [8] (for rats), or a reverse primer from murine factor IX [16] (for mouse experiments). The PCR sensitivity was 1 copy per 300 haploid genomes (500 copies/0.5 μg genomic DNA).

For experiments in dogs, we injected four hemophilic dogs with an AAV vector encoding canine F.IX as described [15] and multiple semen samples were collected 7 days to 18 months following vector injection. The PCR reaction was carried out using primers based on AAV-CMV-canine F.IX vector sequences with the upstream primer located in the chimeric intron (5′-ATAGCAGCTACAATCCAGCTACCATTCTGC-3′) and the downstream primer in canine factor IX (5′-TGGTATCCCGTAGTACAGGAACAAACCACC-3′). DNA (1 μg) extracted from semen samples was used as a template in each PCR reaction, and all samples were analyzed in triplicate. The sensitivity of detection was 30 vector genome copies per μg of DNA. To insure that the substrate DNA was intact, a fragment of the B domain of canine F.VIII was amplified with an upstream primer (5′-CTGGGGCTAAATCATGTGTCAAAT-3′) and a downstream primer (5′-GGGTTCCCTGGGCCAAATAGTT-3′), and, to exclude inhibition of the PCR reaction, 1 μg of semen DNA was "spiked" with 100 copies of plasmid pk9 [15]. All reactions were performed as follows: after an initial denaturation step of 94°C for 10 min, 35 cycles of denaturation (94°C, 1 min), annealing (54°C, 1 min), and extension (72°C, 1.5 min) were carried out followed by a final extension step at 72°C for 10 min.

Production of AAV vectors. The AAV null vector (4.5-kb) contains the β-gal and *neo* genes containing disrupted codons for the initial methionine (ATG → CTG). The genes lack promoters or polyadenylation signals. AAV-hFI.X and AAV-null vectors were produced by Avigen Inc. (Alameda, CA) in an adenovirus-free system in HEK-293 cells using a triple transfection method [17]. Null vector was used to avoid any immune response to a transgene product derived from a different animal species. AAV-cF.IX, mF.IX, and lacZ vectors were produced using identical procedures in a research laboratory (K.A.H.).

IM injection of rabbit hindlimb with AAV-null vector. We injected 12 adult male rabbits 6 months or older (3–4 kg) with a dose of 1×10^{13} vector genomes/kg of AAV null vector distributed over 10 sites in the hindlimbs, at a volume of 300 μl per injection. For each time point, three rabbits were injected with vector and a fourth with a control injection of saline only. Injections were performed under ultrasound guidance (Acuson, Sequoia 512, Mountain View, CA) using ketamine/xylazine administered IM as a general anesthetic. Peripheral blood was obtained by ear vein puncture at 15 min and 24 h postinjection in all rabbits, and for three animals additional samples were collected 48 h and 7 d after injection.

Collection of rabbit tissues. At the appropriate time points, semen was collected with the aid of an artificial vagina (AV) developed at Argus Research Laboratories Inc. (Horsham, PA). The AV is lined with a condom from which the tip is removed and a collection tube is added, and the AV is filled with warm water (50°C) in an outer jacket [18]. For each individual rabbit a new condom and collection tube were used. Following semen collection at time points 7, 30, 60, and 90 d postinjection, animals were euthanized (using 125 mg/kg of sodium pentobarbital intravenously), and testis and injected muscle were harvested using fresh sterile instruments for each tissue sample.

DNA analysis. Semen, testis, and injected muscle samples were incubated overnight with proteinase K before isolating total genomic DNA from samples using the QIAamp Tissue Kit (Qiagen, Chatsworth, CA). DNA from serum samples was isolated with the QIAamp Blood Kit. A fragment of 600 bp of the AAV null vector was amplified by PCR using "Ready To Go" PCR beads (Amersham Pharmacia Biotech, Piscataway, NJ) at a $MgCl_2$ concentration of 2 mM. The forward primer βGal-P7 (5′-CGACTTCCAGTTCAACATCAGC-CGCTACAG-3′) is located in the β-gal sequence and the reverse primer Neo-P8 (5′-GCATCAGAGCAGCCGATTGTCTGTTGTGCC-3′) is located in the *neo* sequence. After an initial denaturation step of 95°C for 12 min, 35 cycles of denaturation (95°C, 30 s), annealing (60°C, 30 s), and extension (72°C, 1 min) were carried out followed by a final extension step at 72°C for 7 min. For semen DNA test samples, triplicate experiments were carried out using 1 μg of genomic DNA per PCR. To determine the sensitivity of the PCR, 10-fold serial dilutions of the plasmid encoding the AAV null vector were mixed with 1 μg genomic rabbit DNA before amplification. The lower limit of detection of this assay was one copy per microgram of rabbit genomic DNA.

