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Original Article

Biodistribution of a Low Dose of Intravenously Administered AAV-2, 10, and 11 Vectors to Cynomolgus Monkeys

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SUMMARY: In gene therapy trials, adeno-associated virus (AAV) vectors are injected directly into target tissues such as muscle and liver. Direct injection can lead to the introduction of a low level of the vector into blood circulation. To determine the systemic effects of the vector released in the blood, we extensively examined the biodistribution of intravenously administered AAV serotype 2 (AAV2) vector in cynomolgus monkeys. Although the vector distribution pattern varied from monkey to monkey, the vector DNA was maintained in the various tissues beyond 7 months post-inoculation (pi). The vector DNA was detected in the lymphoid tissues, particularly in the spleen, more frequently and at a much higher level than in the other tissues tested (i.e., brain, lung, liver, heart, gallbladder, pancreas, colon, kidney, ovary, uterus, etc.). The expression of a transgene was detected in the lymph nodes at 3 months pi. The distribution of two pseudotyped vectors, AAV2/10 and AAV2/11, was similar to that of the AAV2 vector. The present results suggest that when introduced intravenously, the AAV vector DNA persists and may induce transgene expression in various monkey tissues. Thus, the possibility of inadvertent gene transfer to various non-target tissues should be considered in a gene therapy strategy with an AAV vector.

INTRODUCTION

Adeno-associated virus (AAV), a nonenveloped small DNA virus belonging to the genus *Dependovirus* of the family *Parvoviridae*, has been engineered for use as a gene-transfer vector in gene therapy (1). Expression of a transgene introduced into target cells by the AAV vector is expected to last for a long period of time. Fundamental methods of AAV vector production, purification, and quality control have been developed by using the human AAV serotype 2 (AAV2) vector as a model system (2). Pseudotyped AAV2 vectors, in which the AAV2 vector genomes are packaged with capsids from AAVs of the other serotypes, have recently been developed (3). Some of the pseudotypes have shown organ tropism that differs from that of the AAV2 vector (3).

The AAV vector genome, which encodes a transgene, is a single-stranded 4.7 kb DNA. At each end of the vector genome is a 145-base region (inverted terminal repeat [ITR]) containing the viral origin of DNA replication and the packaging signal (1). Since the vector genome lacks the viral rep gene, the product of which mediates viral DNA replication and integration of the viral DNA into the AAVS1 region in human chromosome 19, the vector DNA is not replicable and is randomly integrated into host cell chromosomes or is maintained as episomes of the circularized intermediates (4,5). The vector capsid, an icosahedral particle with a diameter of 25 nm, is composed of the AAV capsid proteins (VP1, VP2, and VP3).

Since AAV vectors are highly stable and can infect various organs, AAV vectors are considered to be suitable for in vivo administration. However, the direct injection of an AAV vector into the target tissue leads to the infection of distant non-target tissues with the vector via blood circulation in non-human primates (6-11). The AAV2 vector, when administered by instillation to the bronchial epithelium of rhesus monkeys, is distributed to the heart, liver, jejunum, kidney, lymph nodes, spleen, pancreas, and brain (6). The AAV2 vector, when injected into the liver of rhesus fetuses, is distributed to the lymph node, liver, skin, spleen, lung, and esophagus of human infants (8). The AAV2 vector, when injected into the muscle, is distributed to the liver and lymph nodes (7). Thus, detailed evaluation of vector biodistribution to various tissues is a necessary part of the assessment of the safety of the vector in the context of administering a gene therapy strategy with in vivo administration.

