JOURNAL OF VIROLOGY, Mar. 2000, p. 2420–2425 Vol. 74, No. 5
0022-538X/00/$04.00+0

Humoral Immunity to Adeno-Associated Virus Type 2 Vectors following Administration to Murine and Nonhuman Primate Muscle

NARENDRA CHIRMULE,[1,2] WEIDONG XIAO,[2] ALEMSEGED TRUNEH,[3] MICHAEL A. SCHNELL,[1] JOSEPH V. HUGHES,[1] PHILIP ZOLTICK,[2] AND JAMES M. WILSON[1,2,4]*

Institute for Human Gene Therapy,[1] Departments of Medicine and Molecular and Cellular Engineering, University of Pennsylvania,[2] and The Wistar Institute,[4] Philadelphia, Pennsylvania 19104, and SmithKline Beecham Pharmaceuticals, King of Prussia, Pennsylvania 19406[3]

Received 30 July 1999/Accepted 3 December 1999

Adeno-associated virus (AAV) is being developed as a vector capable of conferring long-term gene expression, which is useful in the treatment of chronic diseases. In most therapeutic applications, it is necessary to readminister the vector. This study characterizes the humoral immune response to AAV capsid proteins following intramuscular injection and its impact on vector readministration. Studies of mice and rhesus monkeys demonstrated the formation of neutralizing antibodies to AAV capsid proteins that persisted for over 1 year and then diminished, but this did not prevent the efficacy of vector readministration. More-detailed studies strongly suggested that the B-cell response was T cell dependent. This was further evaluated with a blocking antibody to human CD4, primatized for clinical trials, in a biologically compatible mouse in which the endogenous murine CD4 gene was functionally replaced with the human counterpart. Transient pharmacologic inhibition of CD4 T cells with CD4 antibody prevented an antivector response long after the effects of the CD4 antibody diminished; readministration of vector without diminution of gene expression was possible. Our studies suggest that truly durable transgene expression (i.e., prolonged genetic engraftment together with vector readministration) is possible with AAV in skeletal muscle, although it will be necessary to transiently inhibit CD4 T-cell function to avoid the activation of memory B cells.

Adeno-associated virus (AAV) has been engineered for use as a vector in human gene therapy. Replication-defective versions of AAV devoid of all viral open reading frames can be isolated. AAV vectors are capable of efficient and prolonged transgene expression in a number of tissues including skeletal muscle (2). Clinical applications are being considered for the treatment of primary neuromuscular disorders, such as limb-girdle muscular dystrophy, and diseases where muscle serves as a site for secretion of a therapeutic protein such as factor IX for hemophilia B and erythropoietin (EPO) for anemia (6, 9, 14, 17, 19).

For AAV to be useful in the treatment of chronic diseases, it will be necessary to achieve durable expression for the life of the individual. The experience with AAV vectors in skeletal muscle of both small and large animals has been encouraging, with levels of expression persisting for 1 to 2 years, which is a substantial improvement over previous vector systems. Two aspects of AAV biology contribute to the prolongation of transgene expression. First, it appears that the vector genome replicates as a large concatemer, which either exists stably as a large episome or integrates randomly into the host genome (3). In addition, the vector avoids activation of destructive cytotoxic T-lymphocyte responses to antigenic transgene products, a problem previously observed with naked DNA and adenoviral vectors. This may be explained by the restricted tropism of AAV and the resistance of dendritic cells to inadvertent AAV transduction, which is felt to be important in the initial activation of T cells (10).

The stability of transgene expression obtained with AAV vectors in skeletal muscle is impressively prolonged, although it is not permanent; most studies have shown little diminution in transgene expression (i.e., two- to fivefold) over a 1- to 2-year period. The reason for this shallow but steady decline in expression is unclear. Treatment of a chronic disease with AAV vectors over the life of the patient, therefore, will require readministration of vector. A number of investigators have detected antibodies to AAV capsid proteins following vector administration in preclinical models (8, 11). This study evaluates the nature of the humoral immune response to AAV vector in both mice and nonhuman primates and suggests a strategy to allow efficient and repeated readministration of vector.

MATERIALS AND METHODS

Animals and specimen collection. Wild, caught juvenile rhesus monkeys were purchased from Southwest Foundation for Biomedical Research (San Antonio, Tex.) and underwent full quarantine. The monkeys weighed approximately 3 to 4 kg and were serologically negative for simian immunodeficiency virus, simian T-cell leukemia virus, other simian retroviruses, and human adenovirus. Animals were bled for immunological analyses at 7% of the body weight over a 3-week period. C57BL/6, BALB/c, nude BALB/c, RAG1 KO, and CD40LKO mice were purchased from Jackson Laboratories (Bar Harbor, Maine). Mice deficient for the murine CD4 gene and transgenic for human CD4 (HuCD4) were bred in a specific-pathogen-free facility under contract at Charles River (Wilmington, Mass.). HuCD4 mice have previously been described (5). The protocols were approved by the Infection Control Committee of The Hospital of the University of Pennsylvania and the Environmental Health and Safety Office, the Institutional Biosafety Committee, and the Institutional Animal Care and Use Committee of the University of Pennsylvania.

