

Induction of Robust Immune Responses against Human Immunodeficiency Virus Is Supported by the Inherent Tropism of Adeno-Associated Virus Type 5 for Dendritic Cells[†]

Ke-Qin Xin,¹ Hiroaki Mizukami,² Masashi Urabe,² Yoshihiko Toda,¹ Kaori Shinoda,¹
Atsushi Yoshida,¹ Kenji Oomura,¹ Yoshitsugu Kojima,¹ Motohide Ichino,³
Dennis Klinman,⁴ Keiya Ozawa,² and Kenji Okuda^{1*}

Departments of Molecular Biodefense Research¹ and Immunology,³ Yokohama City University Graduate School of Medicine, Yokohama 236-0004, Japan; Division of Genetic Therapeutics, Center for Molecular Medicine, Jichi Medical School, Tochigi-ken 329-0498, Japan²; and Center for Biologics Evaluation and Research, U.S. Food and Drug Administration, Bethesda, Maryland 20892⁴

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The ability of adeno-associated virus serotype 1 to 8 (AAV1 to AAV8) vectors expressing the human immunodeficiency virus type 1 (HIV-1) Env gp160 (AAV-HIV) to induce an immune response was evaluated in BALB/c mice. The AAV5 vector showed a higher tropism for both mouse and human dendritic cells (DCs) than did the AAV2 vector, whereas other AAV serotype vectors transduced DCs only poorly. AAV1, AAV5, AAV7, and AAV8 were more highly expressed in muscle cells than AAV2. An immunogenicity study of AAV serotypes indicates that AAV1, AAV5, AAV7, and AAV8 vectors expressing the Env gp160 gene induced higher HIV-specific humoral and cell-mediated immune responses than the AAV2 vector did, with the AAV5 vector producing the best responses. Furthermore, mice injected with DCs that had been transduced *ex vivo* with an AAV5 vector expressing the gp160 gene elicited higher HIV-specific cell-mediated immune responses than did DCs transduced with AAV1 and AAV2 vectors. We also found that AAV vectors produced by HEK293 cells and insect cells elicit similar levels of antigen-specific immune responses. These results demonstrate that the immunogenicity of AAV vectors depends on their tropism for both antigen-presenting cells (such as DCs) and non-antigen-presenting cells (such as muscular cells) and that AAV5 is a better vector than other AAV serotypes. These results may aid in the development of AAV-based vaccine and gene therapy.

Recombinant adeno-associated viruses (AAVs) have been widely used as gene delivery vectors in animal models (17, 18), and these have entered human clinical trials (34). AAVs have been found in many animal species, including nonhuman primates, canines, fowls, and humans. An increasing number of AAV serotypes have been reported. AAV2, AAV3, and AAV5 are found in humans, while AAV4, AAV7, and AAV8 are found in nonhuman primates (21, 45, 50). The reservoir for AAV1 is unclear because these viruses have not been primarily isolated from tissues; however, reactive antibodies (Abs) against AAV1 have been found to exist in both humans and nonhuman primates (9). AAV6 appears to be a recombinant between AAV1 and AAV2 (60). Most of the current studies involving AAV are based on AAV2 since it was the first available infectious clone (51). The use of AAV2 as a vector to introduce exogenous genes encoding immunogenic proteins for the purpose of vaccination has been explored in several studies conducted by us (65, 66) and other researchers (17, 30, 46, 47). An *ex vivo* experiment demonstrated that the AAV2 vector can transduce dendritic cells (DCs) and that these cells

then present the AAV-encoded antigen to T cells (46, 65). Other AAV serotypes may have advantages as vaccine vectors because AAV serotypes differ in their tissue and/or cell tropism (23, 25, 30, 47). For example, AAV1 and AAV7 are more efficient than AAV2 for the transduction of skeletal muscle (21, 60), while AAV3 is superior for the transduction of megakaryocytes (27). AAV5 and AAV6 infect apical airway cells more efficiently (24, 68). AAV2, AAV4, and AAV5 infect cells of the central nervous system; however, differences with regard to the distribution and target cell types exist among these three serotypes (68).

AAV is a small, single-stranded DNA virus that lacks an envelope. This virus requires a helper virus to facilitate efficient replication. The genome of wild-type AAV is known to integrate into the human genome at a specific site on chromosome 19q (36). However, in nondividing cells, AAV vector genomes mostly adopt the form of concatameric circular episomes that comprise active transcriptional units (16, 54). AAV is currently the only nonpathogenic viral vector that has been shown to mediate long-term gene expression without causing toxicity *in vivo*. Using this vector system, exogenous genes have been efficiently transferred into a number of tissues, including brain (18), muscle (29), lung (19), gut (17), liver (55), and eye (37). A human clinical trial of AAV2 has been conducted (34, 41).

The objectives of the present study were (i) to compare the immunogenicities of AAV serotypes (1 to 8) expressing human

* Corresponding author. Mailing address: Department of Molecular Biodefense Research, Yokohama City University Graduate School of Medicine, 3-9 Fukuura, Kanazawa-ku, Yokohama 236-0004, Japan. Phone: 81(45)787-2602. Fax: 81 (45)787-2851. E-mail: kokuda@med.yokohama-cu.ac.jp.

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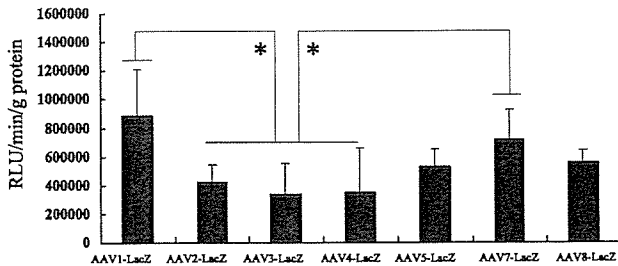
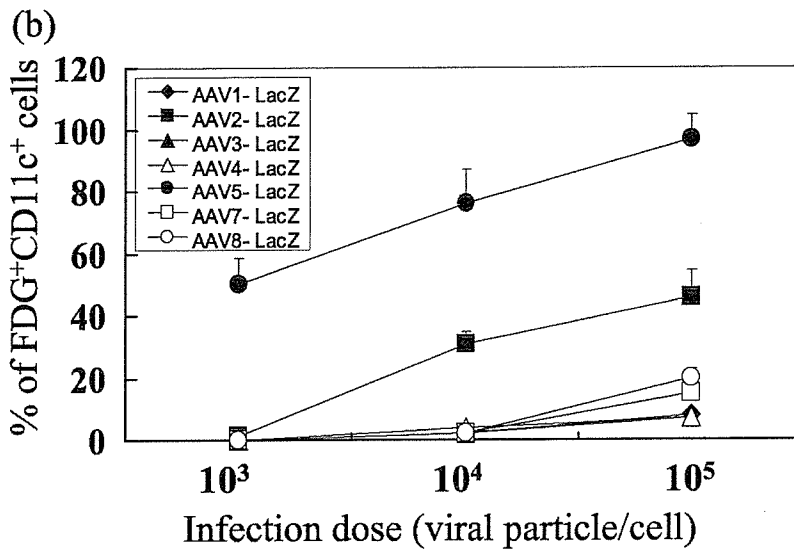
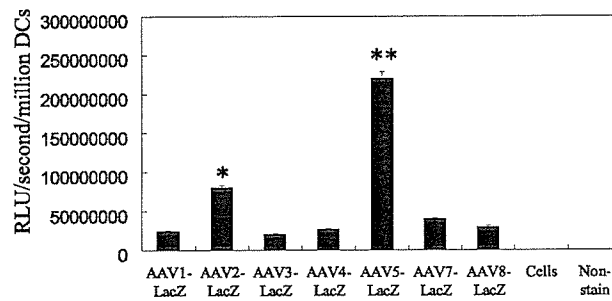
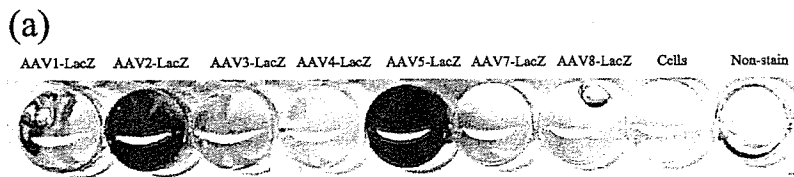


FIG. 1. Transduction of mouse muscle by AAV serotype vectors. AAV LacZ-expressing vectors (10^{10} vp) were injected intramuscularly into five mice/group. β -Galactosidase activity in the muscle was examined 2 weeks later using the Beta-Glo Assay System. An asterisk indicates a significant difference between the two groups ($P < 0.05$). RLU, relative light units.

immunodeficiency virus (HIV) Env gp160 in BALB/c mice, (ii) to compare the immunogenicities of AAV serotype vectors produced by HEK293 cells and insect cells, and (iii) to evaluate the mechanisms involved in the observed responses.

MATERIALS AND METHODS

AAV production. AAV vectors were generated as described previously (65, 66). In brief, a *lacZ* gene or a fragment containing HIV Env gp160 and Rev coding genes, which were derived from the HIV IIIIB strain, was subcloned into a shuttle vector containing the cytomegalovirus (CMV) promoter, poly(A), and the AAV2 inverted terminal repeat (the AAV5 inverted terminal repeat was used for construction of the AAV5 vector; the AAV2 inverted terminal repeat was used for other AAV serotype vectors). We included a Rev coding gene in the construct because expression of HIV Env gp160 is dependent on Rev protein. This approach resulted in increased HIV Env gp160 protein expression in vitro and enhanced immune responses against HIV Env gp160 in vivo (33). The recombinant shuttle vector was packaged by triple transfection of HEK293 cells with an adenovirus helper plasmid, a chimeric packaging construct in which the AAV2 Rep gene was fused to the cap gene derived from either AAV serotype, and a shuttle vector plasmid to produce pseudotypes AAV2-based AAV1-LacZ, AAV2-LacZ, AAV3-LacZ, AAV4-LacZ, AAV5-LacZ, AAV7-LacZ, and AAV8-LacZ or AAV1-HIV, AAV2-HIV, AAV3-HIV, AAV4-HIV, AAV5-