In this study, the biodistribution of intravenously injected AAV2 vectors and pseudotyped AAV2/10 and 2/11 vectors in cynomolgus monkeys was examined in more extensive detail than that of previous studies (6-11). These studies show that the entry of a portion of the vector into the blood vessels is unavoidable after direct inoculation of the vector into the target tissue. Given the doses of AAV vectors used in clinical trials (2×10^{12} genome copies [gc]/kg weight), we chose AAV vector doses of 2×10^{9} gc to 5×10^{10} gc/kg weight for intravenous injection into the monkeys, assuming that 1/100 to 1/1,000 of the inoculate could leak into the bloodstream from the tissue that had received the vector injection. The AAV vector DNA in the tissue samples was measured semi-quantitatively by agarose gel electrophoresis of a PCR-amplified vector DNA. The results were expected to provide basic data required to evaluate the safety of in vivo administration of the AAV vectors.

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MATERIALS AND METHODS

AAV vectors: The vector genomes used in this study are shown in Figure 1. The FLAG-tagged beta-galactosidase gene (beta-galF) was produced by PCR with the reverse primer possessing a FLAG-tag sequence (5′-GATTACAAGGATG ACGACGATAAG) and previously produced pAAVbeta-gal (12) as the template. The beta-galF was inserted between the cytomegalovirus immediate early enhancer/promoter and SV40 polyA signal to produce the genome of AAV2(beta-galF). The fusion gene of EGFP and human alpha-tubulin (EGFPtub) was obtained from pEGFP-Tub (BD Bioscience Clontech, Palo Alto, Calif., USA) by cleavage with *Nhe*I and *Bam*HI. The EGFPtub was inserted between the human elongation factor 1 alpha (EF1alpha) promoter and SV40 polyA signal to produce the genome of AAV2(EGFPtub). By using PCR G at nucleotide 157 (nt157) (A at the first ATG of the EGFP gene is designated as nt1) was changed to T to produce a novel *Hin*dIII cleavage site to obtain EGFPatub. Similarly, G at nt 129 was changed to T to produce a *Hin*dIII cleavage site to obtain EGFPbtub. The amino acid sequences of EGFPatub and EGFPbtub were not affected by the nt substitutions. Each vector genome was flanked with the ITR sequence of AAV2.

beta-galF or EGFPtub was packaged into the AAV2 capsid in human 293 cells as described previously (12). Similarly, EGFPatub and EGFPbtub were packaged into the AAV10 and AAV11 capsids, respectively. The AAV2 vector stocks used in Experiments I, II, and III (Table 1) were purified by heparin affinity column chromatography (13), and the vector stocks used in Experiment IV were purified by CsCl equilibrium centrifugation (12). The infectivity of the AAV2 vector purified by CsCl centrifugation was comparable to that of the AAV2 vector purified by heparin column chromatography. Extract from 293 cells that had not been transfected with plasmids for vector production was similarly processed by heparin affinity column chromatography and was used as a mock inoculant.