Vectors. AAV type 2 (AAV2) vectors expressing α1 antitrypsin (α1AT) were constructed. Under the control of a cytomegalovirus promoter (AAV-α1AT), AAV green fluorescent protein (AAV-GFP), rhesus monkey EPO (AAV-EPO), and rhesus monkey growth hormone (AAV-GH) were made by transfection-infection protocols described earlier (7, 16). Briefly, the *cis* plasmid (with AAV-ITR), the *trans* plasmid (with the AAV *rep* gene and *cap* gene), and a helper plasmid (pFΔ13; with an essential region from the adenovirus genome) were

* Corresponding author. Mailing address: 204 Wistar Institute, 3601 Spruce St., Philadelphia, PA 19104-4268. Phone: (215) 898-3000. Fax: (215) 898-6588. E-mail: wilsonjm@mail.med.upenn.edu.

cotransfected into 293 cells in a ratio of 1:1:2 by calcium phosphate precipitation. pFΔ13 has an 8-kb deletion in the adenovirus E2B region and most of the late genes. The cells were harvested 96 h posttransfection and subsequently purified by two rounds of CsCl gradient. For the generation of recombinant AAV (rAAV) based on AAV2, p5E18 was used as the *trans* plasmid since it greatly improved the rAAV yield. Large-scale production of the virus was according to good manufacturing practices, and virus was obtained from the Human Applications Laboratory of the Institute for Human Gene Therapy.

Antibody. Clenoliximab, a primatized monoclonal antibody (MAb) to human CD4, contains the variable domains from a MAb generated in cynomolgus macaque and human immunoglobulin G4 (IgG4) constant domains. It also contains two single-residue substitutions, the first in the hinge region and the second within the C_H domain, designed to enhance heavy chain dimer formation and reduce Fc receptor binding (A. Truneh and M. Reddy, unpublished data). This MAb has no C1q binding or complement-fixing activity and has a dramatically reduced Fc receptor binding activity. The preparation of purified Clenoliximab used in this study was provided by the Department of Pharmaceutical Technologies, SmithKline Beecham Pharmaceuticals, King of Prussia, Pa. In one experiment, a human IgG1 isotype antibody of irrelevant specificity was used as a control.

LPR assays. Lymphoproliferative (LPR) responses were performed for AAV antigens, using methods optimized for adenovirus antigens described earlier (4, 5). Peripheral blood mononuclear cells (PBMC) harvested from heparinized blood from rhesus monkeys and splenocytes harvested from mice were isolated following Ficoll-Hypaque density gradient centrifugation, washed in phosphate-buffered saline (PBS), and resuspended in RPMI 1640 supplemented with 10% fetal calf serum, penicillin, streptomycin, and 10^{-5} M 2-mercaptoethanol. Triplicate cultures of PBMC (100 μl of medium at 10^6 cells/ml) were cultured with either rAAV (multiplicity of infection in particles = 100) supplemented with 100 ng of staphylococcus enterotoxin B (SEB; Toxin Technologies, Sarasota, Fla.)/ml or medium alone. Proliferation was measured by a standard 16-h [^{3}H]thymidine (1 μCi/well) pulse on a liquid scintillation counter (Wallach, Gaithersburg, Md.). Results are presented as stimulation indexes, which denote the ratio of counts per minute of stimulated cultures to counts per minute of unstimulated cultures.

Cytokine release assays. PBMC from rhesus monkeys or splenocytes from mice were cultured with or without antigen (i.e., rAAV) for 48 h in a 24-well plate. Cell-free supernatants were collected and analyzed for the presence of interleukin-2 (IL-2), IL-4, gamma interferon (IFN-γ), and IL-10 by commercial enzyme-linked immunosorbent assay (ELISA) kits (BioSource, Camarillo, Calif.) using the manufacturer's protocols. The sensitivities of the kits for human cytokines were as follows: IL-2, 5 pg/ml; IFN-γ, 4 pg/ml; IL-4, 2 pg/ml; IL-10, 5 pg/ml; those for murine cytokines were as follows: IL-2, 13 pg/ml; IFN-γ, 1 pg/ml; IL-4, 5 pg/ml; IL-10, 13 pg/ml.

AAV2-specific Igs. Serum samples from mice and rhesus monkeys were analyzed for AAV2-specific, isotype-specific Igs (IgM, IgG1, IgG2, and IgG4 for rhesus monkeys; IgM, IgG1, IgG2a, and IgG3 for mice) by ELISA as described earlier (5). For the ELISA, 96-well flat-bottom, high-binding ELISA plates (Costar, Cambridge, Mass.) were coated with 100 μl of AAV2 vector antigen (5×10^{10} particles/ml) in PBS overnight at 4°C, washed four times with PBS containing 0.05% Tween, and blocked in PBS containing 1% bovine serum albumin for 1 h at 37°C. Appropriately diluted samples were added to antigen-coated plates and incubated for 4 h at 37°C. Plates were washed four times with PBS containing 0.05% Tween and incubated with peroxidase-conjugated goat anti-human IgM, IgG1, IgG2, or IgG4 (for rhesus monkey samples, the dilution was 1:2,000; Sigma Chemical Co., St. Louis, Mo.) or biotin-conjugated rat anti-mouse IgM, IgG1, IgG2a, IgG3 (for mice, the dilution was 1:1,000; PharMingen, San Diego, Calif.) for 2 h at 37°C. For the mouse ELISA, plates were washed and a 1:20,000 dilution of alkaline phosphatase-conjugated avidin was added. Plates were washed as described above, and ABTS [2,2′-azinobis(3-ethylbenzthiazoline-sulfonic acid)] substrate for peroxidase (Kirkegaard and Perry, Gaithersburg, MD) or *p*-nitrophenyl phosphate in diethanolamine buffer substrate for alkaline phosphatase (Sigma) was added. Optical densities were read at 405 nm on a microplate reader (Dynatech Laboratories, Chantilly, Va.).