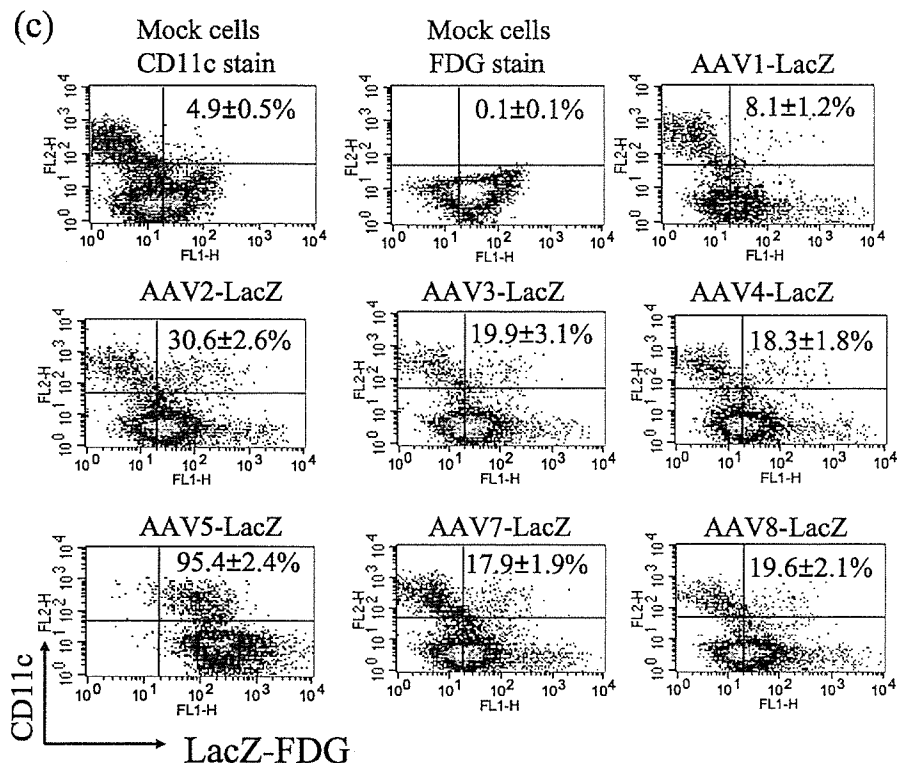


FIG. 2. Transduction of mouse purified DCs by AAV serotype vectors. (a) Mouse CD11c⁺ DCs were transduced with LacZ-expressing AAV vectors at 10⁴ vp/cell (triplicate for each sample). Two days after infection, the cells were stained with X-Gal (upper panel), and the β -galactosidase activity was measured using the Beta-Glo Assay System (bottom panel). The data presented were averaged from three separate experiments. The asterisk indicates a significant difference when AAV2-LacZ-transduced cells were compared to AAV1-LacZ-, AAV3-LacZ-, AAV4-LacZ-, AAV7-LacZ-, and AAV8-LacZ-transduced cells and mock-transduced cells; the double asterisk indicates a significant difference when AAV5-LacZ-transduced cells were compared to AAV2-LacZ-transduced cells. RLU, relative light units. (b) Mouse CD11c⁺ DCs were transduced with AAV-LacZ vectors at 10³ to 10⁵ vp/cell (triplicate for each sample). Two days after transduction, the cells were stained with X-Gal, and the percentages of LacZ-expressing DCs (FDG⁺ CD11c⁺ DCs) were determined. The data presented were averaged from three separate experiments. (c) Enriched immature DCs were transduced with 10⁵ vp/cell of various AAV-LacZ vectors (triplicate for each sample). Two days after transduction, the cells were treated with PE-conjugated anti-mouse CD11c Ab and FDG followed by flow cytometric analysis. The data provided represent the fraction of FDG/CD11c dual-positive cells as a percentage of the total population of CD11c⁺ cells. The data presented were averaged from three independent experiments.

HIV, AAV7-HIV, and AAV8-HIV vectors. The AAV vectors were purified by the standard cesium chloride sedimentation method (65, 66). The titer was determined by quantitative DNA dot blot hybridization.

The insect cell-produced AAV vectors (BacAAVs) were generated as described previously (58). The fragment containing the CMV promoter, HIV Env gp160 and Rev coding genes, and poly(A) was excised from the AAV shuttle vector by using NotI; the resulting fragment was inserted into the corresponding site of a baculovirus transfer plasmid between the serotype 2 or 5 inverted terminal repeats. Recombinant baculoviruses were generated by using the Bacto-Bac baculovirus expression system (Invitrogen, Carlsbad, CA). Recombinant baculoviruses containing the HIV Env gp160 and Rev coding genes; an AAV Rep of serotypes 1, 2, and 5; and an AAV1, AAV2, or AAV5 capsid were used to infect insect cells in order to produce BacAAV1-HIV, BacAAV2-HIV, and BacAAV5-HIV vectors, respectively. The AAV vectors produced were purified by two rounds of ultracentrifugation with a standard cesium chloride density gradient (65, 66). The titer was determined by quantitative DNA dot blot hybridization.

In vivo expression of β -galactosidase. The AAV-LacZ vector (10¹⁰ viral particles [vp]/mouse) was injected into mouse muscle. Two weeks after the administration, the mouse was sacrificed and the β -galactosidase activity in the muscle was monitored periodically from 1 week through 6 months after administration by using the Beta-Glo Assay System (Promega, Madison, WI).

Mouse DC preparation. DCs were isolated from BALB/c mouse bone marrow, as described previously (7, 62). In brief, the bone marrow was obtained from the tibia and femur of BALB/c mice. The DCs at a density of 5 × 10⁵ cells/ml were

cultured in RPMI 1640 medium containing 10% fetal calf serum, 1 ng/ml recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF; Kirin Beer Corp., Tokyo, Japan), and recombinant interleukin-4 (IL-4) for 6 days.

Transduction of AAV vectors to mouse cells. Immature mouse DCs were stained with phycoerythrin (PE)-conjugated anti-mouse CD11c antibody (clone N418; eBioscience, Boston, MA). The CD11c⁺ DCs were sorted using a MoFlo Cell Sorter (Takara Bio Corp., Tokyo, Japan). The cells were transduced with LacZ-expressing serotype AAV vectors at 37°C at 10³ to 10⁵ vp/cell for 2 days. The transduced cells were washed with phosphate-buffered saline (PBS) and stained with 40 mM X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) in staining buffer [5 mM K₃Fe(CN)₆, 5 mM K₄Fe(CN)₆, 2 mM MgCl₂ in PBS] at 37°C for 2 h. The β -galactosidase activity was detected by using the Beta-Glo Assay System (Promega). To count LacZ-expressing cells, the sorted CD11c⁺ DCs were treated with 1 μ M of fluorescein digalactoside (FDG; Molecular Probes, Eugene, OR) followed by flow cytometric analysis (the data are shown in Fig. 2b). The enriched, unsorted DCs were transduced with LacZ-expressing serotype AAV vectors at 37°C at 10⁵ vp/cell for 2 days. The cells were stained with anti-mouse CD11c antibody and treated with FDG followed by flow cytometric analysis (the data are shown in Fig. 2c).

The mouse DCs used in this study were derived from GM-CSF- and IL-4-treated bone marrow cells. To explore the efficiencies of transduction of AAV serotype vectors to hematopoietic cells, unpurified mouse splenocytes and bone marrow cells were transduced with LacZ-expressing serotype AAV vectors at 10⁵ vp/cell at 37°C for 2 days. The bone marrow cells were treated with 1 μ M of FDG, and splenocytes were treated with 1 μ M of FDG and PE-conjugated

anti-mouse CD3e (clone 145-2C11), PE-conjugated anti-mouse CD11b (clone M1/70), or PE-conjugated anti-mouse CD19 (clone MB19-1) (all antibodies were from eBioscience) followed by flow cytometric analysis for staining T cells, macrophages, and B cells, respectively.

Human DC preparation. To explore the transduction efficiencies of AAV serotype vectors to human cells, human peripheral blood mononuclear cells (PBMCs) and an enriched population of PBMC-derived immature DCs were prepared as previously described (67). Briefly, human PBMCs were isolated from the blood of a healthy person using Lymphopar I (Immune Biological Laboratory, Gunma, Japan) according to the manufacturer's instructions. Fresh human PBMCs at 3×10^6 cells/ml in RPMI 1640 medium were dispensed into individual wells of 12-well plates (1 ml/well), which had been previously coated with autologous plasma for 30 min at 37°C. The PBMC cultures were allowed to incubate at 37°C for 1 h. After gentle washing with serum-free RPMI 1640 medium, the adherent cells were cultured in Iscove's modification of Dulbecco modified Eagle medium (2 ml/well) containing human GM-CSF (500 ng/ml) and IL-4 (200 µg/ml) (both from Pierce Biotechnology, Rockford, IL) for 5 days.

Transduction of AAV vectors to human cells. The human PBMCs and an enriched population of PBMC-derived immature DCs were transduced with LacZ-expressing serotype AAV vectors at 10^5 vp/cell at 37°C for 2 days. The AAV-transduced DCs were treated with 1 µM of PDG, and AAV-transduced PBMCs were treated with PDG and PE-conjugated anti-human CD11c (clone 3.9), PE-conjugated anti-human CD3 (clone HIT3a), PE-conjugated anti-human CD11b (clone ICRF44), or PE-conjugated anti-human CD19 (J4-166) (all antibodies were from eBioscience) followed by flow cytometric analysis for staining DCs, T cells, macrophages, and B cells, respectively.

Western blot analysis. To confirm the expression of HIV Env gp160, HEK293 cells were transduced with the AAV vectors encoding the HIV gene in a six-well plate. Two days posttransduction, the cells were washed in PBS and lysed with 0.1 M Tris-HCl (pH 7.8) and 0.125% Nonidet P-40. The cell lysates were mixed with an equal volume of 2× sodium dodecyl sulfate buffer (125 mM Tris-HCl, pH 6.8, 4% sodium dodecyl sulfate, 20% glycerol, 0.01% bromophenol blue, and 10% β-mercaptoethanol) and boiled for 10 min. Then the cell lysates were loaded on an 8% polyacrylamide gel and transferred to a Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech, Buckinghamshire, England). The HIV gp160 and β-actin proteins were detected using a mouse anti-HIV gp120 monoclonal Ab (MAB) (hybridoma 902; AIDS Research and Reference Reagent Program, National Institutes of Health, Maryland) and an anti-human β-actin MAB (Sigma), respectively. An affinity-purified horseradish peroxidase-labeled anti-mouse immunoglobulin (Ig; ICN Pharmaceuticals, Inc., Solon, OH) was used as the secondary Ab. Protein was detected using the ECL Plus Western Blotting Detection System (Amersham Pharmacia Biotech, Uppsala, Sweden).

Animals and immunization. Eight-week-old BALB/c female mice were purchased from Japan SLC, Inc. (Hamamatsu, Japan). The mice were housed in the animal center located at Yokohama City University and maintained on a 12-h day-night cycle. The mice were intramuscularly (i.m.) immunized three times with 10^{10} particles of the AAV vector at 2-week intervals.

Enzyme-linked immunosorbent assay. The enzyme-linked immunosorbent assay was performed as described elsewhere (63, 65). To summarize, 96-well microtiter plates were coated with 1 µg/ml of HIV_{SF2} gp120 protein (donated by the AIDS Research and Reference Reagent Program, National Institutes of Health) and incubated overnight at 4°C. The wells were blocked with PBS containing 1% bovine serum albumin (BSA) for 2 h at room temperature. They were then treated with 100 µl of serially diluted antisera and incubated for an additional 2 h at 37°C. The bound immunoglobulin was quantified using an affinity-purified horseradish peroxidase-labeled anti-mouse antibody (Sigma). The mean antibody titer was expressed as the reciprocal of the serial serum dilution that exceeded the assay background by 2 standard deviations.

IFN-γ ELISPOT assay. Two weeks after the final immunization, a gamma interferon (IFN-γ) ELISPOT assay was performed as described previously (62). In brief, MultiScreen-IP plates (Millipore, Bedford, MA) were coated with 50 µl of 10-µg/ml purified rat anti-mouse IFN-γ antibody (XMG1.2; PharMingen, San Diego, CA) in PBS overnight at 4°C. The plate was then blocked with PBS containing 5% BSA and 0.025% Tween 20 for 2 h at room temperature. Lymphocytes (1×10^5 to 10×10^5) isolated from the spleen were added to each well in triplicate. The spleen cells were stimulated with or without 10 µg/ml of the HIV V3 peptide (RGPGRAFVTI) for 24 h at 37°C. After incubation, the cells were removed and incubated with 0.5 µg/ml of biotinylated anti-mouse IFN-γ antibody (PharMingen) for 2 h at 37°C, followed by the addition of 100 µl/well of 0.2% alkaline phosphatase-streptavidin (Vector Laboratories, Burlingame, CA) in PBS containing 0.05% Tween 20 and 5% BSA for 1.5 h. Finally, the plate was treated with 50 µl/well of 5-bromo-4-chloro-3-indolylphosphate-nitroblue tetrazolium membrane phosphatase (Kirkegaard and Perry Laboratories,

Gaithersburg, MD) at room temperature for 20 min, and the reaction was terminated by holding the plate under running distilled water. The number of spots was counted using a computer-assisted video image analyzer. The results were expressed as spot-forming cells (SFC) per million cells.

