Evidence for gene transfer and expression of factor IX in haemophilia B patients treated with an AAV vector

Mark A. Kay[1,2], Catherine S. Manno[4,5], Margaret V. Ragni[7], Peter J. Larson[4,5], Linda B. Couto[8], Alan McClelland[8], Bertil Glader[1], Amy J. Chew[4], Shing Jen Tai[4], Roland W. Herzog[4], Valder Arruda[4], Fred Johnson[8], Ciaran Scallan[8], Erik Skarsgard[3], Alan W. Flake[4,6] & Katherine A. High[4,5]

Pre-clinical studies in mice and haemophilic dogs have shown that introduction of an adeno-associated viral (AAV) vector encoding blood coagulation factor IX (F.IX) into skeletal muscle results in sustained expression of F.IX at levels sufficient to correct the haemophilic phenotype[1,2]. On the basis of these data and additional pre-clinical studies demonstrating an absence of vector-related toxicity, we initiated a clinical study of intramuscular injection of an AAV vector expressing human F.IX in adults with severe haemophilia B. The study has a dose-escalation design, and all patients have now been enrolled in the initial dose cohort (2×10^{11} vg/kg). Assessment in the first three patients of safety and gene transfer and expression show no evidence of germline transmission of vector sequences or formation of inhibitory antibodies against F.IX. We found that the vector sequences are present in muscle by PCR and Southern-blot analyses of muscle biopsies and we demonstrated expression of F.IX by immunohistochemistry. We observed modest changes in clinical endpoints including circulating levels of F.IX and frequency of F.IX protein infusion. The evidence of gene expression at low doses of vector suggests that dose calculations based on animal data may have overestimated the amount of vector required to achieve therapeutic levels in humans, and that the approach offers the possibility of converting severe haemophilia B to a milder form of the disease.

Haemophilia B is the bleeding diathesis resulting from mutations in the gene encoding F.IX (*F9*), a proenzyme required for generation of a fibrin clot. The clinical severity of haemophilia B correlates closely with circulating levels of F.IX: individuals with less than 1% of normal activity are severely affected, whereas those with levels 1–5% of normal generally have a more moderate course. Current treatment is based on intravenous infusion of clotting factor concentrates; regimens in which factor is infused prophylactically, with a goal of maintaining factor levels greater than 1% at all times, have been shown to prevent most of the joint damage and life-threatening bleeding complications of the disease[3,4]. Thus, the goal of gene therapy for haemophilia B is the sustained expression of F.IX at levels more than 1% of normal. This goal has been achieved in mice and haemophilic dogs by introducing an AAV vector expressing F.IX into skeletal muscle. Intramuscular injection of an AAV vector expressing human F.IX into immunodeficient mice caused expression of F.IX at 5–7% of normal human plasma levels for more than 12 months (vector dose of 1×10^{13} vector genomes (vg)/kg; ref. 1). Subsequently, intramuscular injection of an AAV vector expressing canine F.IX in dogs with haemophilia B resulted in expression of F.IX at levels up to 1.4% of normal (vector dose of 8.5×10^{12} vg/kg; ref. 2). The levels of expression in these haemophilic dogs are currently stable more than 2.5

Table 1 • Clinical data for patients A, B and C

	A	B	C
Age	38	23	67
Race	European	Asian	European
Baseline F.IX activity level	<1%	<1%	<1%
Baseline F.IX antigen level	24%	<1%	<1%
Mutation	Arg-4→Leu nt 6,365 CGG→CTG	Ala 351→Pro nt 31,172 GCT→CCT	Gly 114→Arg nt 17,755 GGA→CGA
Viral status			
HIV	positive	negative	negative
hepatitis C	positive	positive	negative
hepatitis B	negative	negative	positive
hepatitis A	negative	negative	negative
Significant medical history	s/p GI bleed; s/p seizures secondary to bilateral epidural haematomas; s/p eosiniphilic granuloma R parietal skull; s/p knee synovectomy and arthroscopy	s/p GI bleed; s/p nephrectomy secondary to iliopsoas bleed mellitus	s/p GI bleed; adult onset diabetes
Current medications	ritonavir, lamivudine, stavudine, oxycodone prn	oxycodone prn	glyburide