Anti-AAV2 NAbs. Neutralizing antibody (NAb) titers were analyzed by determining the ability of serum antibody to inhibit transduction of reporter virus AAV-GFP into E4-expressing 293 cells (84-31 cells) cells as described earlier (7). Various dilutions of antibodies preincubated with the reporter virus for 1 h at 37°C were added to 90% confluent 84-31 cell cultures. The lowest dilution used in the assay was 1:20. Cells were incubated for 16 h, and expression of GFP was analyzed by fluoroimaging (Molecular Dynamics, Sunnyvale, Calif.). The neutralizing titer of antibody was calculated as the highest dilution with which 50% of the cells turned green.

Human α1AT assay. The concentration of human α1AT in mouse serum was measured using ELISA (16). The coating antibody was rabbit anti-human α1AT (Sigma), and goat anti-human α1AT (Sigma) was used as the detection antibody. The sensitivity of this assay was around 0.3 ng/ml.

Study design for HuCD4 mice. Particles (10^{11}) of AAV-GFP vector were administered intramuscularly in a volume of 25 μl to HuCD4 mice. For this purpose, animals were anesthetized and a skin incision was made on the leg. Muscles were exposed, and vector was injected into the tibialis anterior using a 27-gauge needle. The skin incision was then closed using a 4-0 vicryl suture material. Clenoliximab was administered intraperitoneally in a volume of 100 μl as several doses, at 2 mg/dose/mouse, on days −3, 1, 4, 8, 15, 22, and 28 as described earlier (5). AAV-α1AT was readministered on day 56 or day 180. Blood was drawn by retro-orbital bleeding on days 56 and 180 for AAV-NAb analyses, on days 70 and 194 for determining α1AT levels, and on day 56 for Ig isotype analyses.

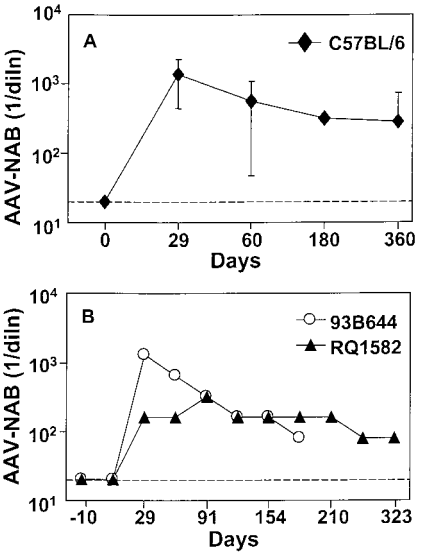

FIG. 1. Development of NAbs to AAV. (A) C57BL/6 mice were injected with 10^{11} particles of AAV-GFP into the tibialis anterior. Serum samples drawn on various days were analyzed for AAV-NAbs. Each point represents a mean ± standard deviation for five to eight animals per group. diln, dilution. (B) Rhesus monkeys were administered AAV-EPO (animals 93B644 and RQ1582) intramuscularly as described in Materials and Methods. Sera were obtained on various days, and NAbs were measured. The neutralizing titers of the sera were calculated as the highest dilutions with which 50% of the cells turned green. The dashed lines indicate the lowest dilution in the assays (1:20).

RESULTS

Humoral immune responses to AAV in mice. Figure 1A shows that C57BL/6 mice administered AAV vectors intramuscularly induce NAbs, which peak on day 29 and persist through 360 days. A more detailed characterization of the nature and relevance of AAV-NAbs is presented in Fig. 2. AAV-GFP or PBS was administered to different strains of mice on day 1, followed by readministration of AAV encoding human α1AT on day 28. Analysis of serum for human α1AT levels on day 56 provided a quantitative readout of vector readministration. Figure 2 shows the α1AT levels (on day 56) and AAV-NAb titers (on day 28) in these mice. Immunodeficient RAG1 KO mice, CD40LKO mice, and nude mice failed to generate AAV-NAbs, and AAV-α1AT was successfully administered to immunized animals at levels equivalent to that achieved in naive animals (i.e., animals that initially received PBS). AAV-α1AT gene transfer was diminished relative to gene transfer in naive mice but was still detectable in BALB/c (4-fold decrease) and C57BL/6 (10-fold decrease) mice when mice were challenged with vector 1 month after immunization with AAV-GFP, at a time when significant NAbs are present in serum. These observations indicate that the AAV-NAb response following intramuscular administration of AAV vectors is T cell dependent and that NAbs substantially inhibit, but do not completely block, vector uptake.

The qualitative nature of AAV-specific antibody responses was assessed by measuring Ig isotypes. Isotype analysis has been shown to be associated with the nature of the T- and B-cell responses (1). In mice, IgM and IgG3 responses are