Tetramer assay. The tetramer assay was performed as described previously (62–65). The H-2D^d/p18 tetramer (RGPGRAFVTI) labeled with PE was prepared by ProImmune Limited (Oxford, United Kingdom). In brief, mouse splenocytes were incubated with 4% healthy mouse serum in PBS for 15 min at 4°C. The cells were stained with fluorescein isothiocyanate (FITC)-labeled anti-mouse CD8α (Ly-2; PharMingen) at 0.5 µg/10⁶ cells for 30 min at 4°C. After two washes in staining buffer (3% fetal calf serum, 0.1% NaN₃ in PBS), the cells were incubated with the tetramer reagent for 30 min at 4°C and analyzed by flow cytometry.

In vivo CTL assay. The assay was performed as previously described (52). Briefly, cytotoxic T-lymphocyte (CTL) target cells were derived from naïve splenocytes and then pulsed with 10 µg/ml of the HIV V3 peptide (RGPGRAFVTI) or with no peptide at 37°C for 1 h. Peptide-pulsed cells were labeled with a high concentration of carboxyfluorescein diacetate succinimidyl ester (CFSE; 5 µM; Molecular Probes, Eugene, Oreg.), or unpulsed cells were labeled with a low concentration of CFSE (0.5 µM). Cells were then washed and enumerated. For the in vivo CTL assay, 5×10^6 pulsed and 5×10^6 unpulsed cells were combined into a final 200 µl of PBS and intravenously injected into vaccinated mice. Mice were killed 24 h after in vivo target cell incubation. The mouse splenocytes were analyzed by flow cytometry. The percent in vivo killing of CFSE-labeled target cells was defined as the relative loss of such cells after in vivo incubation and thus was taken as the measure of CTL. It was calculated using the formula % killing = $[1 - (\text{unpulsed cells/peptide-pulsed cells from unimmunized control}) / (\text{unpulsed cells/peptide-pulsed cells from immunized group})] \times 100$.

Surface expression of HIV gp120 and ex vivo transduction. To investigate whether the AAV-HIV-transduced DCs could present the HIV peptide to T cells, mouse bone marrow-derived DCs were transduced with AAV-HIV vectors at 10^5 vp/cell at 37°C for 2 days. To explore whether HIV gp160 is expressed on the surface of DCs, the AAV-HIV-transduced DCs were stained with HIV gp120 MAb (hybridoma 902) followed by FITC-conjugated rabbit anti-mouse IgG and PE-conjugated anti-mouse CD11c antibody (clone N418; eBioscience). The cells were analyzed by flow cytometry. Enriched mouse DCs were transduced with the AAV-HIV vectors for 2 h and then washed with PBS, and 10^5 cells were intravenously administered to recipient BALB/c mice. One week after the administration, HIV-specific responses were detected by a tetramer assay.

Data analysis. All the values are expressed as means ± standard errors. Statistical analysis of the experimental data and controls was conducted using one-way factorial analysis of variance. Statistical significance was defined as a value of $P < 0.05$.

RESULTS

Different expression levels of AAV serotype vectors in mouse muscle. Muscular cells are one of the major targets transduced when viral vectors are delivered via the i.m. route. In this study, the relative efficiency with which various AAV serotype vectors were transduced and expressed in vivo was explored. Mice were injected i.m. with AAV serotype vectors carrying the *lacZ* gene. β-Galactosidase activity was monitored periodically from 1 week through 6 months after administration. Results show that the AAV1 and AAV7 vectors elicited significantly higher β-galactosidase activity than the AAV2, AAV3, and AAV4 vectors, with AAV5 and AAV8 yielding intermediate levels of expression at 2 weeks after administration (Fig. 1). We found that AAV expression was maximal 2 to 4 weeks after AAV vector administration, although β-galactosidase activity remained elevated for up to 6 months (data not shown).

Strong tropism of AAV5 for mouse DCs. The ability of each AAV serotype to transduce DCs was then examined. Enriched CD11c⁺ DCs were purified and incubated for 2 days with 10^4 vp/cell of each AAV serotype. Some cells were stained with X-Gal, and others were examined for β-galactosidase expression. As shown in Fig. 2a, very strong X-Gal staining was

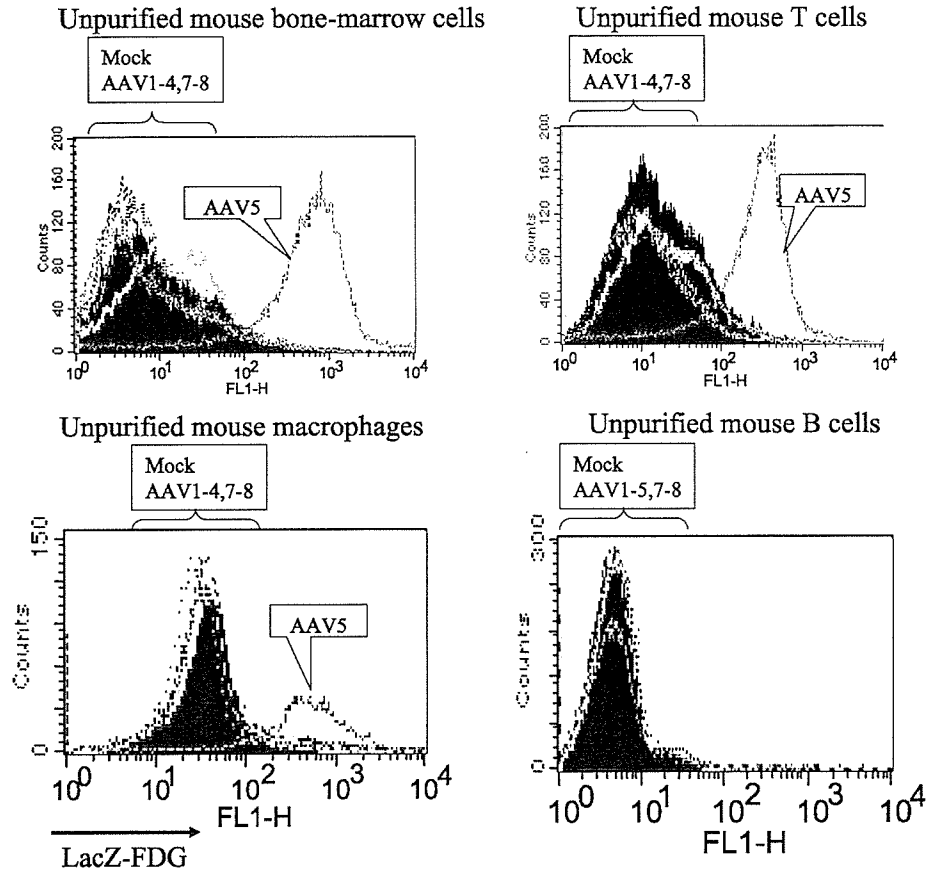


FIG. 3. Transduction of mouse unpurified bone marrow cells and splenocytes by AAV serotype vectors. Mouse bone marrow cells and splenocytes were transduced with 10⁵ vp/cell of AAV-LacZ vectors. Two days after transduction, the cells were treated with FDG and the splenocytes were stained with anti-mouse CD3 (T cells), anti-mouse CD11b (macrophages), or anti-mouse CD19 (B cells) antibody followed by flow cytometric analysis. Panels show results from one of three independent experiments.

observed in cells treated with AAV5 vector, weaker staining in those treated with AAV2, and none in cells exposed to the other AAV serotypes. Similar results were obtained when purified CD11c⁺ DCs were incubated with 10³ to 10⁵ vp/cell, and X-Gal expression was monitored by flow cytometry. As the dose of vector increased, up to 96.5% of DCs were transduced by the AAV5 vector and 45.7% by the AAV2 vector, but <10% for all other vectors (Fig. 2b). Similar results were observed using enriched bone marrow-derived immature DCs (Fig. 2c). Of note, the AAV5 vector strongly transduced both bone marrow-derived CD11c⁺ cells and CD11c⁻ cells (Fig. 2c). Further study demonstrated that AAV5 transduced most unpurified bone marrow cells, whereas AAV2 was much less effective and other AAV serotypes largely ineffective (Fig. 3). AAV5 was also effective at transducing T cells (>85% of CD3⁺ cells) and macrophages (>30% of CD11b⁺ cells), but less than 10% of T cells and macrophages were transduced by other AAV serotypes (Fig. 3). However, there was no significant difference in the abilities of various vectors to transduce B cells (<15%).

Strong tropism of AAV5 for human DCs. To explore the transduction efficiency of AAV5 for human target cells, DCs were prepared from human PBMCs. At an infection dose of

10⁵ vp/cell, 26.8% of enriched human CD11⁺ DCs were transduced by AAV5, but less than 5% of DCs were transduced by other serotypes (Fig. 4). The cellular tropism of AAV5 was also examined by flow cytometry. AAV5 infected 56.4% of CD3⁺ T cells (Fig. 4) and 23.5% of CD11b⁺ macrophages (Fig. 4). However, other serotype vectors were poorly transduced into both T cells and macrophages (<5%, Fig. 4). No significant difference in transduction of CD19⁺ B cells was observed among AAV serotype vectors (<10% of B cells). In general, human DCs, macrophages, and T cells were transduced less effectively by AAV5 than were murine cells, perhaps reflecting differences in AAV5 receptor expression between these two species.

Similar immunogenicities of AAV vectors produced by HEK293 cells and insect cells. Difficulty in producing high-titered AAV stocks has limited the clinical utility of this class of vectors. To resolve the problem, AAV vectors have been produced utilizing baculovirus in insect cells (42, 56, 58) and herpes simplex virus (8, 14). In this study, HEK293 cells were infected with 10⁵ vp/cell of AAV-HIV vectors and the expression of AAV-HIV vectors was confirmed by Western blotting (Fig. 5a). HIV gene expression by AAV1 and AAV2 vectors was slightly stronger than that by other serotype vectors. The

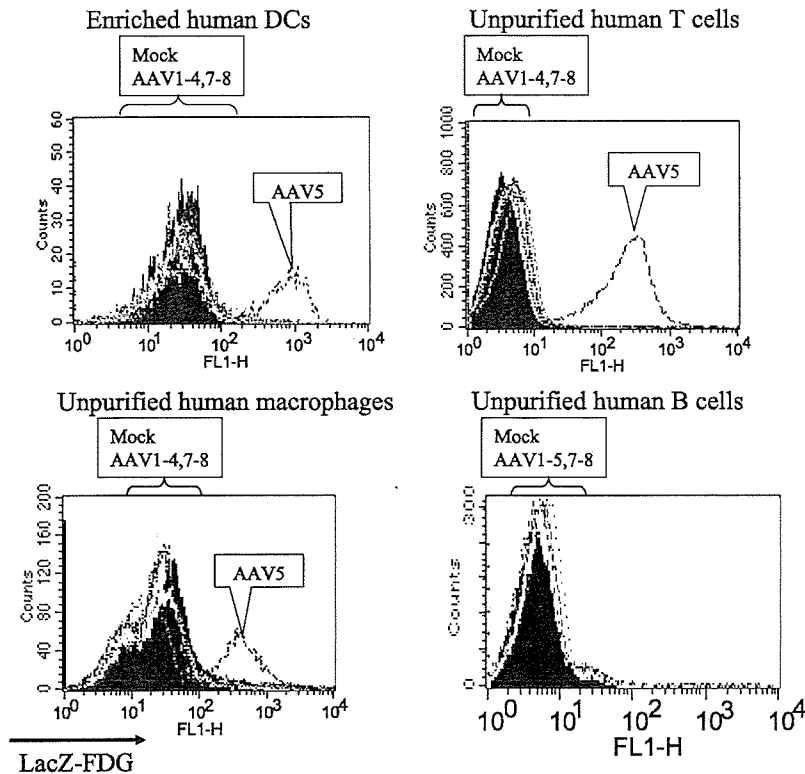


FIG. 4. Transduction of enriched human DCs and unpurified PBMCs by AAV serotype vectors. Human enriched DCs or PBMCs were transduced with 10^5 vp/cell of AAV-LacZ vectors. Two days after transduction, the DCs were treated with FDG and anti-human CD11c Ab, and PBMCs were treated with FDG and anti-human CD3 (T cells), anti-human CD11b (macrophages), or anti-human CD19 (B cells) antibody followed by flow cytometric analysis. Panels show results from one of three independent experiments.

abilities to produce three AAV serotypes (AAV1, AAV2, and AAV5) in insect cells and in HEK293 cells were compared. Expression levels of insect cell-produced AAV vectors similar to those of HEK293-produced corresponding AAV vectors were observed. Again, as seen in Fig. 6, similar cell-mediated immune responses were elicited by the corresponding AAV vectors. However, AAV vectors derived from insect cells were slightly more effective at inducing humoral immune responses than vectors derived from HEK293 cells (Fig. 5b). This may be due to vector contamination by baculovirus components, since baculovirus itself can have an adjuvant effect on vaccine-induced immune responses (1, 22).