Departments of [1]Pediatrics, [2]Genetics and [3]Surgery, Stanford University School of Medicine, Palo Alto, California, USA. [4]The Children's Hospital of Philadelphia, Philadelphia, Pennsylvania, USA. Departments of [5]Pediatrics and [6]Surgery, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania, USA. [7]Department of Medicine, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, USA. [8]Avigen, Inc., Alameda, California, USA. Correspondence should be addressed to K.A.H. (e-mail: high@email.chop.edu).

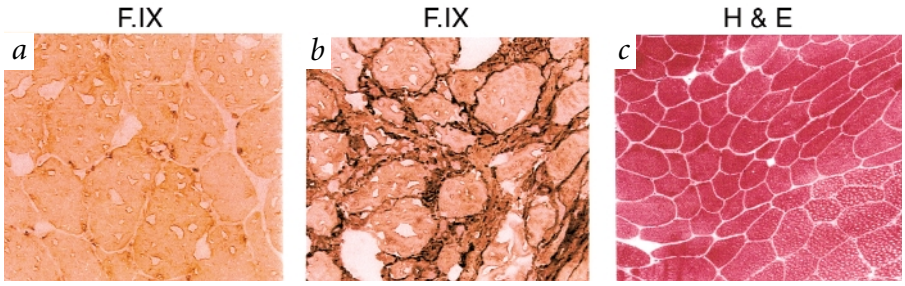


Fig. 1 Histochemical analysis of muscle biopsy. Immunoperoxidase staining of F.IX is shown for cross-sections of muscle tissue of a negative control (***a***) and a vector-injected patient (***b***). The dark brown staining for F.IX is seen in the extracellular matrix surrounding muscle fibres. Original magnification ×200. ***c***, Haematoxylin-and-eosin–stained cross-section of muscle tissue from a patient injected with vector. Original magnification ×100. Muscle biopsies were performed 2–3 months after vector administration.

years after the initial and only injection (R.W.H., K.A.H. and T. Nichols, unpublished data).

Clinical data on our first three patients are shown (Table 1). Evidence for gene transfer and expression following vector administration was sought directly by muscle biopsy and indirectly by measurement of circulating F.IX levels and assessment of bleeding episodes and frequency of clotting factor infusion. We performed muscle biopsies 8–12 weeks after vector administration; PCR on DNA extracted from injected muscle was positive for vector sequences in all three patients (data not shown). Immunohistochemical staining of skeletal muscle was positive for F.IX in the extracellular space, a pattern that had been documented in pre-clinical studies for F.IX secreted by muscle fibres[1] (Fig. 1*a*,*b*). Additional sections analysed by routine histology showed no evidence of inflammation or muscle injury (Fig. 1*c*, and data not shown). Results of coagulation assays and records of factor usage for patients A and B are shown (Fig. 2 and Table 2). Patient A, who was documented to have a baseline F.IX level of less than 1% by three clinical coagulation laboratories, demonstrated a level of more than 1% (also documented by three clinical coagulation laboratories) on multiple occasions beginning approximately 8 weeks after vector administration. These levels were drawn at time points at least 14 days after the most recent factor infusion, eliminating the possibility that the levels reflected a contribution from residual infused factor. Patient B showed a small change in F.IX level, remaining less than 1% of normal (Table 2), but both patients showed a reduction in clotting factor consumption following treatment with the AAV vector (Fig. 2). The treatment time lines are given in 20-day intervals; the first half of the time line (pre-AAV treatment) serves as a control for the second half (post-AAV treatment). Patient A has experienced a 50% reduction in factor usage sustained over a period of more than 100 days, and patient B has experienced an 80% reduction in factor usage also sustained over a period of more than 100 days. Patient C, despite a F.IX level of less than 1%, treats himself infrequently (so-called 'mild-severe'[5]), typically less than four times per year. Since vector injection five months ago, he has had no change in clinical status or F.IX levels (data not shown). Gene transfer and expression, however, were documented by Southern blot on DNA extracted from a muscle biopsy specimen, which showed approximately one vector genome copy per diploid genome, and by RT–PCR, which was positive for F.IX expression (data not shown).