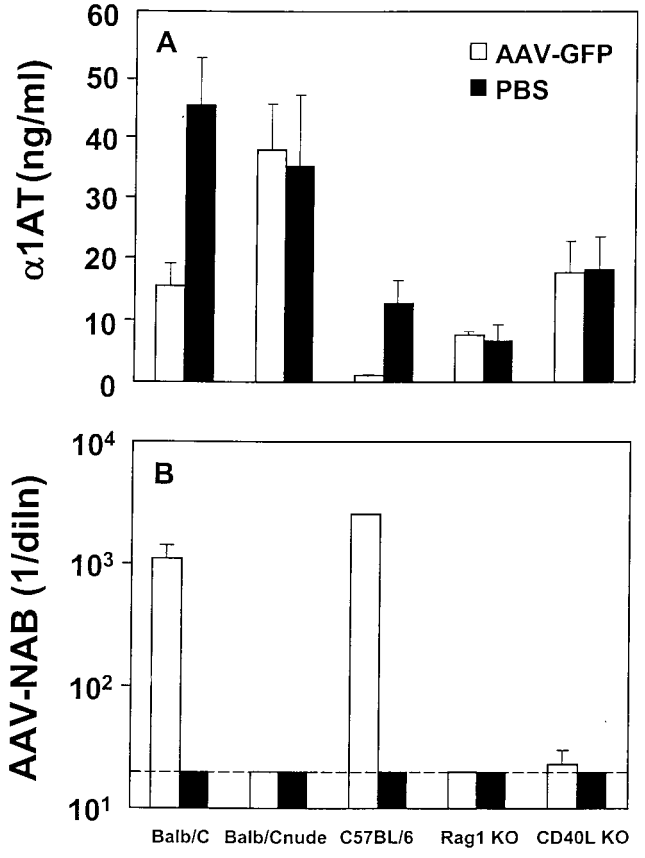

FIG. 2. Readministration of AAV in mice. Various strains of mice were administered either PBS or 10^{11} particles of AAV-GFP into the tibialis anterior of the left leg on day 1. C57BL/6 and BALB/c mice represent immunocompetent mice; BALB/c nude mice and CD40LKO mice have B cells but lack functional T cells; RAG1 KO mice lack both mature T and B cells. On day 28, these mice were readministered AAV-α1AT into the tibialis anterior of the right leg. α1AT levels in sera (A) were measured on day 56 (28 days following the second vector administration) by ELISA. NAbs in sera (B) obtained on day 28 were measured. Each bar represents a mean and standard deviation for three to five animals per group. The dashed line indicates the lowest dilution in the assay (1:20).

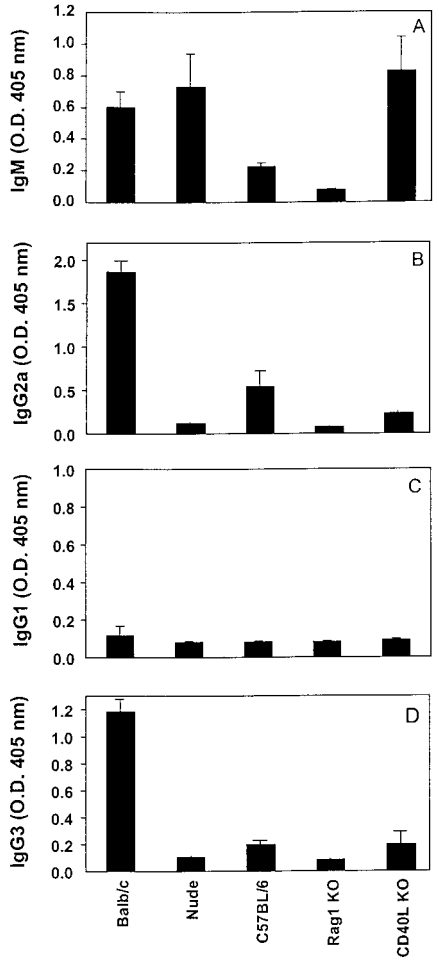

FIG. 3. AAV vector-specific Ig isotypes in sera following intramuscular administration of adenovirus vectors to mice. Sera obtained from mice administered AAV-GFP were analyzed for the presence of AAV-specific Ig isotypes (IgM, IgG1, IgG2a, and IgG3) by ELISA, as described in Materials and Methods. Results are expressed as optical densities (O.D.). Each bar represents the mean and standard deviation for three to five animals per group.

T-cell-independent responses, while IgG2a and IgG1 have been implicated in Th1 and Th2 responses, respectively (12). Figure 3 shows the AAV-specific IgM, IgG2a, IgG1, and IgG3 levels in various strains of mice. RAG1 KO mice, which are deficient in mature T and B cells, failed to elicit AAV antibodies. CD40LKO and nude mice generated strong IgM responses and minimal or absent IgG3, IgG2a, or IgG1 isotype responses. C57BL/6 and BALB/c mice generated equivalent IgM responses; IgG2a and IgG3 responses were present in both although greater in BALB/c mice.

LPR assays of splenocytes from immunocompetent mice failed to demonstrate significant activation of T cells to AAV capsids. Cytokine secretion profiles for IFN-γ, IL-2, and IL-4 were unremarkable, although moderate IL-10 responses were seen (Table 1).

Humoral immune responses to AAV in nonhuman primates. AAV-mediated gene transfer to rhesus monkey skeletal muscle was studied with a vector that expresses rhesus monkey EPO from a cytomegalovirus promoter (AAV-EPO). This provides a quantitative assessment of transgene engraftment by direct measure of serum EPO by ELISA and the resulting elevation in hematocrit. Intramuscular administration of AAV-encoding EPO to rhesus monkeys resulted in prolonged

TABLE 1. LPR responses and cytokine secretion profile following AAV vector administration to mouse muscle[a]

Mouse type	LPR response (cpm) in:			AAV-induced level (pg/ml) of cytokine:			
	Medium	AAV	SEB	IL-2	IFN-γ	IL-4	IL-10
BALB/c	201	211	9,081	<13	<1	<5	209
BALB/c nude	212	220	240	<13	<1	<5	<5
C57BL/6	155	208	10,264	<13	<1	<5	106
RAG1 KO	101	102	101	<13	<1	<5	<5
CD40LKO	166	229	490	<13	<1	<5	<5

[a] Various strains of mice were administered AAV-GFP intramuscularly as described in Materials and Methods. Spleens were obtained 28 days following vector administration (similar results were obtained on day 10; not shown). Splenocytes were isolated by Ficoll-Hypaque density gradient centrifugation and cultured in medium alone or in the presence of AAV antigens or SEB for LPR responses. LPR responses were measured by [^{3}H]thymidine incorporation. For induction of cytokines, splenocytes obtained on day 28 were cultured with AAV antigens for 48 h. Culture supernatants were collected and measured in duplicate for the presence of IFN-γ, IL-2, IL-4, and IL-10 using commercial ELISA kits (BioSource International), as described by the manufacturer's protocol.