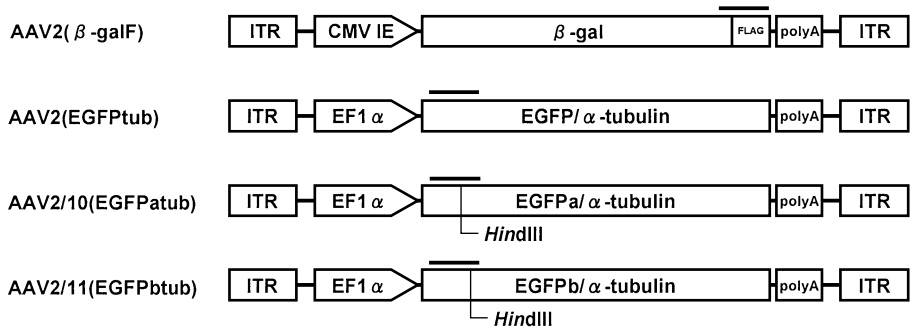

Fig. 1. Schematic representation of the vector genomes. AAV2(beta-galF) is the type 2 vector which has the FLAG-tagged beta-galactosidase gene driven by the cytomegalovirus immediate early promoter (CMV IE). AAV2(EGFPtub) is the type 2 vector which has the fusion gene of enhanced green fluorescent protein (EGFP) and alpha-tubulin driven by the human elongation factor I alpha promotor (EF1 alpha). AAV2/10(EGFPatub) is the type 10 pseudotyped vector which has the fusion gene of EGFPa and alpha-tubulin driven by EF1 alpha. EGFPa has a cleavage site of *Hin*dIII at the nucleotide (nt) 157 (A at the first ATG of EGFP cording region is numbered as nt 1). AAV2/11(EGFPbtub) is the type 11 pseudotyped vector which has the fusion gene of EGFPb and alpha-tubulin driven by EF1 alpha. EGFPb has a cleavage site of *Hin*dIII at the nt 124. The horizontal bars represent PCR amplicons used for detection of the vector genomes. ITR, inverted terminal repeat of AAV2; polyA, SV40 poly adenylation signal.

Table 1. Study design for vector administration in cynomolgus monkeys

Experiment	Monkey No.	Gender	AAV vector	Dose (genome copies/animal)	Time of sample collection
I	#1	FM	Mock	–	2 days
	#2	FM	AAV2(β-galF)	2.5×10^{10}	2 days
	#3	FM	AAV2(β-galF)	2.5×10^{10} [1)]	3 months
	#4	M	AAV2(β-galF)	2.5×10^{10}	3 months
	#5	FM	AAV2(β-galF)	2.5×10^{10}	3 months
II	#6	M	AAV2(EGFPtub)	2.5×10^{11}	3 months
	#7	FM	AAV2(EGFPtub)	2.5×10^{11}	3 months
	#8	M	AAV2(EGFPtub)	2.5×10^{11}	3 months
	#9	FM	AAV2(EGFPtub)	2.5×10^{11}	3 months
III	#10	FM	AAV2(EGFPtub)	1.0×10^{11}	5 months
	#11	FM	AAV2(EGFPtub)	1.0×10^{11}	5 months
	#12	M	AAV2(EGFPtub)	1.0×10^{11}	5 months
	#13	M	AAV2(EGFPtub)	1.0×10^{11}	5 months
IV	#14	FM	Mock	–	3 months
	#15	FM	AAV2, 2/10, 2/11 [2)]	1.0×10^{10} each	3 months
	#16	M	AAV2, 2/10, 2/11	1.0×10^{10} each	3 months
	#17	FM	AAV2, 2/10, 2/11	1.0×10^{10} each	7 months

[1)]: #3 was received second injection of AAV2(beta-galF) (5×10^{10} gc) at 60 days after the first injection.
[2)]: Mixture of AAV2(EGFPtub), AAV2/10(EGFPatub), and AAV2/11(EGFPbtub).

The DNase-resistant vector DNA in the vector stock was measured by Real-Time PCR (Applied Biosystems, Foster City, Calif., USA) with TaqMan probes (CCCAACGAGAAG CGCGATCACA) hybridized to EGFP DNA.

Animal experiments: Cynomolgus monkeys (4 to 5 years of age and weighing 3 to 5 kg) were obtained from the Tsukuba Primate Research Center of the National Institute of Biomedical Innovation (Ibaraki, Japan). The monkeys were sedated during all procedures by the administration of ketamine (10 mg/kg). The AAV vectors or the mock inoculant in 5 ml of physiological saline were administered intravenously into the femoral vein of the monkeys. The dose of the AAV vectors and the time of necropsy are indicated in Table 1. The monkeys were bled every 2 weeks until they were sacrificed. All animal studies were performed in accordance with the guidelines for animal experiments in National Institute of Infectious Diseases, Tokyo, Japan.

Extraction of DNA from tissues: Monkey tissues were harvested at necropsy and stored at −80°C until use. Before necropsy, blood was extensively drawn to avoid contamination of the tissues by blood. DNA was extracted from each frozen tissue type (approximately 25 mg) by using QIAamp DNA extraction kit (Qiagen GmbH, Hilden, Germany).