Major safety issues to be addressed here include the risk of formation of inhibitory antibodies to the transgene product, which can block treatment by conventional protein therapy, and the risk of inadvertent germline transmission of vector sequences. Evidence for formation of anti-F.IX antibodies was sought by two different methods, the standard Bethesda assay and a western-blot method. Bethesda assay performed weekly through the first eight weeks, then biweekly through the next four months, showed no evidence of inhibitor formation (data not shown). Western-blot analysis, which detects both inhibitory and non-inhibitory antibodies, has also been consistently negative for evidence of antibodies (Fig. 3*a*). The clinical responses of the patients to infused factor, and a pharmacokinetic study completed in one patient (data not shown), support these laboratory studies, because all patients continue to exhibit excellent responses to clotting factor concentrates.

Pre-clinical biodistribution studies in mice and rabbits carried out at doses 50-fold higher than those used here demonstrated that AAV vector introduced into sites in skeletal muscle remains largely confined to that tissue. Specifically, there is no evidence of distribution of vector into the semen (ORDA web site, http://www.nih.gov/od/orda/3-99RAC.htm) despite transient low-level positive signals in serum 24 hours after injection.

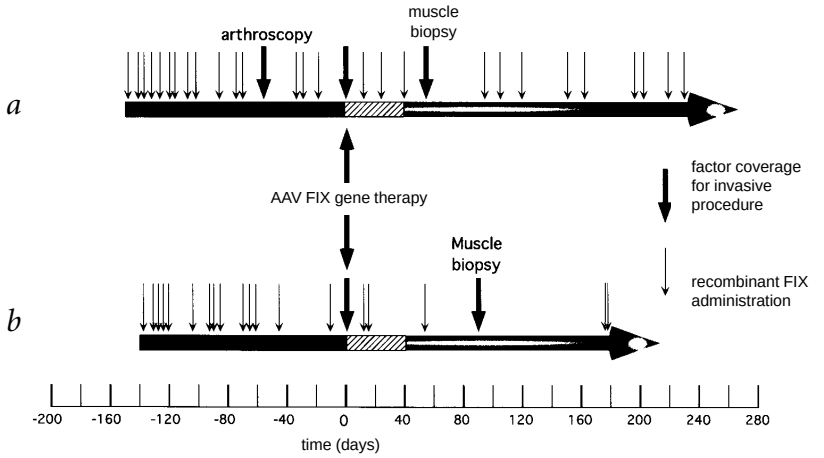


Fig. 2 Factor usage for patients A and B. The horizontal line denotes time; the scale at the bottom is marked in 20-day increments. Arrows denote infusion of F.IX concentrate for spontaneous bleeds (thin arrows) or invasive procedures (thick arrows). The thick vertical arrow in the middle of the chart denotes the date of vector infusion. The hatched bar on the timeline denotes the initial six-week period during which transgene expression is expected to be low based on animal studies[2]. All patients have baseline F.IX levels <1%. ***a***, For patient A, F.IX was documented to be 1%, with an activated partial thromboplastin time (aPTT) of 61 s, when he presented for muscle biopsy at 8 weeks following injection. On the day of and 1 d after muscle biopsy, the patient received F.IX concentrate; after 17 d, with no intervening factor treatment, the F.IX level was 1.6% with an aPTT of 48 s. Ten days later, the F.IX level was determined to be 1.4% with an aPTT of 47 s, again with no intervening factor treatment. Ten days later the patient treated himself with concentrate for atypical knee pain, and a F.IX level drawn after 4 d was 3.7% with an aPTT of 41 s, reflecting the recent protein infusion. A blood sample drawn 14 d after a subsequent treatment showed a F.IX level of 1.3% with an aPTT of 50 s. Over the ensuing weeks the factor level was measured in the 0.5–1.0% range, with aPTTs in the range of 50 s. Factor infusion is reduced ~50% from baseline. ***b***, The baseline F.IX level of patient B is <0.3%; his baseline factor infusion is ~2–5 times/month. Despite no substantial change in F.IX level, patient B's factor consumption has decreased by >80%.