AAV-HIV immunization stimulates a humoral immune response. To explore the immunogenicity of the AAV vectors, BALB/c mice were immunized three times with 10^{10} vp of various AAV-HIV vectors. The resultant HIV-specific serum Ab response was examined 1 month after the last boost. The strongest HIV-specific serum IgG response was generated in mice immunized with the AAV5-HIV vector; the poorest humoral responses were observed in mice immunized with AAV3-HIV or AAV4-HIV (Fig. 5b). AAV1-HIV, AAV7-HIV, and AAV8-HIV induced intermediate humoral responses (Fig. 5b).

AAV-HIV immunization stimulates a cellular immune response. The induction of HIV-specific IFN- γ -secreting cells was then examined in the immunized mice. As observed for humoral immunity, animals immunized with the AAV5-HIV

vector generated the strongest cellular immune response (1,500 SFC/million cells). The AAV1, AAV2, AAV7, and AAV8 HIV vectors also stimulated >300 IFN- γ -secreting SFC/million spleen cells, whereas the AAV3- and AAV4-HIV vectors elicited significantly weaker cellular responses ($P < 0.05$, Fig. 6a). The AAV5-HIV produced by either HEK293 or insect cells induced significantly higher cellular immune responses than the AAV1-HIV produced by corresponding cells.

A tetramer binding assay was used to identify major histocompatibility complex class I-restricted HIV-specific T cells (3). Mice immunized with the AAV1-, AAV5-, and AAV7-HIV vectors induced strong HIV-specific CD8⁺ T-cell responses ($>2\%$, Fig. 6b), and AAV2-HIV was intermediate (1.2%), while AAV3- and AAV4-HIV elicited only 0.4% HIV-specific CD8⁺ T cells.

An *in vivo* CTL assay was used to detect vaccine-elicited cell-mediated immune responses. Whereas the AAV3-, AAV4-, and AAV8-HIV vectors elicited approximately 40% CTL activity, the AAV1-, AAV5-, and AAV7-HIV vectors were significantly more effective (generally $>50\%$ activity), with the AAV2-HIV vector being intermediate (Fig. 6c).

DCs present HIV peptide to T cells *in vivo*. To examine whether DCs play a role in AAV-HIV-induced immune responses, DCs were enriched from the bone marrow of naïve mice. Enriched DCs were then treated with AAV5-LacZ, AAV1-HIV, AAV2-HIV, or AAV5-HIV vectors *in vitro* (10^5

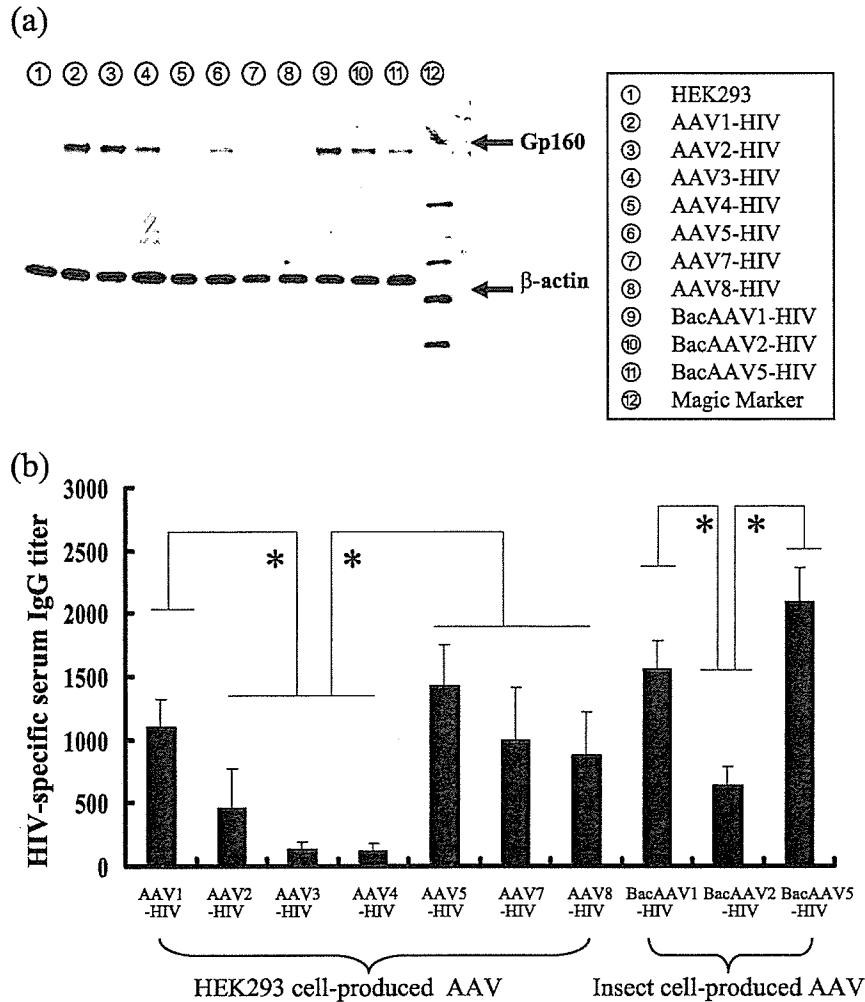


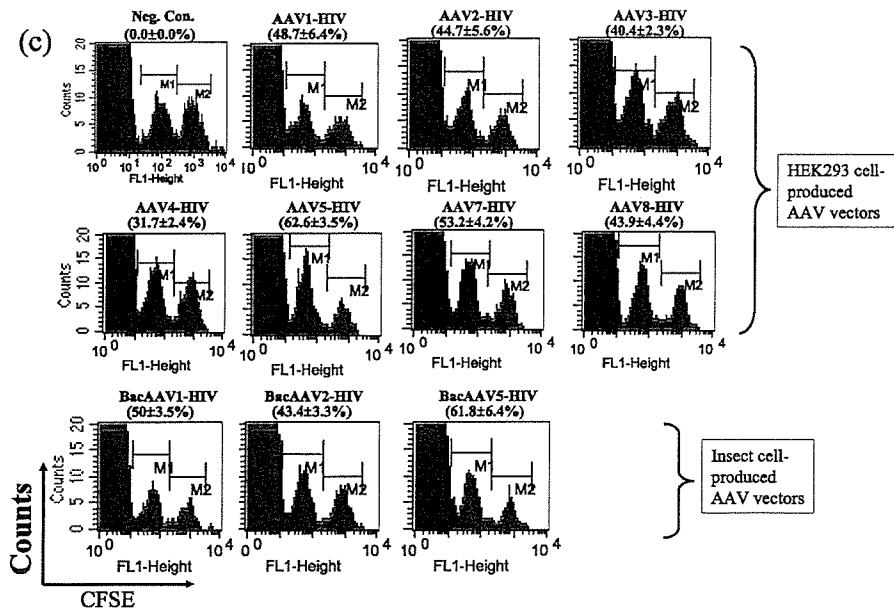
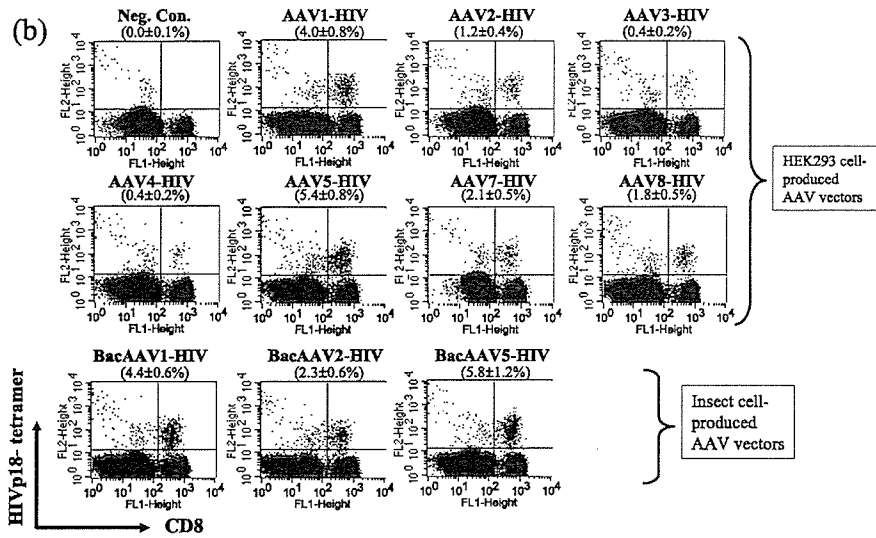
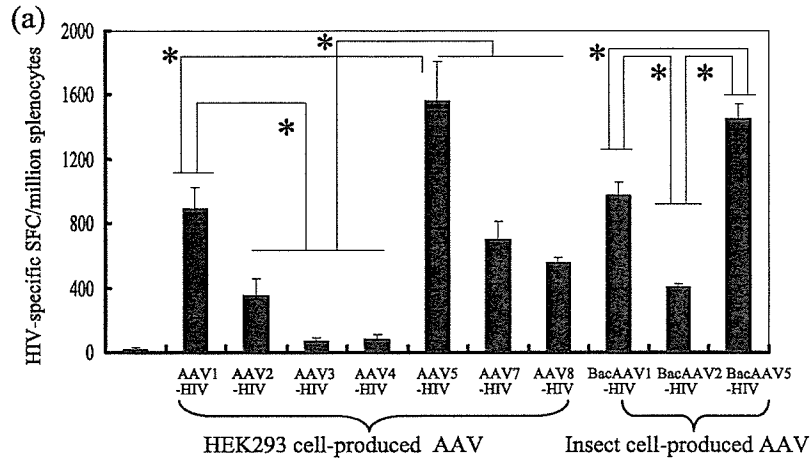
FIG. 5. Expression of HIV gp160 and HIV-specific serum IgG titer by AAV serotype vectors. (a) HEK293 cells were transfected with AAV vectors carrying the HIV Env gp160 gene. Two days after infection, cell lysates were analyzed by Western blotting using anti-HIV Env MAb and anti-human β -actin MAb. (b) BALB/c mice (five mice/group) were immunized with AAV-HIV vectors on days 0, 14, and 28. HIV-specific serum IgG was measured 1 month after the final immunization. The BacAAV vectors were generated in insect cells, whereas other vectors were produced in HEK293 cells. The asterisks indicate significant differences between the two groups ($P < 0.05$).

vp/cell). The expression of HIV gp160 protein on the surface of mouse DCs was confirmed by anti-mouse HIV gp120 monoclonal antibody and anti-mouse CD11c antibody staining followed by flow cytometric analysis. HIV gp160 protein was detected in 13.5%, 19.4%, and 65.2% of CD11c⁺ DCs which were transfected with AAV1-HIV, AAV2-HIV, and AAV5-HIV vectors, respectively (Fig. 7a). On the other hand, enriched mouse DCs were transfected with AAV-HIV vectors and injected into naive recipients. Seven days later, HIV-specific tetramer binding CD8⁺ cells were quantified using the tetramer assay. Recipients of the AAV2-HIV (0.6%)- and AAV5-HIV (2.1%)-transfected cells had significantly higher HIV peptide-binding CD8⁺ T cells than the AAV-LacZ-treated control (0.0%) (Fig. 7b). In contrast, a weak immune response was observed in recipients of AAV1-HIV vector-transfected cells (0.1%).