In a set of control reactions, a fragment of the growth hormone gene was amplified by PCR using forward primer GHF (5′-GGGGCAGCGC-TACTCCATCCAGAA-3′) and reverse primer GHR (5′-GTCGC-CGCGCAGGTTTGTGTCA-3′) [19]. The PCR product of 700 bp was obtained using the same reaction conditions as described above. PCR amplification products were separated by 1% agarose gel electrophoresis and visualized by ethidium bromide staining.

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To analyze faint bands seen on ethidium bromide-stained gels, PCR products (10 μl) from rabbit semen DNA and from positive control samples (1 μg rabbit genomic DNA spiked with 1 to 100 copies of plasmid DNA) were transferred to a nitrocellulose membrane and hybridized to a 3-kb fragment of null vector plasmid containing β-galactosidase and neo genes radioactively labeled using random primed reactions with the Prime-it II Kit (Stratagene, La Jolla, CA). The membrane was placed on an X-ray film for 4 to 16 h at –80°C.

Histological analysis and fluorescent in situ hybridization (FISH). We carried out FISH using a series of gonadal or muscle tissue samples embedded in paraffin or 3–5 μm cryosections obtained from injected and non-injected animals. Plasmid DNA (2 μg) of 8.4 kb in length containing recombinant AAV used for the AAV-null vector construct was labeled with digoxigenin dUTP by nick translation. FISH was performed using 400 ng labeled probe per sample as described [20] and detected by an antibody to digoxigenin tagged with rhodamine. Samples were evaluated using a fluorescent microscope with filter sets for DAPI (Sigma Chemical Co, St. Louis, MO) and rhodamine anti-digoxigenin (Oncor, Inc., Gaithersburg, MD) and representative photographs were prepared.

Immunofluorescence staining was carried out on cryosections of gonadal tissue using a mouse anti-AAV intact particle monoclonal antibody (American Res. Prod, Inc., Belmont, MA) detected by rhodamine labeled rabbit anti-mouse IgG (Chemicon Int., Inc. Temecula, CA) and rat anti-heparan sulfate proteoglycan monoclonal antibody (Chemicon Int., Inc. Temecula, CA) detected by FITC labeled mouse anti-rabbit IgG (Sigma Chemical Co, St. Louis, MO).

Transduction of murine spermatogonia and Sertoli cells by AAV vector encoding LacZ. Spermatogonial cell isolation and culture were carried out as described [21]. Briefly, Sertoli cells and spermatogonia were isolated from testis of 3-day-old B6D2F1 mice (The Jackson Laboratory, Bar Harbor, ME). At this age the only germ cells that are present in the testis are spermatogonia, half of them at stage A_0, which can be identified by immunohistochemistry using antibody anti-GCNA as described [10]. The cells were plated onto 24-well plates covered with a thin layer of Matrigel (Collaborative Research Products) and cultured in DMEM/F-12 as described [21]. The day after plating, the cultures were transduced with AAV-CMV-LacZ at a MOI of 5000 for 16 h in serum-free medium. X-gal histochemistry was performed after 3, 5, and 7 d of culture [22]. Human embryonic kidney cells (293T cell line) or murine fibroblasts cells (STO cell line) were similarly transduced with AAV-CMV-LacZ as controls for the transduction experiments.

Biodistribution studies in rats and dogs following hepatic artery administration of AAV vectors. As part of a preclinical toxicity study, 36 male Sprague Dawley rats (Charles River Laboratories, Kingston, NY) received AAV vector following a midline incision and visualization of the hepatic artery. Vector was infused directly into the hepatic artery via a 30-gauge needle attached to a syringe. Rats received AAV vector expressing human F.IX under the control of a liver-specific promoter as described [23] at doses of 1×10^{11}, 1×10^{12}, and 1×10^{13}vg/kg. The animals were sacrificed at 50 and 90 d following injection and gonadal tissue harvested. A TaqMan assay was developed using serial dilutions of plasmid pAAV-hFIX15 in a background of DNA extracted from rat liver, with a sensitivity of detection of 10 copies per 1 μg of rat genomic DNA.

Three dogs received AAV vector by direct infusion into the hepatic artery following introduction of a catheter under fluorographic guidance that allows the vizualization and direct infusion into hepatic artery. AAV null vector was administered at doses of 3.7 3 10^{12}, 5×10^{12}, and 7×10^{12} vg/kg and semen samples were collected at 7, 30, 60, and 90 d following injection. The animals were sacrificed and gonadal tissues harvested. PCR sensitivity was determined by 10-fold serial dilutions of the plasmid encoding the AAV null vector mixed with 1 μg of genomic canine DNA before amplification; the limit of detection of this assay was 100 copies/μg DNA.

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Note added in proof. To achieve greater sensitivity in the analysis of PCR products from DNA extracted from semen of dogs injected with vector via the hepatic artery, PCR products were electrophoresed, transferred to a nitrocellulose membrane, and probed with a radiolabeled fragment corresponding to the PCR product. The Southern blot was also negative for vector-derived sequences.

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