Table 2 • Coagulation data[a] for patients A and B

	Patient A[b]			Patient B[bc]		
	F.IX	aPTT		F.IX	aPTT	
Baseline	<1%			<0.3%		
Week 6	<0.3%	82.9				
Week 8	1%	61		<0.3%	102	
Week 10	1.6%	48		0.3%	91.2	
Week 12	1.4%	46.8		0.3%	102.3	
Week 14	3.7%	41.0	(post-F.IX infusion)	3.0%	52.6	(post-F.IX infusion)
Week 17	1.3%	50.6		0.4%	72	
Week 18	0.8%	49.4				
Week 20	0.5%	54.1		0.4%	107	
Week 22	0.9%	53.7				
Week 24	0.5%	52.1		0.8%	65.5	

[a]Unless otherwise noted, all data points were drawn at least 14 d after the most recent factor transfusion. [b]Data generated in CHOP Clinical Coagulation Laboratory. [c]Data generated in Stanford University Clinical Coagulation Laboratory.

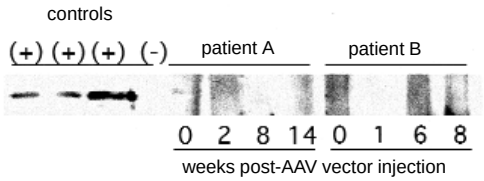
PCR analysis for vector sequences in body fluids from patients (data not shown) is in agreement with the pre-clinical studies, as serum samples were positive for vector sequences at 24 and 48 hours after injection, but were negative thereafter. Saliva samples were also positive at 24 hours after injection, but were subsequently negative, and one patient had a positive urine sample at 24 hours with all subsequent urine samples being negative. All remaining samples, including serial semen samples collected out to 48 days, 56 days and 59 days after injection, were negative for vector sequences.

The effect on transduction efficiency in skeletal muscle of neutralizing antibodies against AAV serotype 2 is unknown[6]. All patients enrolled in this study had detectable titres of neutralizing antibodies against AAV before treatment, with the titre varying over a range of two logs, from 1:10 to 1:1,000 (Fig. 3*b*). The rise in neutralizing antibody titre following vector administration varied from 10- to 1,000-fold. The highest pre-treatment antibody titre was in patient B, whose post-injection muscle biopsy is positive for F.IX expression by immunohistochemical staining (Fig. 1*b*), arguing against any inhibitory effect of the antibodies on skeletal muscle transduction. Additional laboratory studies, including serial complete blood counts and serum chemistries, disclosed no treatment-related abnormalities (data not shown).

Despite promising pre-clinical data, clinical experience with AAV vectors is limited; our study is the first in which AAV vectors have been introduced into skeletal muscle. On the basis of these initial patients, the approach appears to be safe, with no evidence at this dose for toxicity related to vector administration, inadvertent germline transmission of vector sequences or formation of inhibitory antibodies to the transgene product. Moreover, biopsy of injected sites shows evidence of gene expression by immunofluorescence staining. Notably, one of the patients in the initial low-dose cohort showed detectable circulating levels of F.IX above 1%. On the basis of studies in mice and haemophilic dogs[1,2], we had predicted that the patients in the low-dose group would not show measurable levels of F.IX expression (Table 3). Our observations suggest that the vector may be more efficient in humans than in mice or dogs; indeed, we have observed this to be the case in tissue culture, where we have measured as much as a 2-log difference in copy number of the donated gene in primary cultures of human versus mouse muscle cells (unpublished data). Because the vector is engineered from a virus that infects humans but not rodents, the processes of vector binding and entry[7,8] may be more efficient in human cells than in those of other species. A similar consideration applies to the CMV promoter-enhancer used in the vector; because CMV infects humans but not other species, the promoter may have evolved to express most efficiently in the setting of human transcription factors. An objective of dose escalation will be to identify a dose at which all patients express F.IX levels of more than 1%.