Detection of AAV vector DNA in tissue DNA: PCR was designed to amplify the 285-bp region of beta-galF (forward primer: 5′-GCGACTTCCAGTTCAACATC, reverse primer: 5′-TTACGCGAAATACGGGCAGA) and 323-bp region of EGFP (forward primer: 5′-ACAAGTTCAGCGTGTCCGGC, reverse primer: 5′-CCTCCTTGAAGTCGATGCCC) in the vector genomes. PCR consisted of an initial heating step at 94°C for 5 min, 37 cycles of incubation at 94°C for 30 s and at 68°C for 1 min, and incubation at 68°C for 5 min. The vector DNA fragment in the tissue DNA sample was amplified by PCR. The DNA sample contained 0.5 μg DNA (equivalent to 10^5 cells). For comparison, the DNA fragment in standard DNA solution, which contained a known amount of plasmid DNA having beta-galF or EGFPtub genes (10^2 to 10^5 copies) and DNA extracted from the liver of monkey (#1) that had received the mock inoculant were amplified by PCR in a similar manner. To verify the quality and quantity of DNA in the samples, a portion of G3PDH gene was amplified with the previously described primers (12). The PCR products were analyzed by electrophoresis on a 2% agarose gel followed by ethidium bromide staining. When the DNA sample contained more than 100 gc of the vector DNA, the amplified DNA fragment was clearly detected (Fig. 2).

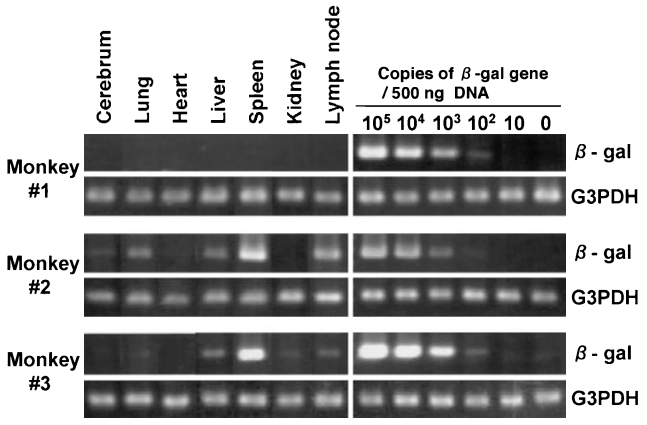

Fig. 2. Detection of AAV vector DNA in monkey tissues. DNA samples were extracted from the various tissues of monkey #1, injected with mock inoculate, and of monkeys #2 and 3, injected with AAV2(beta-galF). The segment of the vector genome in the DNA samples was amplified by PCR. G3PDH gene was amplified for references. Representative results of the agarose gel electrophoresis of the PCR products were presented.

To determine the serotype of the vector in Experiment IV, PCR products were digested with *Hin*dIII, and the size of the resultant DNA fragment was analyzed with an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, Calif., USA). The *Hin*dIII digestion produced 324-bp, 243-bp, and 276-bp fragments from the genomes of AAV2(EGFPtub), AAV2/10(EGFPatub), and AAV2/11(EGFPbtub), respectively.

Immunoblot detection of EGFP/alpha-tubulin fusion protein in the tissue extracts: Monkey tissue (50 mg) was homogenized in a lysis buffer (0.1M MES, 2 mM EGTA, 1 mM $MgCl_2$, pH 6.8) and the homogenate was centrifuged at 100,000 g for 30 min at 4°C. The supernatant was mixed with Taxol (Paclitaxel; Sigma-Aldrich, St. Louis, Mo., USA) (20 μM) and GTP (Sigma-Aldrich) (0.5 mM), and was warmed to 37°C for 30 min in order to allow the tubulin to assemble into microtubules. Then, the supernatant was chilled on ice for 10 min and was centrifuged at 10,000 × g for 30 min at 4°C to precipitate the microtubules. The pellet was resuspended in PBS and electrophoresed in SDS-polyacrylamide gel. The proteins in the gel were transferred to a Hybond-P nylon membrane (Amersham Biosciences Corp., Piscataway, N.J., USA). After blocking the membrane with 5% skim milk, the EGFP/tubulin fusion protein on the membrane was allowed to bind with anti-EGFP rabbit polyclonal antibody (#632376; BD Bioscience Clontech, Palo Alto, Calif., USA) and anti-alpha-tubulin mouse monoclonal antibody (T-9026; Sigma-Aldrich). Horseradish peroxidase conjugated anti-rabbit and anti-mouse IgG goat antibodies (SC-2030 and SC-2031, respectively; Santa Cruz Biotechnology, Inc., Santa Cruz, Calif., USA) and an ECL Western Blotting Detection System (Amersham Biosciences) were used to detect rabbit and mouse IgGs on the membrane. Fluorescence was detected using a Storm Phosphor Imager (Amersham Biosciences).