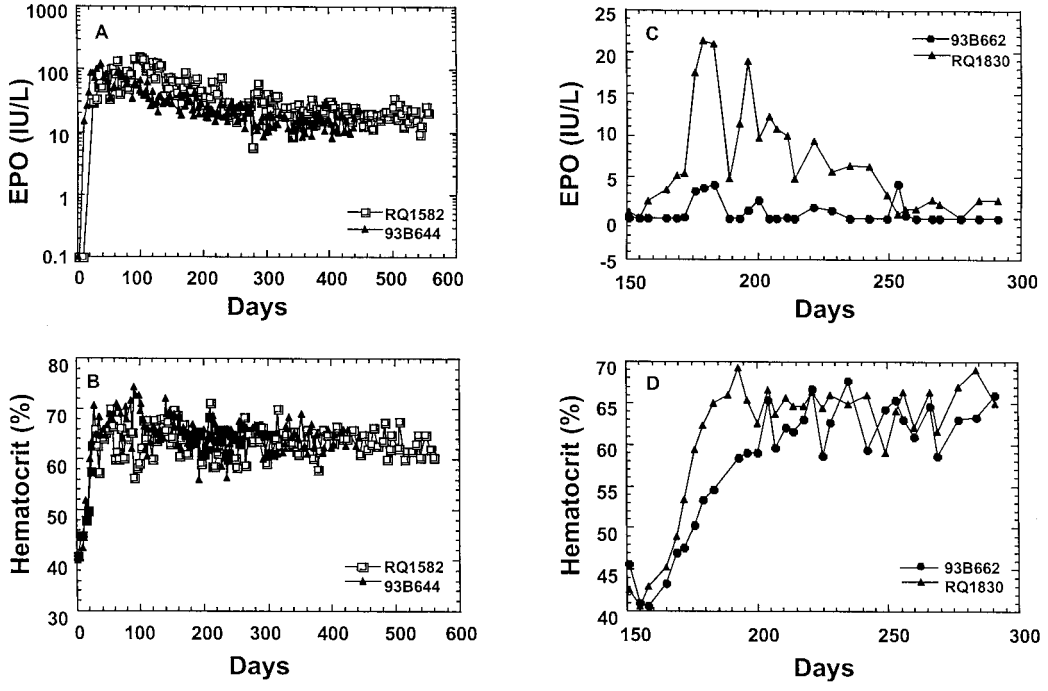

FIG. 4. Serum EPO and hematocrit levels following intramuscular injection of AAV vectors in rhesus monkey muscle. Rhesus monkeys RQ1582 and 93B644 were administered AAV-EPO intramuscularly on day 1. Rhesus monkeys 93B662 and RQ1830 were administered AAV-GH on day 1 and readministered AAV-EPO on day 151. The AAV-NAb titers of these animals are shown in Fig. 1B and 5A. Sera were analyzed for the presence of EPO (A and C) by ELISA, and for hematocrits (B and D) by centrifugation of whole blood. Rhesus monkeys were bled for therapeutic reasons when hematocrits increased over 65%.

expression of the transgene, as evidenced by supraphysiological serum EPO concentrations (Fig. 4A) in the setting of a persistently elevated hematocrit (Fig. 4B).

The kinetics of EPO expression is quite interesting and reproducible between two animals dosed with equivalent quantities produced using different methods. Peak expression occurred about 60 days after gene transfer; the decay was biphasic, with a gradual decline to 10 to 20 IU/liter during the first 300 days followed by little, if any, further decrease in EPO from days 300 to 600. Hematocrits stayed elevated, requiring repeated therapeutic phlebotomies. A preliminary description of these two animals was previously presented (18).

The nonhuman primates that received AAV-EPO had a 2-log-unit increase of AAV-NAb, which peaked on day 29 and decreased 10-fold over the next year (Fig. 1B). The ability to readminister AAV to nonhuman primate muscle was studied with animals administered AAV-EPO 151 days after intramuscular administration of AAV-GH. The expression of EPO from the second vector administration did diminish 5- to 10-fold over 300 days, although it persisted at levels sufficient to maintain significant polycythemia (Fig. 4D). Serum EPO was evaluated 60 days after AAV-EPO administration in AAV-GH-pretreated animals; peak expression, shown in Fig. 5, was compared to levels achieved in two separate naive monkeys administered equivalent doses of AAV-EPO. Both rhesus monkeys administered AAV-GH generated strong AAV-NAb responses (Fig. 5A). The presence of the NAb apparently diminished, but did not abrogate, the initial expression of EPO in AAV-EPO-treated animals compared to historical controls (Fig. 5B). The NAb response to AAV was boosted by at least 1 log unit following the second vector administration (Fig. 5A).