DISCUSSION

The present study demonstrates that AAV5 is a superior vector for achieving humoral and cellular immune responses, and the immunogenicity of AAV vectors depends on their tropism for both antigen-presenting cells (APCs) (such as DCs) and non-antigen-presenting cells (such as muscular cells).

Consistent with previous studies, AAV-HIV vectors of different serotypes had distinct effects on the induction of HIV-specific humoral and cell-mediated immune responses (10, 17, 20, 28, 31, 38, 40, 53, 65, 66). However, most of these studies examined the durability of transgene expression after i.m. administration of the AAV vector but did not typically monitor cell-mediated immune responses against the encoded antigen (19, 32, 61). In contrast to studies in which the AAV vector encoded a self protein (as in gene therapy) or in which a



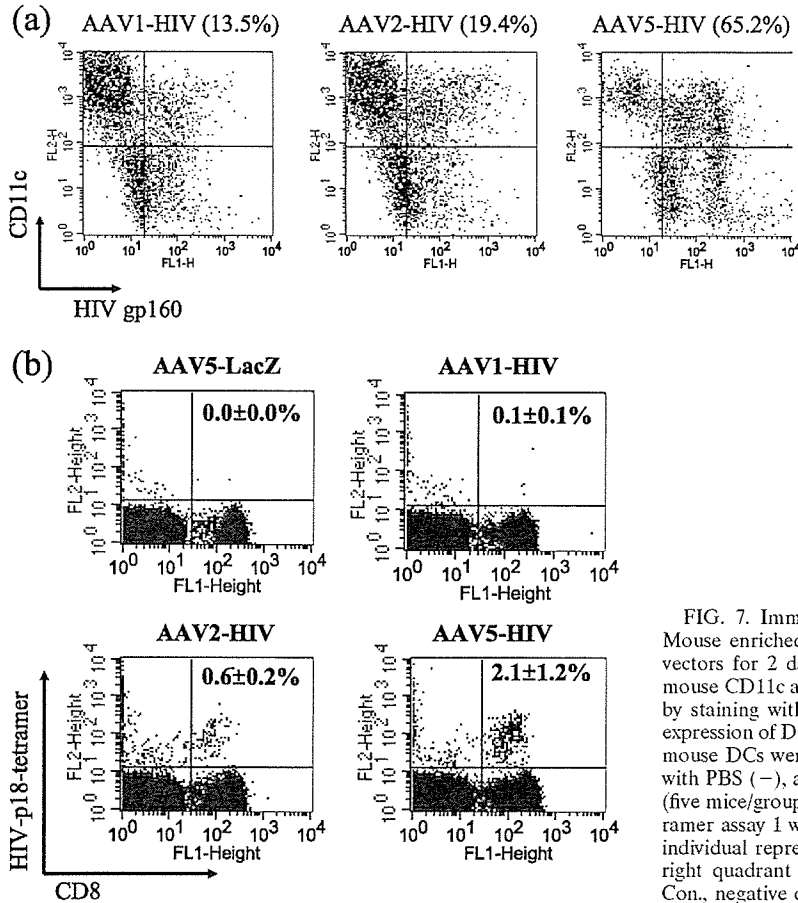


FIG. 7. Immune responses induced by ex vivo DC transduction. Mouse enriched DCs were transduced with 10^5 vp/cell of AAV-HIV vectors for 2 days. The cells were stained with PE-conjugated anti-mouse CD11c antibody plus mouse anti-HIV gp120 antibody followed by staining with FITC-conjugated anti-mouse IgG. The HIV gp160 expression of DC surface was analyzed by flow cytometry (a). Enriched mouse DCs were transduced with AAV-HIV vectors for 2 h, washed with PBS (-), and injected intravenously into recipient BALB/c mice (five mice/group). HIV-specific responses were detected using the tetramer assay 1 week after administration. Dot plots show results from individual representative animals, while the data shown in the upper right quadrant represent the averages of five mice/group (b). Neg. Con., negative control.

neoantigen was used to evaluate long-term expression (such as β -galactosidase), the immune response elicited by our HIV Env gp160-encoding vector was quite strong (10, 17, 20, 28, 31, 38, 40, 53, 65, 66).

Most currently used AAV vectors for vaccine or gene therapy are based on the AAV2 serotype (10, 17, 20, 28, 31, 38, 40, 53, 65, 66). The current work compared the immunogenicity of AAV2-HIV to that of other AAV serotype-based vectors and found that the AAV5 vector induced the strongest cellular and humoral immune responses. This finding may help in the development of novel AAV vector-based vaccines, particularly because natural immunity to AAV5 is rare (30). Of note, while we used a prime/boost regimen involving repeated administration of the same AAV serotype vector, it may be even more effective to prime with one serotype and boost with another (since neutralizing Abs raised against the first vector are unlikely to cross-react with a different serotype) (48).

Following vaccination with the AAV vector, antigen-specific immune responses can be induced through at least two pathways. In one of the pathways, the vaccine vector directly infects APCs, such as DCs, where the encoded antigen is expressed. The antigen is then processed and presented by APCs to T cells (Fig. 7) (65). The percentage of HIV gp160⁺ CD11c⁺ cells in Fig. 7a was lower than the percentage of FDG⁺ CD11c⁺ cells in Fig. 2c. This may be due to the presentation of HIV gp160 peptides by DCs present in culture. In the other pathway, the vaccine vector infects non-APCs, such as muscle cells. APCs take up the antigen expressed by the infected cells and present peptides from the exogenous antigen to T cells by major histocompatibility complex class I-restricted cross-presentation. It has been known that adenovirus vectors may use both pathways; however, AAV2 relies on the latter pathway due to a low efficiency of transfection of DCs (53). Current findings indicate that AAV1,

FIG. 6. HIV-specific cell-mediated immune responses induced by AAV vector vaccination. BALB/c mice (five mice/group) were immunized with AAV-HIV vectors on days 0, 14, and 28. Five mice per group for the IFN- γ ELISPOT and tetramer assay and five mice per group for the in vivo CTL assay were used. The HIV-specific IFN- γ ELISPOT assay was performed (a), the percentage of HIV-specific tetramer binding CD8⁺ cells was determined (b), and the in vivo CTL assay was performed (c) 2 weeks after the final immunization. BacAAV vectors were generated in insect cells, whereas other AAV vectors were generated in HEK293 cells. Asterisks indicate significant differences between the two groups ($P < 0.05$). Panels b and c show results from individual representative animals, while the data represent the averages of five mice/group.

AAV7, and AAV8 vectors preferentially infect muscle cells, whereas the AAV5 vector optimally infected DCs (Fig. 1 to 4 and 7). Following i.m. administration, all four vectors induced higher HIV-specific immunogenicity than the AAV2 vector (Fig. 5 and 6).

DCs are primarily responsible for stimulating resting naive T lymphocytes and initiating a CTL response (5). Immature DCs residing in the peripheral tissues capture foreign antigens, mature, and then migrate to secondary lymphoid organs, where the processed antigen is presented. To determine whether DCs might contribute to the immune responses induced by the AAV-HIV vector, enriched DCs were transduced with the AAV5-HIV vector and transferred into naive mice. As seen in Fig. 7b, recipients generated HIV-specific tetramer binding CD8⁺ T cells. These results demonstrate that DCs can present vector-encoded antigen to naive T cells efficiently. In this context, the levels of immune response induced by the AAV1-HIV, AAV2-HIV, and AAV5-HIV vectors correlated with their tropism for DCs (70).

A number of studies show that recombinant AAV vectors can be used to transduce DCs (2, 13, 32, 35, 39, 46, 49, 53, 70). Current results document that AAV5 can transfect mouse and human DCs much more efficiently than other AAV serotype vectors (Fig. 2 to 4). In addition, AAV5 was efficiently transduced into T cells and macrophages (Fig. 3 and 4), which may be useful for gene therapy of diseases involving hematopoietic cells (4, 26, 43, 57, 59, 69).

Eight AAV serotypes (AAV1 to AAV8) have been described, with each serotype having unique binding and cell tropism characteristics (6, 11, 12, 21, 44, 50, 60). A primary coreceptor for AAV5 is sialic acid, while the platelet-derived growth factor receptor is involved in the binding and cell entry of AAV5 (15). Platelet-derived growth factor receptor is mainly expressed on fibroblasts, smooth muscle cells, glial cells, and chondrocytes but not on hematopoietic cells. Surprisingly, we found that AAV5 was efficiently transduced into hematopoietic cells (Fig. 3 and 4), suggesting that another receptor(s) may be used by AAV5 for cell entry.

The AAV2 vector can be produced in insect cells by using baculovirus expression vectors in suspension culture, a strategy that is amenable to easy scale-up (58). In this study, AAV1, AAV2, and AAV5 vectors expressing the HIV gp160 gene were prepared using either insect cells or HEK293 cells. Similar immune responses were obtained using vectors produced by either method (Fig. 5b and 6), indicating that insect cell-produced AAV vectors should be considered for the large-scale preparation of AAV vectors.

In summary, this is the first report that systematically compares DC tropism and immunogenicity of AAV serotype vectors. Results show that AAV5 vectors can efficiently transduce DCs and produce stronger antigen-specific immune responses than other AAV vectors. Thus, AAV5-based vectors deserve further consideration for clinical vaccine development and immune therapy.

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Adipose Tissue as a Novel Target for *In Vivo* Gene Transfer by Adeno-Associated Viral Vectors

HIROAKI MIZUKAMI,¹ JUN MIMURO,² TSUYOSHI OGURA,¹ TAKASHI OKADA,¹ MASASHI URABE,¹ AKIHIRO KUME,¹ YOICHI SAKATA,² and KEIYA OZAWA¹

ABSTRACT

Traditionally, skeletal muscle and liver are the preferred target organs for gene transfer to supply a transgene product into the systemic circulation. In this respect, adipose tissue presents a number of attractive features. However, adipose tissue transduction *in vivo* has not been feasible by conventional methods. To solve this issue, we tested the utility of excipients in adeno-associated virus (AAV) vector-mediated gene transfer and found that Pluronics are suitable for this purpose. In a histological analysis of adipose tissue in *db/db* mice, Pluronic F88 showed the greatest augmentative effect on β -galactosidase expression in combination with the AAV1 vector. When the vector encoding mouse erythropoietin (Epo) was used in the same manner, increased plasma Epo concentrations were observed (230 ± 80 versus 58 ± 14 mU/ml). Moreover, the plasma Epo concentration returned to the normal level after the surgical removal of transduced adipose tissue. No damage was observed in the transduced tissue. Our results indicate that the proposed method is safe and efficient for gene transfer into adipose tissues, thus providing an alternative for supplemental gene therapy.