The fact that F.IX levels of just above 1% in patient A were associated with a reduction in factor use is consistent with the findings of the Swedish prophylaxis studies, which showed a reduction in haemorrhages when concentrate was dosed to maintain nadir levels of approximately 1% (refs 3,4). The reduction in bleeding seen in patient B raises the question of whether levels of F.IX less than 1% can also result in a reduction in clinical bleeding episodes. More data will be required to resolve this issue. The difference in factor levels seen among patients A, B and C may be accounted for by biologic variation, but another factor that may be important is the presence or absence of circulating F.IX antigen (Table 1). The volume of distribution of F.IX includes both the intravascular and extravascular space, where F.IX can bind tightly to collagen IV (ref. 9). Saturation of these potential binding sites in individuals with circulating F.IX protein may result in higher levels of the donated gene product in the

a



b

time post vector injection	patient A	patient B	patient C
pre-treatment	1:100	1:1,000	1:10
1 week	1:1,000	1:10,000	1:1,000
2 weeks	1:5,000	1:10,000	1:10,000
4 weeks	NS	1:10,000	1:10,000
6 weeks	NS	1:10,000	NS
8 weeks	1:5,000	1:10,000	NS
12 weeks	1:1,000	NS	NS

Fig. 3 Immune responses to AAV-CMV-hF.IX. ***a***, Western-blot analysis of anti-human F.IX in serum samples of haemophilia B patients. Plasma-derived human F.IX is transferred to a membrane, which is incubated with serum samples from patients. Lanes 1,2, positive control (+) (patient with inhibitory anti-F.IX) diluted 1:2,000; lane 3, positive control (+) diluted 1:1,000; lane 4, negative control (–); lanes 5–8, samples from patient A pretreatment (0 weeks, lane 5) and 2 weeks (lane 6), 8 weeks (lane 7) and 14 weeks (lane 8) following AAV-vector injection; lanes 9–12, samples from patient B pretreatment (0 weeks, lane 9) and 1 week (lane 10), 6 weeks (lane 11) and 8 weeks (lane 12) post-injection. ***b***, Neutralizing antibody titres against AAV before and after treatment with AAV-CMV-hF.IX. NS, no sample available at time of assay.

Table 3 • Predicted levels of circulating F.IX in humans

Dose	F.IX level in mice[a]	F.IX level in dogs[b]	Predicted level in humans[c]	Predicted % normal levels in humans
2×10^{11} vg/kg	6 ng/ml	2–4 ng/ml	2–6 ng/ml	≤0.1%
2×10^{12} vg/kg	60 ng/ml	16 ng/ml	16–60 ng/ml	0.3–1.2%
1×10^{13} vg/kg	300 ng/ml	80 ng/ml	80–300 ng/ml	1.8–6%

[a]Predicted plasma F.IX level in mice based on mouse experimental data[1]. [b]Predicted plasma F.IX level in dogs based on canine experimental data[2]. [c]Extrapolated from studies in animals.

circulation. The data gathered so far indicate that AAV-mediated gene therapy for haemophilia B is safe and has the potential to demonstrate efficacy, although testing at higher doses will be required to confirm this interpretation. This treatment strategy thus offers the possibility of converting severe haemophilia B to a milder form of the disease through a relatively non-invasive procedure. In the broader context of gene-based treatment of inherited diseases, the record so far has been discouraging, with no clear-cut evidence of success with *in vivo* gene therapy. Our results indicate that *in vivo* administration of viral vectors offers the possibility of improving the clinical course of genetic diseases that affect many individuals worldwide.