Quantitative analyses of the humoral immune response to AAV were performed by measuring the AAV-specific isotype antibodies. Figure 6 shows that all four monkeys administered

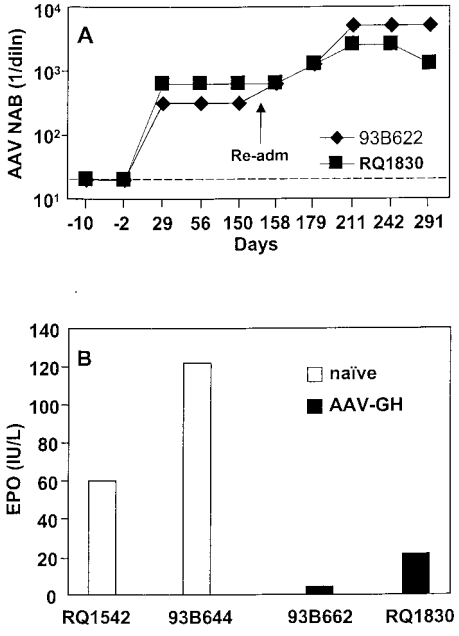

FIG. 5. Readministration of AAV2 in nonhuman primates. Rhesus monkeys were administered AAV-GH (animals 93B662 and RQ1830) intramuscularly as described in Materials and Methods on day 1 and readministered AAV-EPO on day 151. Animals RQ1542 and 93B644 represent animals expressing EPO following administration of AAV-EPO to naive animals. Sera were obtained from rhesus monkeys on various days. (A) Serum AAV-NAb levels following administration of AAV-GH (on day 1) and AAV-EPO (on day 151) are shown as reciprocal dilutions (diln) of sera. (B) Serum EPO levels measured 60 days after administration of AAV-EPO to either naive rhesus monkeys or AAV-GH-treated monkeys, as measured by ELISA.

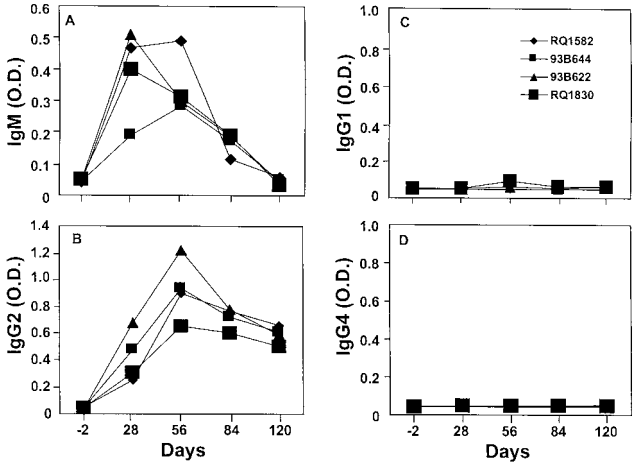

FIG. 6. AAV vector-specific Ig isotypes in sera following intramuscular administration of AAV vectors in nonhuman primates. Sera obtained from rhesus monkeys administered AAV-GH (93B622 and RQ1830) or AAV-EPO (93B644 and RQ1582) intramuscularly were analyzed for the presence of AAV-specific Ig isotypes (IgM, IgG1, IgG2, IgG4) by ELISA, as described in Materials and Methods. Results are expressed as optical densities (O.D.).

AAV-EPO or AAV-GH generated a strong IgM response, which peaked at day 28 and decreased to almost baseline levels by day 120. On the other hand, IgG2 responses peaked on day 56 and persisted up to day 120; no IgG1 or IgG4 responses could be measured. Analysis of PBMC revealed little proliferation to AAV and insignificant amounts of secreted IFN-γ, IL-2, and IL-4; two of four monkeys showed modest IL-10 responses (Table 2).

Primatized anti-CD4 antibodies inhibit AAV-mediated NAbs and permit vector readministration. In an attempt to prevent the B-cell response to capsid proteins we studied transient inhibition of T-cell function at the time of vector administration. A primatized blocking antibody to human CD4, called Clenoliximab, was tested in a biologically compatible murine model, the HuCD4 mouse (5). The endogenous murine CD4 gene has been functionally replaced in this mouse with a HuCD4 gene. Animals were treated with AAV-GFP in skeletal muscle in the presence or absence of a short course of the CD4 antibody and injected 2 or 6 months later with AAV-α1AT (Table 3). Engraftment of the second vector was diminished when the vector was administered on day 56 (threefold reduction) and day 180 (twofold reduction) after immunization with AAV-GFP. Treatment of animals with CD4 antibody at the time of administration of the first vector prevented the formation of NAbs and allowed efficient vector readministration (equivalent to that seen in naive animals) long after the initial doses of CD4 antibody had waned (Table 3). Analyses of Ig secretion patterns, at the time of AAV-α1AT administration on day 56, are also presented in Table 3. HuCD4 mice treated with AAV-GFP alone on day 1 (group A) generated IgM, IgG2a, and IgG1 responses. IgG3 responses were not significantly induced. Clenoliximab treatment had no effect on IgM responses, but IgG1 and IgG2a responses were significantly inhibited.

DISCUSSION

Durable gene expression will be required to effectively treat many chronic diseases such as inherited diseases manifested during childhood. This can be accomplished through the combination of very prolonged gene engraftment in combination with efficient vector readministration when the previous treatment wanes.

Expression of AAV-encoded genes in skeletal muscle persists for at least 1 to 2 years in a variety of animal models including mice, dogs, and nonhuman primates (6, 9, 14, 17, 19). Quantitative analysis of these models has shown modest declines in transgene expression over this period, indicating that vector readministration may be necessary at intervals of several years. Mechanisms responsible for the steady decline in transgene expression have yet to be determined, although a number of hypotheses have been entertained including promoter shutoff, degradation of the vector genome (if it indeed persists as an unintegrated concatemer), and turnover of the transduced muscle fiber (15). The last mechanism is likely if in vivo transduction is restricted to muscle fibers as opposed to the satellite cells, which are progenitors of the muscle fibers.