OVERVIEW SUMMARY

Adipose tissue holds promise as an alternative depot organ in gene transfer approaches. However, no efficient method of gene transfer into adipose tissue *in vivo* has been established. In this study, we explored the utility of excipients to augment gene transfer into the adipose tissue of mice and found that Pluronic F88 was useful for this purpose when combined with AAV serotype 1 vectors. The improvement was also demonstrated with vectors encoding murine erythropoietin, and the mice became polycythemic. Moreover, after removing transduced adipose tissue, plasma erythropoietin levels returned to normal, which suggests the unique advantage of this method.

INTRODUCTION

IN SUPPLEMENTAL GENE THERAPY, skeletal muscle and the liver have been the preferred targets for gene transfer to supply transgene products into the systemic circulation. However, adipose tissue presents a number of attractive features. Adipose

tissue can be found throughout the body and is easily accessible for vector injection. Increasing evidence supports the notion that adipocytes are designed to secrete numerous factors into the systemic circulation (Mohamed-Ali *et al.*, 1998). Further, the majority of adipocytes are considered to be nondividing, which is suitable for achieving long-term expression of transferred genes by the use of nonintegrating vectors such as adeno-associated virus (AAV) vectors (Russell and Kay, 1999). Moreover, the transduced tissue can be safely removed when unexpected events occur, thus adding a unique feature to safety considerations. However, adipose tissue transduction has not been feasible through conventional methods, and few studies have investigated its efficacy *in vivo* (Nagamatsu *et al.*, 2001; Ogata *et al.*, 2004). To overcome these limitations and develop a more practical method, we tested the usefulness of excipients for gene transfer. To achieve efficient and widespread gene transfer, it is essential to assure that the vectors stay within the target tissue for a certain period of time. For this purpose, non-ionic surfactants are promising because they have low toxicity and unique features that help stabilize the membrane (American Pharmaceutical Association [AphA] and Royal Pharmaceutical Society of Great Britain [RPSGB], 1986). Moreover,

¹Division of Genetic Therapeutics and ²Division of Cell and Molecular Medicine, Center for Molecular Medicine, Jichi Medical University, Tochigi 329-0498, Japan.

improved gene delivery with Pluronic compounds was demonstrated in various applications (Kabanov and Alakhov, 2002; Kabanov *et al.*, 2005). Therefore, we tested the effects of these compounds on *in vivo* gene transfer targeting adipose tissue.

MATERIALS AND METHODS

Cells and plasmids for AAV vector preparation

HEK293 cells, a human embryonic kidney cell line, were maintained as described in a previous report (Fan *et al.*, 1998). Murine erythropoietin (Epo)-encoding plasmids have already been described by Mochizuki *et al.* (2004). AAV vectors of serotype 1 through 5, which encode LacZ or Epo and have a cytomegalovirus (CMV) promoter, were prepared by transient transfection, purified and quantitated as described previously (Matsushita *et al.*, 1998; Mochizuki *et al.*, 2004). Usually, the stocks of AAV vectors contained approximately $1-2 \times 10^{10}$ genome copies/ μl .

In vitro assessment of potential toxicity of surfactants

A panel of Pluronics was provided by Asahi Denka (Tokyo, Japan). Tween 80 was obtained from Sigma-Aldrich (St. Louis, MO). The surfactants were dissolved in distilled H₂O to prepare stock solutions at a concentration of 20%. To assess the potential compatibility, we added various concentrations of these surfactants to cultured 293 cells at the time of confluency. After 24 hr, the cells were examined microscopically. The highest concentrations of the surfactants that did not produce any deleterious effect on these cells were tested. The effect of the surfactants on AAV vector capsids was also examined by including a 10% surfactant in the vector stocks (AAV-LacZ with serotype 2 capsid, 2×10^{10} VG/ μl) for 24 hr; subsequently, the vector solutions were added to the cultured 293 cells in a 96-well plate at a dose of 2×10^4 VG/cell. The final concentration of the surfactants within the culture medium was 0.05%. Two days later, the infectivity was assessed by 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) staining (Fan *et al.*, 1998).

Analysis of gene expression and of enhancing effect in vivo

For the *in vivo* experiments, the AAV vector solutions containing various concentrations of Pluronic F68, F88, and Tween 80 were injected into the subcutaneous adipose tissues of C57BLKS/J *db/db* mice (Japan SLC, Hamamatsu, Japan) at the age of 10 to 12 weeks. At the time of injection, the average weight of the animals was more than 50 g, and the subcuta-

neous adipose tissues were well developed. The AAV-CMV-LacZ vectors with various serotypes (serotype 1–5) were tested at a dose of 6×10^{10} VG/body. Two weeks after injection, the mice were killed, and their adipose tissues were enucleated, stained with 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal), cut into pieces, and then frozen with Tissue-Tek optimal cutting temperature (O.C.T.) compound (Sakura Finetek, Torrance, CA) in dry ice–ethanol, as described (Ogata *et al.*, 2004). When the tissue blocks were sliced for microscopic examination, they were also evaluated macroscopically and photographed. A densitometric analysis of the blocks was performed in order to substantiate the difference in the efficiency of transduction of adipose tissue under various injection conditions. In practice, five areas of the corresponding blocks were chosen and quantified, using Image Gauge software (version 3.0; Fuji Photo Film, Tokyo, Japan). After subtracting the background value, these numbers were analyzed and the statistical significance was evaluated. Experiments were performed with AAV-Epo vectors under conditions optimized with the AAV-LacZ vectors. Later, experiments at a higher vector dose (2×10^{11} VG/body) were included. On the basis of the preliminary experiments, the volume of injection was optimized as 100 μl /body (50 μl per lobe) and kept constant thereafter. Whole blood was collected from the tail vein every 2 weeks. Plasma Epo concentrations were quantified with an enzyme-linked immunosorbent assay (ELISA) kit (Roche Diagnostics, Mannheim, Germany). Complete blood counts were performed with a PC-608 particle counter (Erma, Tokyo, Japan).

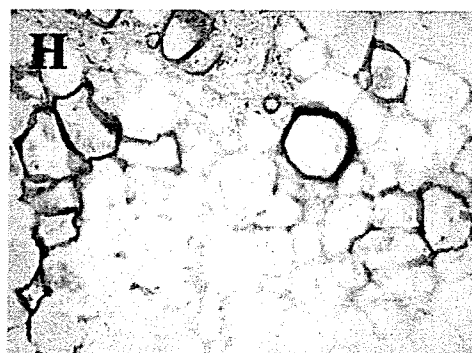
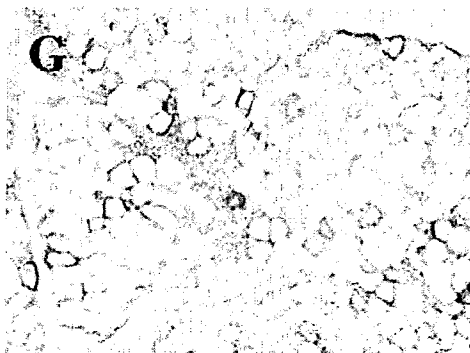
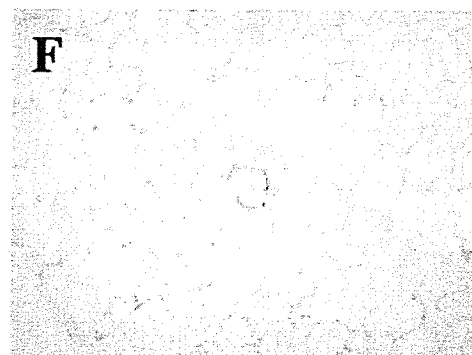
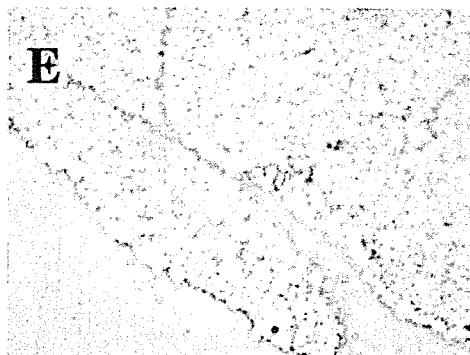
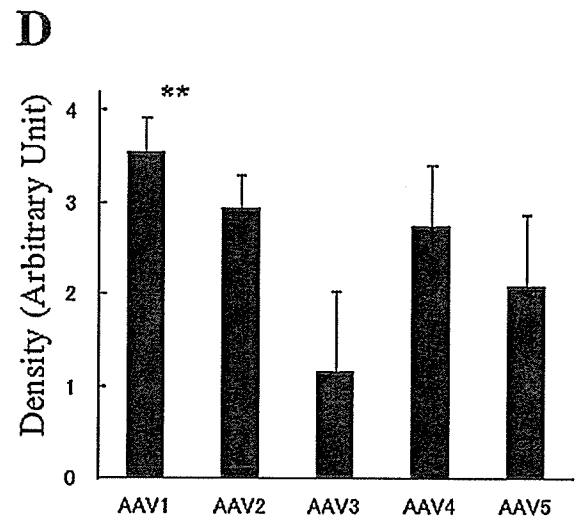
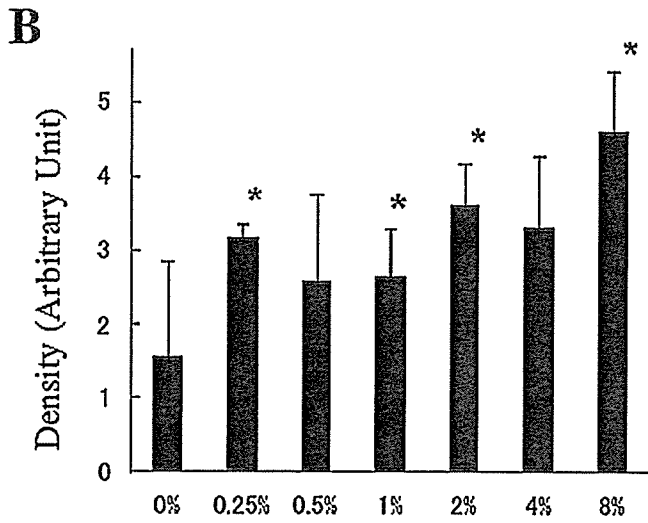
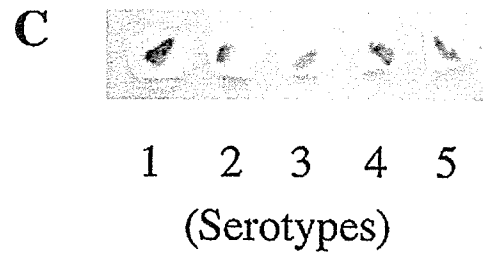
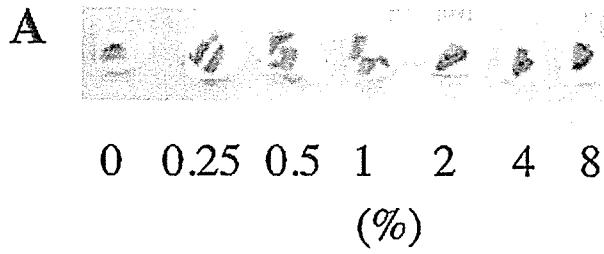
Detection of murine Epo within adipose tissue of db/db mice

Adipose tissues were obtained from *db/db* mice at the time of sacrifice or later by operation. For the immunofluorescence study, adipose tissues were fixed and frozen in the presence of the O.C.T. compound in dry ice–ethanol, following which they were reacted with goat anti-human Epo (Santa Cruz Biotechnology, Santa Cruz, CA) and donkey anti-goat IgG conjugated with Alexa 488 (Invitrogen Molecular Probes, Eugene, OR) at 4°C for 16 hr to visualize murine Epo by fluorescence microscopy, as described in a previous report (Ogata *et al.*, 2004).