Methods

Clinical protocol. Our study was designed as an open-label, dose-escalation Phase I trial. The clinical protocol was reviewed and approved by the Institutional Review Boards of The Children's Hospital of Philadelphia (CHOP), Stanford University and the University of Pittsburgh Medical Center, the Institutional Biosafety Committees at CHOP and Stanford (the institutions where vector is injected), the Center for Biologics Evaluation and Research of the U.S. Food and Drug Administration, and the NIH Office for Recombinant DNA Activities. Inclusion criteria for the study include severe haemophilia B with F.IX level ≤1%, life expectancy of at least one year, male sex, age ≥18 years, >20 days exposure history to F.IX concentrates and ability to give informed consent. Exclusion criteria include acute infectious illness, end-stage renal disease, severe liver disease defined as bilirubin >2 times normal, transaminases >5 times normal or alkaline phosphatase >5 times normal, platelet count <50,000, presence of inflammatory muscle disease, unwillingness to practice birth control until three semen samples are documented to be negative for vector sequences and unwillingness to stop a regimen of prophylactic clotting factor infusion. The mutation in *F9* was determined for each patient by the dideoxynucleotide chain termination method following PCR amplification of the eight exons of *F9* from genomic DNA that had been isolated from patient blood samples.

Preparation of AAV-CMV-hF.IX. Vector was prepared in a GMP facility (Avigen) using a triple-transfection procedure[10,11]. The F.IX expression plasmid is an 11,442-bp plasmid containing the cytomegalovirus (CMV) immediate early promoter, exon 1 of *F9* (ref. 12), a 1.4-kb fragment of *F9* intron 1 (ref. 13), exons 2–8 of *F9*, 227 bp of *F9* 3′ UTR and the SV40 late polyadenylation sequence between two AAV inverted terminal repeats. The rep/cap plasmid pHLP19 and the helper adenovirus plasmid pLadeno5 have been described[10,11]. Recombinant AAV was produced by transfecting the three plasmids into HEK 293 cells by calcium phosphate transfection. Following incubation to allow vector amplification, cells were lysed and treated with nuclease to reduce residual cellular and plasmid DNA. After precipitation, vector was purified by two cycles of isopycnic ultracentrifugation; fractions containing vector were pooled, dialysed and concentrated. The concentrated vector was formulated, sterile filtered (0.22 μM) and aseptically filled into glass vials. Vector genomes were titred by a quantitative dot-blot assay in which the signal from aliquots of test material is compared with a standard curve generated using the linearized F.IX expression plasmid. The vector underwent in-process and final product testing as described[14].

Vector administration. After giving informed consent, patients were admitted to the Clinical Research Center at either CHOP or Stanford University for history, physical examination and baseline laboratory studies. On day 0 of the protocol, patients were infused with F.IX concentrate to bring factor levels up to ~100%, and, under ultrasound guidance, vector was injected percutaneously into 10–12 sites in the *vastus lateralis* of either or both anterior thighs. Injectate volume at each site was 250–500 μl, and sites were at least 2 cm apart. Local anaesthesia to the skin was provided by ethyl chloride or eutectic mixture of local anaesthetics (EMLA). To facilitate subsequent muscle biopsy, the skin overlying several injection sites was tattooed and the injection coordinates recorded by ultrasound. We observed patients in the hospital for 24 h after injection; routine isolation precautions were observed during this period to minimize any risk of horizontal transmission of vector. Patients are then discharged and seen daily in outpatient clinic for the next three days, then weekly at the home haemophilia centre for the next eight weeks, twice monthly up to five months, monthly for the remainder of the year, then annually in follow-up. Patients are instructed to infuse factor as usual for haemorrhagic episodes.