Our study characterized several features of humoral immunity to AAV in skeletal muscle relevant to the challenge of vector readministration. A vibrant humoral response ensues; it declines over a year, but remains substantial. Experiments with mice genetically deficient in T-cell function indicated that the B-cell response is T cell dependent despite an inability to demonstrate significant activation of CD4 T cells exposed to AAV antigens in vitro. Immunocompetent mice developed a strong NAb response. The differences in the Ig isotypes and NAb titers in C57BL/6 and BALB/c mice suggest that some of the anti-AAV antibody made by BALB/c mice was not neutralizing, although direct comparisons between these two assays are difficult. Isotype analyses of Clenoliximab-treated mice suggest that the T-cell-dependent isotypes, IgG1 and IgG2a, contribute to the NAb response. Readministration of vector is possible in mice and nonhuman primates in the presence of maximal NAb responses, although it is diminished 4- to 20-fold with significant strain-specific variation in mice and animal-to-animal variation in nonhuman primates.

The strategy for blocking the humoral response emerged from an understanding that it is T cell dependent and that presentation of capsid antigens by antigen-presenting cells would be short-lived. Sher and coworkers suggested that activated dendritic cells turn over in 7 to 10 days and that therefore the window for inhibition of T-cell activation can be restricted to a 2-week period following vector administration (13). Our experience with an antibody to HuCD4 supports this hypothesis (5). Application of Clenoliximab to gene therapy will be

TABLE 2. LPR responses and cytokine secretion profile following AAV vector administration in nonhuman primate muscle[a]

Animal	LPR response (cpm) in:			AAV-induced level (pg/ml) of cytokine:			
	Medium	AAV	SEB	IL-2	IFN-γ	IL-4	IL-10
RQ1582	498	308	39,077	<5	<4	<2	<5
93B644	426	605	44,633	<5	<4	<2	58
93B622	1,718	2,421	10,432	<5	<4	<2	<5
RQ1830	1,951	1,020	22,571	<5	<4	<2	14

[a] Rhesus monkeys were administered either AAV-GH (animals 93B622 and RQ1830) or AAV-EPO (animals RQ1582 and 93B644) intramuscularly as described in Materials and Methods. Heparinized blood was drawn 28 days following vector administration. PBMC were isolated by Ficoll-Hypaque density gradient centrifugation and cultured in medium alone or in the presence of AAV antigens or SEB for 6 days for LPR responses. LPR responses were measured by [^{3}H]thymidine incorporation. For induction of cytokines, PBMC obtained on day 28 were cultured with AAV antigens for 48 h. Culture supernatants were collected and measured in duplicate for the presence of IFN-γ, IL-2, IL-4, and IL-10 using commercial ELISA kits (BioSource International), as described by the manufacturer's protocol.

TABLE 3. Readministration of AAV vectors in HuCD4 transgenic mice, following immune suppression with anti-human CD4 antibody[a]

Group[b]	Level of indicated Ig (OD), α1AT (ng/ml), or NAb (1/dilution) on day:							
	56						180	
	α1AT	NAb	IgM	IgG3	IgG2a	IgG1	α1AT	NAb
A	14.6 ± 1.4	20 ± 0	0.073 ± 0.021	0.027 ± 0.012	0.040 ± 0.019	0.014 ± 0.004	ND	ND
B	17.6 ± 9.8	20 ± 0	0.373 ± 0.191	0.040 ± 0.022	0.075 ± 0.043	0.035 ± 0.014	38 ± 5	24 ± 8
C	4.6 ± 1.3	840 ± 481	0.349 ± 0.106	0.032 ± 0.014	1.019 ± 0.249	0.100 ± 0.037	7 ± 3	136 ± 111

[a] HuCD4 transgenic mice were administered either PBS or AAV-GFP on day 1. One group of animals was treated with an anti-human CD4 antibody (Clenoliximab) as described in Materials and Methods. Animals were administered AAV-α1AT on day 56 or 180. AAV-NAbs were measured on day 56 and day 180 prior to administration of the second vector. The α1AT levels in these mice were measured 14 days later (days 70 and 194) by ELISA. The level for the animals administered PBS is 1:20, the lowest titer in the assay. Ig isotypes against AAV were measured by ELISA on day 56. Values are means ± standard deviations for five animals per group. ND, not done.

[b] For group A, vector 1 was PBS; for groups B and C, it was GFP. For each group, vector 2 was α1AT. The MAb for group B was CD4.

greatly facilitated by the extensive safety data being generated on its application for patients with a variety of autoimmune diseases, such as rheumatoid arthritis and psoriasis.

In summary, readministration of AAV to skeletal muscle is indeed possible in the face of high NAb levels, although efficiencies are strongly diminished. It is possible that NAbs may eventually wane to noninhibiting levels over an interval, measured in years, that is less than or equal to the duration of therapeutic gene expression. In this scenario, inhibiting the B-cell response is not necessary. We believe it is prudent to anticipate that the interval of gene expression will be less than the duration of NAbs, requiring a strategy to eliminate or substantially reduce the humoral response. A course of treatment with Clenoliximab, a nondepleting anti-CD4 antibody, is one way that such a strategy could be evaluated in humans. Realize, however, that this has been modeled in recipients who are naive to vectors at the time of gene transfer, which will apply to two-thirds of humans before the absence of preexisting NAb and T-cell responses to AAV2. Different strategies may be required to modify secondary responses.

ACKNOWLEDGMENTS

N.C. and W.X. contributed equally to this work.