Detection of murine Epo transcript within adipose tissue by reverse transcription-polymerase chain reaction

Total RNA was isolated from adipose tissue with an RNeasy lipid tissue kit (Qiagen, Hilden, Germany) and was converted to

FIG. 1. Distribution of LacZ expression within adipose tissues. AAV-CMV-LacZ vectors of various serotypes (serotypes 1–5) were tested ($n = 3$ for each serotype) at a dose of 6×10^{10} VG/body. Two weeks after injection, adipose tissues were removed, stained with X-Gal, and resected in pieces. Tissues were frozen with Tissue-Tek and then cut into 20- μm -thick slices for microscopic analysis. Blocks were also evaluated macroscopically, photographed, and analyzed by densitometry. (A) Representative blocks of adipose tissue injected with various concentrations of Pluronic F88. (B) Densitometric analysis of the corresponding blocks with various concentrations of Pluronic F88. Asterisks indicate statistical significance ($p < 0.05$) compared with values of tissues without F88 (0% data). (C) Blocks of adipose tissue injected with serotypes of AAV-LacZ vectors with 2% F88. (D) Densitometric analysis of blocks corresponding to the serotypes of AAV-LacZ vectors. Double asterisks indicate statistical significance ($p < 0.05$) relative to values obtained with the rest of the serotypes. Microscopic analysis of adipose tissue sections transduced with AAV1-LacZ without excipients is shown at low (E) and high (F) magnifications. Adipose tissues transduced with AAV1-LacZ in the presence of 2% Pluronic F88 are shown at low (G) and high (H) magnifications.



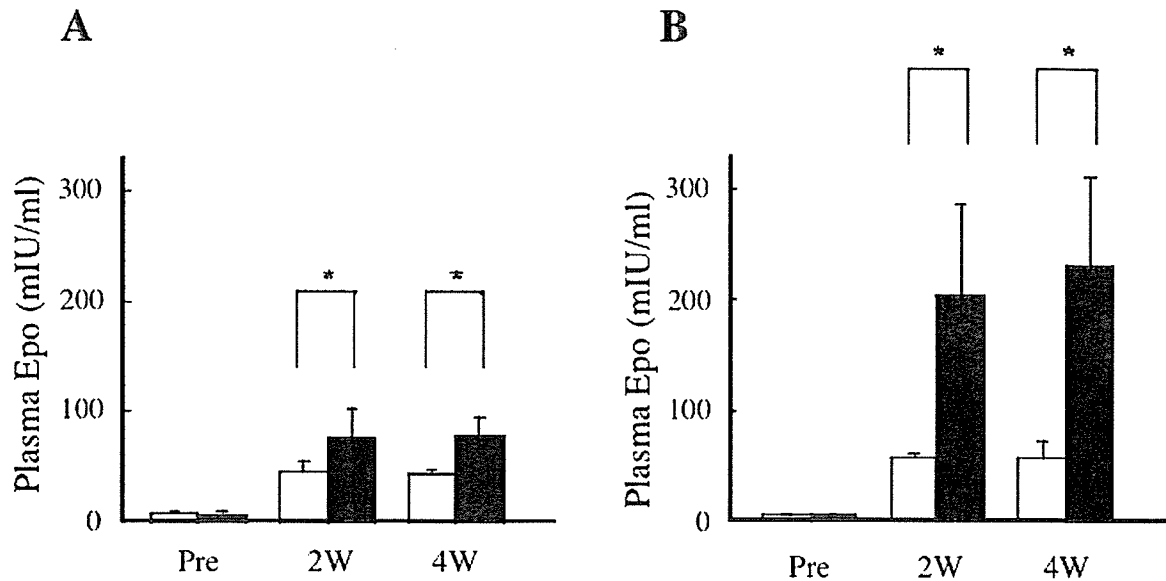


FIG. 2. Plasma Epo concentrations in *db/db* mice after injection of AAV1-Epo vectors into adipose tissue at a dose of (A) 6×10^{10} or (B) 2×10^{11} VG/body. Open and solid columns represent groups without and with 2% Pluronic F88, respectively, at the time of vector injection into adipose tissue. Each column and error bar indicate, respectively, the mean and SD of the group ($n = 5$). Asterisks indicate significance ($p < 0.05$).

cDNA with reverse transcriptase (SuperScript; Invitrogen, Carlsbad, CA) and oligo(dT) primers in a 20- μ l mixture after DNase I (amplification grade; Invitrogen) treatment according to the manufacturer's instructions. Subsequent polymerase chain reaction (PCR) amplification was carried out with 1 μ l of cDNA solution in a 50- μ l reaction mixture containing 5 units of *Taq* polymerase, 10 mM Tris-HCl (pH 8.5), 50 mM KCl, 1.5 mM MgCl₂, and 100 μ M dNTPs in the presence of specific primer pairs (200 nM) designed to amplify DNA fragments derived from the transcript of the mouse Epo transgene. Each PCR cycle involved denaturation at 94°C for 15 sec, annealing at 56°C for 30 sec, and extension at 72°C for 30 sec. The PCR products were analyzed by agarose gel electrophoresis. The authenticity of the PCR products was confirmed by observing their molecular sizes after agarose gel electrophoresis and by sequencing (ABI PRISM 310 genetic analyzer; Applied Biosystems, Foster City, CA). The primer sequences for mouse Epo were 5'-GTG CAG AAG GTC CCA GAC TGA GTG A-3' and 5'-TTG GCG TAG ACC CGG AAG AGC TTG-3'. The primers for mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Clontech Laboratories (Palo Alto, CA).

Removal of transduced tissue

AAV1-Epo vector solution (2×10^{11} VG/body) including 2% Pluronic F88 was injected into the subcutaneous adipose tissues of *db/db* mice. After 4 weeks, the adipose tissues were removed by standard surgical techniques under anesthesia. Plasma Epo concentrations were followed up 2 weeks thereafter. Four animals were analyzed and monitored.

Data and statistical analysis

Data are presented as means \pm SD and were analyzed by Student *t* test after confirming an insignificant difference in

variance between groups. $p < 0.05$ was considered statistically significant.

RESULTS

Screening of excipients in vitro

First, *in vitro* screening experiments were performed using nonionic surfactants. We tested Pluronic F68, F88, L72, P85, and Tween 80 for their effects on cell culture. Pluronic F68 and F88 were innocuous to cultured 293 cells at concentrations of up to 10 and 0.3%, respectively. The rest of the surfactants showed deleterious effects on the cultured cells when included at concentrations of 0.1% or higher. Next, we incubated AAV2-LacZ vectors with up to 10% Pluronic F68 and F88 solutions for 1 hr and checked their infectivity in 293 cells at 1×10^5 VG/cell. There were no differences in the infectivity of the vectors treated with these excipients, as assessed by X-Gal staining (data not shown).

Assessment of LacZ expression by use of Pluronics

On the basis of general safety data and *in vitro* experiments, we selected Pluronic F68, Pluronic F88, and Tween 80 as candidates for vector injection into *db/db* mice. Because preliminary experiments indicated the usefulness of Pluronic F88 combined with the AAV1 vector, we tested the usefulness of F88 at various concentrations. Comparison of blocks showed that there appeared to be an augmentation of LacZ expression with increasing concentration (Fig. 1A). The enhanced expression was substantiated by densitometric analysis of the blocks (Fig. 1B). On the basis of the result, we compared the usefulness of serotypes 1 through 5 combined with 2% Pluronic F88. The result showed that AAV1 was the most suitable serotype to trans-

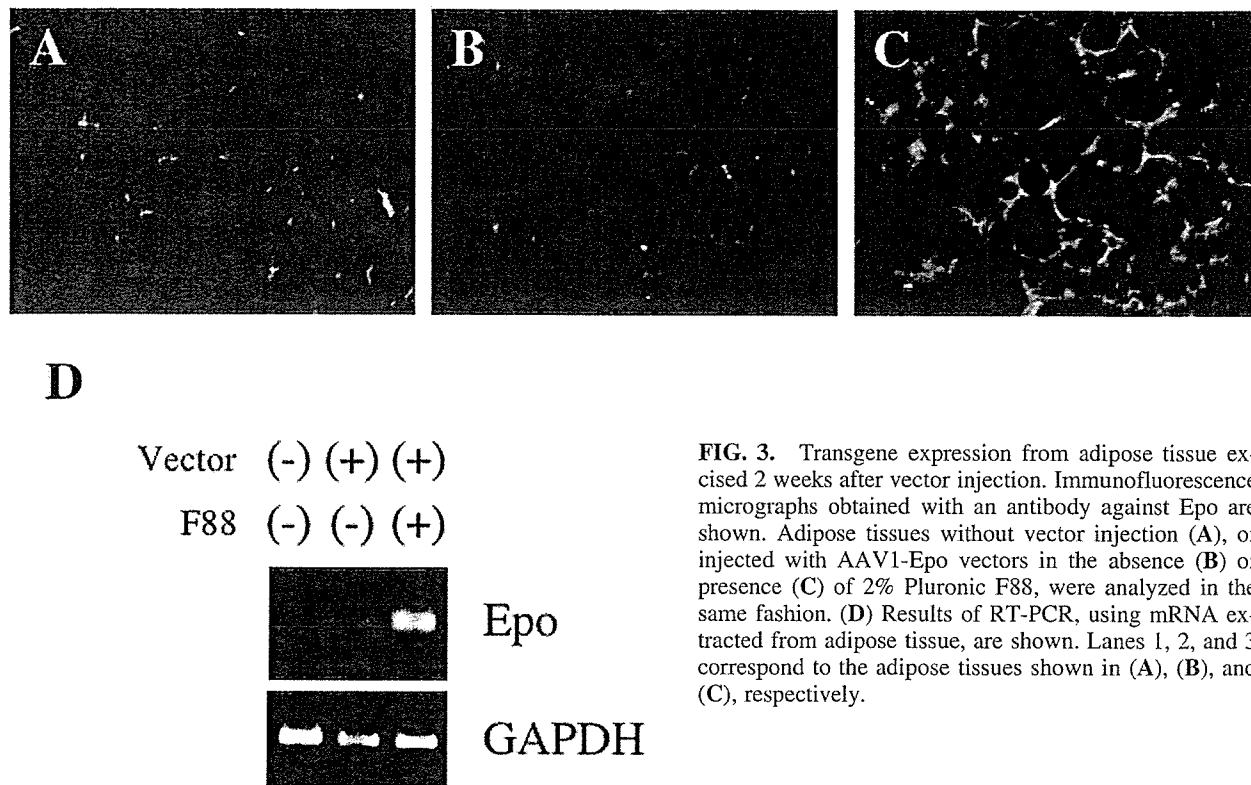


FIG. 3. Transgene expression from adipose tissue excised 2 weeks after vector injection. Immunofluorescence micrographs obtained with an antibody against Epo are shown. Adipose tissues without vector injection (A), or injected with AAV1-Epo vectors in the absence (B) or presence (C) of 2% Pluronic F88, were analyzed in the same fashion. (D) Results of RT-PCR, using mRNA extracted from adipose tissue, are shown. Lanes 1, 2, and 3 correspond to the adipose tissues shown in (A), (B), and (C), respectively.

duce adipose tissue *in vivo* (Fig. 1C and D). Enhancement of LacZ expression was also observed microscopically in the presence of Pluronic F88 (Fig. 1G and H) when compared with that of the vector alone (Fig. 1E and F).