A HeLa cell clone expressing EGFPtub was newly produced and used as a model for immunoblot detection of endogenous tubulin and EGFPtub. HeLa cells, cultured in growth medium (DMEM supplemented with 10% fetal bovine serum) in a 10-cm dish, were transfected with 2 μg of pEGFP-Tub (BD Bioscience Clontech) with the transfection reagent Effectene (Qiagen). After incubation of the cells for 48 h, they were passaged at a split ratio of 1 to 10 and cultured in growth medium containing a selective drug, G418 (500 μg/ml). Drug-resistant cell clones were obtained by two successive single colony isolations. One clone (HeLa/EGFPtub) that stably expressed EGFPtub was selected. Endogenous tubulin and EGFPtub were extracted from HeLa/EGFPtub and were used as markers for tubulin and EGFPtub.

Neutralization of AAV vectors with serum antibody: The neutralizing activities of the monkey serum were examined by testing the inhibition of the transduction of COS-1 cells by AAV vectors expressing beta-gal. COS-1 cells (2×10^4 cells/well) were seeded in 96-well plates at 6 h before inoculation. Fifteen microliters of vector solution containing 4×10^3 transducing unit/ml was mixed with 18 μl of serum that had been serially diluted from 1:10 to 1:6,250 with PBS and incubated for 1 h at 37°C. The number of vector genome copies required for 4×10^3 transducing unit with COS-1 were 10^6 gc, 10^8 gc, and 10^8 gc for the AAV2,

AAV2/10, and AAV2/11 vectors, respectively. Then, the sample was mixed with 30 μl of DMEM containing 10% FBS and was used to inoculate the cells in two wells (30 μl/well). After incubation of the cells with occasional rocking for 2 h at 37°C, 70 μl of fresh DMEM containing 10% FBS was added to each well. Two days later, the cells were fixed and stained using an In Situ beta-Galactosidase Staining Kit (Stratagene, La Jolla, Calif., USA). The cells that showed positive staining for beta-gal were counted under a microscope. The neutralizing titer of the antiserum was expressed as the reciprocal of the highest dilution that repressed the number of beta-gal-positive cells to half of the number obtained with the samples mixed with similarly diluted serum from a non-immunized mouse.

RESULTS

Distribution of AAV2(beta-galF) in cynomolgus monkeys: In Experiment I (Tables 1 and 2), one (#1) and four monkeys (#2, 3, 4, and 5) were intravenously injected into the femoral vein with a mock inoculate and 2.5×10^{10} gc of AAV2(beta-galF) (Fig. 1), respectively. No clinical symptoms were observed among these monkeys, indicating that the AAV2 vector particle did not induce any acute toxic effects, as has also been reported previously (4,14). In urine collected between 0 and 24 h post-inoculation (pi), a very low level of the vector DNA was found in monkey #2 sample (10^2 gc/30 μl), but this was not observed in the samples from the other monkeys. The vector DNA was not detected in the urine collected between 24 and 48 h pi from any of the five monkeys. The results indicated that excretion of the injected AAV2 vector was very limited. In whole blood samples obtained at 1 day pi, a very low level of the vector DNA was found in samples from monkeys #2, 3, 4, and 5 (10^2 gc/10 μl). Since the body weight of these monkeys was approximately 3 kg, the total blood of each monkey could be estimated as 250 ml. Therefore, only 1/10,000 of the vectors initially administered is thought to have remained in the circulating blood at 1 day pi. In whole blood samples from monkeys #3, 4, and 5 obtained at 1 week pi, no vector DNA was detected (data not shown).