We thank the members of the Wilson lab for helpful discussions. Support from the Animal Models Group (Marcia Houston-Leslie, Rosalind Barr, Holly Clouse, Jeanna Stabinski), Vector Core (Guangping Gao), Cell Morphology Core, Immunology Core (Ruth Qian, George Qian, and Parag Dhagat), and Animal Services Unit (Ernest Glover, Lisa Stephens) of the Institute for Human Gene Therapy is greatly appreciated.

This work was funded by grants from the NIH (P30 DK47757-05), NIH/NIAMS (P01 AR/NS43648-04), MDA, and Genovo, Inc., a biotechnology company Dr. Wilson founded and has equity in.

REFERENCES

1. **Abbas, A. K., K. M. Murphy, and A. Sher.** 1996. Functional diversity of helper T lymphocytes. Nature **383:**787–793.
2. **Blau, H. M., and M. L. Springer.** 1995. Muscle-mediated gene therapy. N. Engl. J. Med. **23:**1554–1556.
3. **Carter, B. J., and T. R. Flotte.** 1996. Development of adeno-associated virus vectors for gene therapy of cystic fibrosis. Curr. Top. Microbiol. Immunol. **218:**119–144.
4. **Chirmule, N., J. V. Hughes, G.-P. Gao, S. E. Raper, and J. M. Wilson.** 1998. Role of E4 in eliciting CD4 T-cell and B-cell responses to adenovirus vectors delivered to murine and nonhuman primate lungs. J. Virol. **72:**6138–6145.
5. **Chirmule, N., A. Truneh, S. E. Haecker, J. T. Tazelaar, G.-P. Gao, S. E. Raper, J. V. Hughes, and J. M. Wilson.** 1999. Repeated administration of adenoviral vectors in lungs of human CD4 transgenic mice treated with a non-depleting antibody. J. Immunol. **163:**448–455.
6. **Fisher, K. J., K. J. Jooss, J. Alston, Y. Yang, S. E. Haecker, K. High, R. Pathak, S. E. Raper, and J. M. Wilson.** 1997. Recombinant adeno-associated virus for muscle directed gene therapy. Nat. Med. **3:**306–312.
7. **Gao, G.-P., G. Qu, L. Faust, R. K. Engdahl, W. Xiao, J. V. Hughes, P. Zoltick, and J. M. Wilson.** 1998. High-titer adeno-associated viral vectors from a rep/cap cell line and hybrid shuttle virus. Hum. Gene Ther. **9:**2353–2362.
8. **Halbert, C. L., T. A. Standaert, C. B. Wilson, and A. D. Miller.** 1998. Successful readministration of adeno-associated virus vectors to the mouse lung requires transient immunosuppression during the initial exposure. J. Virol. **72:**9795–9805.
9. **Herzog, R. W., E. Y. Yang, L. B. Couto, J. N. Hagstrom, D. Elwell, P. A. Fielfs, M. Burton, D. A. Bellinger, M. S. Read, K. M. Brinkhouse, G. M. Podsakoff, T. C. Nichols, G. J. Kurtzman, and K. A. High.** 1999. Long-term correction of canine hemophilia B by gene transfer of blood coagulation factor IX mediated by adeno-associated viral vector. Nat. Med. **5:**56–63.
10. **Jooss, K., Y. Yang, K. J. Fisher, and J. M. Wilson.** 1998. Transduction of dendritic cells by DNA viral vectors directs the immune response to transgene products in muscle fibers. J. Virol. **72:**4212–4223.
11. **Manning, W. C., S. Zhou, M. P. Bland, J. A. Escobedo, and V. Dwarki.** 1998. Transient immunosuppression allows transgene expression following readministration of adeno-associated viral vectors. Hum. Gene Ther. **9:**477–485.
12. **Rajewsky, K.** 1996. Clonal selection and learning in the antibody system. Nature **381:**751–758.
13. **Sousa, C. R., S. Hieny, T. Scharton-Kersten, D. Jankovic, H. Charest, R. N. Germain, and A. Sher.** 1997. In vivo microbial stimulation induces rapid CD40 ligand-independent production of interleukin 12 by dendritic cells and their redistribution to T cell areas. J. Exp. Med. **186:**1819–1829.
14. **Synder, R. O., C. Niao, L. Meuse, J. Tubb, B. A. Donahue, H.-F. Lin, D. W. Stafford, S. Patel, A. R. Thompson, T. Nichols, M. S. Read, D. A. Bellinger, K. M. Brinkhous, and M. A. Kay.** 1999. Correction of hemophilia B in canine and murine models using recombinant adeno-associated viral vectors. Nat. Med. **5:**64–70.
15. **Verma, I. M., and N. Somia.** 1997. Gene therapy—promises, problems and prospects. Nature **389:**239.
16. **Xiao, W. D., N. Chirmule, S. Berta, B. McCullough, G.-P. Gao, and J. M. Wilson.** 1999. Gene therapy vectors based on adeno-associated virus type I. J. Virol. **73:**3994–4003.
17. **Xiao, X., J. Li, and R. J. Samulski.** 1996. Efficient long-term gene transfer into muscle tissue of immunocompetent mice by adeno-associated virus vector. J. Virol. **70:**8098–8108.
18. **Ye, X., V. M. Rivera, P. Zoltick, F. Cerasoli, M. A. Schnell, P. P. Gao, J. V. Highes, M. Gilman, and J. M. Wilson.** 1999. Regulated delivery of therapeutic proteins after in vivo somatic cell gene transfer. Science **283:**88–91.
19. **Zhou, S., J. E. Murphy, J. A. Escobedo, and V. J. Dwarki.** 1998. Adeno-associated virus-mediated delivery of erythropoietin leads to sustained elevation of hematocrit in nonhuman primates. Gene Ther. **5:**665–670.