Assessment of murine erythropoietin expression by use of Pluronic F88

On the basis of the findings obtained with LacZ vectors, we used vectors encoding murine erythropoietin (Epo) to demonstrate the enhancement of Epo concentration in a more quantitative manner. Increased plasma Epo concentration was observed 2 and 4 weeks after vector injection, and there was enhancement of Epo in the presence of 2% Pluronic F88 at a dose of 6×10^{10} VG/body (Fig. 2A). At a higher dose of 2×10^{11} VG/body, the enhancement effect was more prominent (230 ± 80 versus 58 ± 14 mU/ml at 4 weeks; Fig. 2B).

Assessment of transgene expression within adipose tissue

In the histological analysis of transduced tissues, a significant enhancement of Epo expression was observed by immunofluorescence when 2% F88 was included in the vector solution (Fig. 3A–C). Results of reverse transcription (RT)-PCR also showed enhanced expression of murine Epo in the presence of 2% F88 (Fig. 3D). Tissue damage or cellular infiltrates were not observed in the transduced adipose tissues throughout the histological evaluation (data not shown).

Effect of removal of transduced tissue

Bilateral lobes of abdominal adipose tissue were selected as a target for transduction. Plasma Epo concentrations were monitored after injection of AAV1-Epo vector (2×10^{11} VG/body) with 2% F88. After 4 weeks of observation, both lobes of the adipose tissue were removed *en bloc*, using standard surgical techniques. A significant decrease in plasma Epo concentration was observed 2 weeks after operation, with a return to the baseline level (Fig. 4). In addition, mice after removal of transduced tissue showed extended survival compared with “non-operated” mice. The survival period of *db/db* mice is summarized in Table 1.

DISCUSSION

In this study, we demonstrated the advantages of using excipients in adipose tissue transduction with AAV vectors. In practice, Pluronics have been widely used as excipients, including for administration to humans (Apha and RPSGB, 1986). Generally, one of the most frequent complications associated with administering a surfactant *in vivo* is hemolysis. With regard to this, it is noteworthy that Pluronics have a membrane-protecting effect on erythrocytes and that Pluronic F68 has long been used as a drug to prevent hemolysis and thrombotic events during extracorporeal circulation (Wright *et al.*, 1963). Further, their efficacy for use in the treatment of vasoocclusive disease in sickle cell anemia is currently being evaluated (Gibbs and Hagemann, 2004). Pluronics are also used in

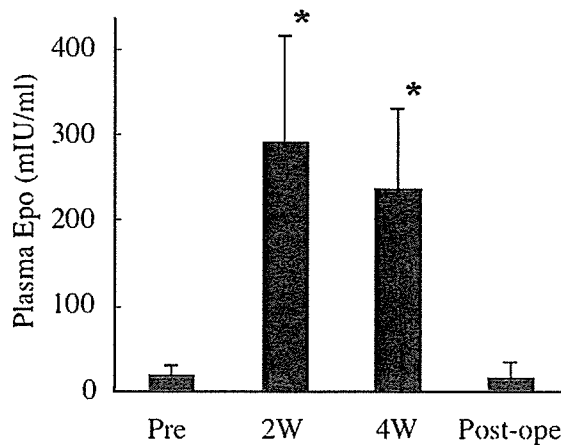


FIG. 4. Plasma Epo concentrations of *db/db* mice after injection and removal of adipose tissue. AAV1-Epo vectors were injected into adipose tissue at 2×10^{11} VG/body with 2% Pluronic F88. Four weeks after injection, transduced adipose tissues were surgically removed and monitored for an additional 2 weeks. Columns and bars indicate, respectively, mean \pm SD of the groups ($n = 4$ each). Asterisks indicate significance ($p < 0.05$) relative to concentrations before injection. No significant differences in concentrations were observed 2 weeks after the operation relative to concentrations before injection.

a variety of applications in gene therapy; for example, they are used to augment gene transfer into cultured cells (Gebhart and Kabanov, 2001), to protect skeletal muscle membranes at the time of electroporation *in vivo* (Lee *et al.*, 1992; Hartikka *et al.*, 2001), and to enhance adenovirus-mediated gene transfer into the lungs (Croyle *et al.*, 2001). In addition, it has been postulated that adding a low concentration (0.01%) of Pluronic F68 into the stocks of AAV vectors prevents vector loss by reducing nonspecific attachment (Sommer *et al.*, 2003).

In the present study, the efficiency of gene transfer into adipose tissue was augmented by the use of Pluronic F88. The mechanisms behind this phenomenon are not clear; however, it is known that Pluronics improve the distribution of a solution and its content (APhA and RPSGB, 1986). In a previous study, one member of the Pluronics family (poloxamer 407) was shown to improve the efficiency of adenovirus-mediated gene transfer to arterial smooth muscle cells (Feldman *et al.*, 1997). A reduction in incubation time from 20 to 10 min to attain the same level of gene transfer by including this excipient was also demonstrated. The following two possible mechanisms were postulated for the increase in efficiency of gene transfer: (1) the formation of a transient local reservoir for the sustained release of adenoviral vectors, or (2) acceleration of the uptake of adenoviral vectors produced by the interaction between poloxamer 407 and the cell membrane. On the basis of our observations concerning LacZ expression within adipose tissues (Fig. 1), it can be said that vector distribution was improved and that uptake of the vectors was facilitated. The augmentation was most significant in the case of AAV1 vectors, as assessed on the basis of both LacZ and Epo. There may be a specific advantage in combining the AAV1 capsid with Pluronic F88 when transducing adipose tissue.

A novel action of Pluronics has been reported (Sriadibhatla *et al.*, 2006). In this literature, transcriptional activation of transgenes driven by the CMV promoter or nuclear factor- κ B (NF- κ B)-responsive elements was demonstrated in the presence of Pluronics. As all the vectors in our current study used the CMV promoter, transcriptional activation through this mechanism might be a concern. Therefore, this issue needs to be taken into consideration. In the literature, all the transcriptional activation was observed in *in vitro* experiments, and Pluronics were continuously present within the culture medium, typically at levels of 0.1% or higher. On the other hand, in our experiments, Pluronics were administered only at the time of vector injection, and the net amount constitutes 0.004% of the total body weight based on the volume and concentration of the vector solution. Moreover, the half-life of Pluronics *in vivo* is estimated as some hours and the majority of the administered material is known to be excreted from the urine within days (APhA and RPSGB, 1986; Gibbs and Hagemann, 2004). Therefore, it is unlikely that transcriptional activation is responsible for gene expression *in vivo* weeks after administration. Nonetheless, this mechanism of action may potentially be useful in order to enhance the outcome of gene therapy approaches *in vivo*. As all the known regulatable gene expression systems share the weakness of toxicity (Goverdhana *et al.*, 2005), safety profiles of Pluronics along with rapid clearance from the body may lead to the development of a novel system for regulatable gene expression *in vivo*. Further studies in this respect may extend the utility of Pluronics in future.

A relatively small number of studies have reported successful gene transfer into adipocytes. There are reports on gene transfer into cultured adipocytes by using viral vectors such as adenovirus (Meunier-Durmort *et al.*, 1996, 1997; Hertzfel *et al.*, 2000), lentivirus (Morizono *et al.*, 2003; Carlotti *et al.*, 2004), and retrovirus (Ito *et al.*, 2005). Regarding efficacy *in vivo*, gene transfer into gonadal adipose tissues, using adenoviral vectors, demonstrated clinical efficacy in treating diabetic conditions (Nagamatsu *et al.*, 2001). Successful transduction of adipose tissue by using either simian immunodeficiency viral vector (Ogata *et al.*, 2004) or herpes simplex viral vector (Fradette *et al.*, 2005) was reported. To our knowledge, this is the first report that demonstrates the efficacy of adipocyte-mediated gene transfer by AAV vectors.

In terms of vector dose, adipocyte-mediated gene transfer required a higher vector dose to achieve the same plasma Epo

TABLE I. LENGTH OF SURVIVAL OF *db/db* MICE

Group	Survival (weeks after injection)
AAV1-Epo (no Pluronic F88) ^a	4, ^b 4, 6, >8, ^c >8 ^c
AAV1-Epo + 2% Pluronic F88 ^d	4, 4, 5, 6, >8 ^c
AAV1-Epo + 2% Pluronic F88 + operation ^e	>24, >24, >24, >24

^aReflects animals in Fig. 2B (open columns).

^bThis animal became paralyzed at the time of blood collection and subsequently died.

^cThese animals were killed at week 8 for tissue analysis.

^dReflects animals in Fig. 2B (solid columns).

^eReflects animals in Fig. 4.

concentration as that obtained by muscle- or liver-mediated gene transfer in our previous study (Mochizuki *et al.*, 2004). At a dose of 6×10^{10} VG/body, which was the standard dose for muscle- and liver-mediated gene transfer, the Epo concentration was less prominent; the plasma Epo concentration became comparable at a dose of 2×10^{11} VG/body. Therefore, even after the addition of F88, transduction efficiency was still low in adipose tissue. Whether there are any better methods to augment the efficiency of transduction, including the use of a higher vector dose or other serotype-derived vectors, needs to be investigated further.

In our series of experiments, all the transduced mice became polycythemic; therefore, transgene-derived Epo was functional (data not shown). Although the Epo concentration was augmented by the addition of Pluronic F88, there was no significant difference in blood hemoglobin levels or red blood cell counts among the groups. This is because the Epo concentrations in the transduced animals were far beyond the physiological dose-response window (Mochizuki *et al.*, 2004), and even modest Epo expression after injecting the vector without Pluronic F88 could result in polycythemic conditions. It is generally difficult to eliminate the possibility that the use of this excipient may alter the tropism of the vector and promote gene transfer to certain remote organ(s). Nonetheless, because removal of the transduced adipose tissue resulted in the elimination of the Epo (Fig. 4), we can exclude this possibility. Whether the tissue specificity of expression is common to all serotypes of AAV is yet to be confirmed. To test the tissue specificity, *db/db* mice are useful because they develop rich adipose tissues and a specific lobe can be completely removed by standard surgical procedures. On the other hand, the limitation of this model lies in the difficulty of long-term transgene expression: these animals were naturally diabetic and susceptible to thromboembolic events when they became polycythemic and eventually lost their lives after 4 weeks (Table 1). In this series of experiments, no clear threshold of Epo level on mortality was recognized, although all the "operated" animals attained long-term survival with normalized values of Epo and blood parameters. Therefore, in order to demonstrate long-term expression, a different transgene needs to be used.

Transducing adipose tissue may have another advantage with respect to immunology. Although the distribution and density of antigen-presenting cells within the adipose tissue remain unknown, it is possible that these cells are relatively scarce in the adipose tissue than in "standard" tissues such as muscle or liver. Therefore, the immune response against transgene product, which is a current hurdle in the field of gene therapy (Zaiss and Muruve, 2005), can partly be overcome by targeting adipose tissue. In our series of experiments, we did not observe any immunological responses to the transgene products or to the transduced adipose tissues. To test this hypothesis, a transgene product that is highly immunogenic to mice should be chosen and the outcome needs to be evaluated.

Adipose tissue is usually abundant in the body, can be easily transduced by simple vector injection, and can be removed safely. For these reasons, it is a potential depot organ for gene transfer. In this sense, there may be a wide range of applications of this method in supplemental gene therapy.

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Address reprint requests to:

Dr. H. Mizukami
 Division of Genetic Therapeutics
 Center for Molecular Medicine
 Jichi Medical University
 3311-1 Yakushiji
 Shimotsuke, Tochigi 329-0498, Japan

E-mail: miz@jichi.ac.jp

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