Monkeys #1 and 2 were sacrificed at 2 days pi. The vector DNA was found in none of the monkey #1 samples, and it was found in various tissues of monkey #2 (Fig. 2 and Table 2). Relatively high levels of the vector DNA were observed in the spleen, tonsil, and axillary lymph node. Low levels of the vector DNA were detected in the brain, ovary, and uterus. Histological examination of formalin-fixed specimens of tissues positive for the vector DNA did not show any abnormalities or inflammatory reactions.

At 2 months pi, monkey #3 received a second intravenous injection with 5×10^{10} gc of AAV2(beta-galF). No clinical symptoms were observed after the injection, indicating that the second injection did not induce any strong acute abnormal immunological reactions.

Monkeys #3, 4, and 5 were sacrificed at 3 months pi. During the 3-month period, the hematological profiles were examined periodically, and no abnormalities were found in the blood samples of monkeys #3, 4, and 5 (data not shown). The vector DNA was present in various tissues (Fig. 2 and Table 2). The highest vector DNA level was detected in the spleen of monkey #3, which had been injected twice with the vector. Among monkeys #3, 4, and 5, the vector distribution pattern to tissues appeared to vary from monkey to monkey. For example, the vector DNA was not detected in the spleen of monkey #5. It was noteworthy that, the vector DNA was found in the ovaries of monkeys #3 and 5, albeit at low levels. The detection of the vector DNA in the spleen, lymph node, and ovary sections by in situ hybridization with PCR was not successful, because it was difficult to find sections positive for the vector DNA. Thus, no vector DNA-positive cell species within the tissues were identified.

Because the vector DNA was readily detected in lymphoid tissues such as the spleen and lymph nodes, we examined the susceptibility of peripheral blood mononuclear cells (PBMCs) to the vector. PBMCs were collected from a cynomolgus monkey that had received no AAV vectors before and were incubated with an AAV2 vector. PBMCs (10^5) or COS-1 cells (5×10^4) in the wells of a 48-well plate were inoculated with 10^9 genome copies of AAV2(EGFP-tub). Three days later, the cells were examined for EGFP expression under a fluorescence microscope. Whereas almost all of the COS-1 cells were positive for EGFP expression, none of the PBMCs were positive for EGFP. It is possible that following the low-dose intravenous injection to the monkeys, the vector did not infect the lymphocytes in the spleen and lymph node.

Table 2. Experiment I: Detection of the vector genomes in the various tissues. Monkeys injected with AAV2(beta-galF) (2.5×10^{10} gc) were sacrificed at 2 days or 3 months after the injection

Tissue	Monkey				
	#1 (Mock)	#2 (2d)	#4 (3m)	#5 (3m)	#3 (3m)[1)]
Cerebrum	−	+	+	−	−
Cerebellum	−	+	+	+	+
Bone marrow	−	++	+	−	−
Retina	−	−	−	−	ND
Skin	−	−	+	−	+
Muscle	−	−	−	−	−
Trachea	−	+	−	+	+
Lung	−	++	−	−	+
Heart	−	−	+	−	−
Liver	−	++	−	−	+
Gallbladder	−	++	+	−	+
Pancreas	−	+	+	+	−
Spleen	−	++++	++	−	+++
Esophagus	−	+	−	−	−
Stomach	−	+	−	−	+
Jejunum	−	ND	−	+	+
Ileum	−	+	+	+	−
Colon	−	+	+	+	ND
Kidney	−	−	+	−	+
Adrenal gland	−	−	−	−	−
Bladder	−	+	−	ND	+
Tonsil	−	+++	+	−	+
Thymus	−	+	−	−	−
Parotid gland	−	+	−	−	−
Submandibular gland	−	+	−	−	−
Thyroid gland	−	++	+	−	+
Axillary lymph node	−	+++	+	−	−
Hilar lymph node	−	+	ND	−	+
Mesenteric lymph node	−	++	−	−	+
Iliac lymph node	−	++	ND	ND	ND
Inguinal lymph node	−	+	+	−	+
Testis/Ovary	−	+	−	+	+
Epididymis/Uterus	−	+	ND	+	−