Laboratory studies. Laboratory studies drawn in follow-up included F.IX level, aPTT, Bethesda assay, anti-AAV neutralizing antibody titre, routine chemistries, muscle enzymes, CBC, urinalysis, HIV viral load for HIV positive patients, fragment 1.2, and collection of serum, semen, urine, saliva and stool for PCR detection of vector sequences. Patients underwent muscle biopsy of injected sites at 2, 6 and 12 months after injection; studies on skeletal muscle included routine haematoxylin and eosin staining, immunohistochemical staining for F.IX expression, PCR for vector sequences on extracted DNA and Southern blot with a vector probe if adequate amounts of muscle DNA were available. Whenever possible, an effort was made to draw blood samples before factor infusion if a haemorrhagic episode required treatment. All studies were performed in routine clinical laboratories (at CHOP and Stanford) using CLIA-approved procedures, except F.IX ELISA, immunostaining of muscle for F.IX expression, anti-AAV neutralizing antibody titre, PCR of body fluids for vector sequences and western blot to detect anti-F.IX antibodies. We carried out F.IX ELISA as described[15]. For immunohistochemical staining, frozen muscle tissue was cryosectioned and stained using a goat anti-human F.IX antibody (Affinity Biologicals; 1:800 dilution) as described[1], except that a biotinylated horse anti-goat IgG was used as a secondary antibody (1:200 dilution) for immunoperoxidase staining using a kit (Vector Laboratories). Sections were counter-stained with Myers haematoxylin stain.

Antibody assays. We determined AAV neutralizing antibody titres by incubating an AAV vector expressing *lacZ* with serial dilutions of patient serum, then used this cocktail to transduce HEK 293 cells. We lysed cells after 24 h and assayed enzymatic activity using the o-nitrophenyl β-D-galactopyranoside (ONPG) assay[16]. Samples were read at OD_{420} to measure β-galactosidase activity; sera were scored as positive for neutralizing AAV antibodies if the OD_{420} was ≤50% that observed when rAAV-lacZ was pre-incubated with negative control mouse sera. Positive samples were titred; AAV neutralizing antibody titres are presented as dilutions that inhibit infection of rAAV-lacZ by 50% based on the ONPG assay. We carried out western-blot analysis to detect anti-F.IX antibodies. Purified human F.IX was electrophoresed on a 7.5% polyacrylamide gel and transferred to a nitrocellulose membrane using an electroblot system (Biorad). The membrane was incubated with a 1:1,000 dilution of the patient's serum sample as primary antibody and 1:10,000 dilution of anti-human IgG peroxidase conjugate using a chemiluminescent substrate (Pierce) as a detecting antibody.

Viral shedding. We used a PCR assay to detect vector sequences in body fluids (serum, urine, saliva, semen and stool) and biopsied muscle. The 5′ primer (5′–AGTCATCGCTATTACCATGG–3′) was derived from the CMV enhancer and the 3′ primer (5′–GATTTCAAAGTGGTAAGTCC–3′) from

intron 1 of human *F9*. Amplified vector sequence yields a PCR fragment of 743 bp. For each sample, a control reaction containing the sample to be tested spiked with vector plasmid (50 copies/μg DNA) was also run to establish that the sample did not inhibit the PCR reaction. For semen, 3 μg of DNA was analysed (1 μg in each of 3 separate reactions); for saliva and biopsied muscle, 1 μg; and for urine, serum and stool, DNA was extracted from a 1–2 ml volume and analysed. The sensitivity of the assay is 50 copies of vector sequence in 1 μg DNA.

Factor IX levels. We determined F.IX levels using an automated analyser (MDA, Organon-Teknika, or MLA-800, Medical Laboratory Automation). Plasma test samples were mixed with F.IX-deficient substrate (George King, Inc.), and results compared with the degree of correction obtained when dilutions of verify reference plasma were added to the same F.IX-deficient substrate. The reference curve was linear down to a lower limit of 0.3%.

The F.IX measurements reported here deserve comment, as the changes are small. Most clinical laboratories do not report a numerical value for clotting factor levels of <1%, but in preparation for this trial, the coagulation laboratories at CHOP and Stanford University Medical Center prepared detailed standard curves for F.IX, which were linear down to levels of ~0.3%. Most authorities would agree that an experienced clinical coagulation laboratory can distinguish between levels <1% and >1%. The values of >1% in patient A were actually repeated and verified by a third clinical laboratory. Thus it appears that these numbers represent an increase from the patient's true baseline, which was also verified to be <1% by three clinical laboratories before the beginning of the trial.

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