[1)]: #3 was received second injection of AAV2(beta-galF) (5×10^{10} gc) at 60 days after the first injection. (−), $<10^2$ gc/0.5 μgDNA; (+), 10^2-10^3 gc/0.5 μgDNA; (++), 10^3-10^4 gc/0.5 μgDNA; (+++), 10^4-10^5 gc/0.5 μgDNA; (++++), $>10^5$ gc/0.5 μgDNA; (ND), Not done.

Distribution of AAV2(EGFPtub) in cynomolgus monkeys and expression of EGFPtub: In Experiment II, 2.5×10^{11} gc of AAV2(EGFPtub) (Fig. 1) was administered into four monkeys (#6, 7, 8, 9) intravenously into the femoral vein, and the monkeys were sacrificed at 3 months pi (Tables 1 and 3). The vector DNA in various tissues was examined by similar procedures to those used in Experiment I. With some variation from monkey to monkey, high levels of the vector DNA (>10^3 gc/0.5 μg DNA) tended to be detected in the spleen, liver, gallbladder, tonsils, and lymph nodes, and low levels of the vector DNA (<10^3 gc/0.5 μg DNA) were present in the cerebrum, bone marrow, muscle, trachea, lung, heart, pancreas, esophagus, colon, kidney, adrenal gland, bladder, and parotid gland. At 3 months after injection, the vector DNA was not detected in the blood samples. Therefore, we concluded that the vector DNA in these specimens was not derived from contamination with blood.

EGFPtub expression was detected in the axillary lymph nodes of monkeys #7, 8, and 9 (Fig. 3). Endogenous tubulin and EGFPtub proteins were extracted from the liver, spleen, tonsils, and axillary lymph nodes of the monkeys. The two proteins were readily co-purified and concentrated by a previously described method (15). The tubulin/EGFPtub complex extracted from approximately 10^7 cells was analysed by SDS-polyacrylamide gel electrophoresis followed by immunoblotting with a mixture of anti-EGFP and anti-alpha-tubulin antibodies. The tubulin/EGFPtub complex extracted from a newly produced HeLa cell clone expressing EGFPtub was used as a size-marker for endogenous tubulin and EGFPtub protein. Comparison with commercially available purified tubulin (Sigma-Aldrich) enabled us to estimate the approximate amounts of EGFPtub (Fig. 3A). Samples for immunoblotting (50 mg) contained approximately 10 to 20 ng of EGFPtub. Under these conditions, transgene expression was detected in 6 out of 23 axillary lymph nodes tested. No transgene expression was detected in the other tissues including the spleen and liver, in which the injected vector DNA was readily found, most likely because the analyzable volume of a single piece of tissue is limited. Therefore, it remains unclear whether the transgene was not expressed in most of the tissues or the cells expressing the transgene were escaped from the sampling.

Table 3. Experiment II: Detection of the vector genomes in the various tissues. Monkeys injected with AAV2(EGFPtub) (2.5×10^{11} gc) were sacrificed at 3 months after the injection

Tissue	Monkey			
	#6 (3m)	#7 (3m)	#8 (3m)	#9 (3m)
Cerebrum	+	−	−	−
Cerebellum	−	−	−	−
Spinal cord	−	−	−	−
Bone marrow	++	++	−	−
Skin	−	−	−	−
Muscle	++	++	−	−
Trachea	+	++	+	−
Lung	−	−	−	+
Heart	+	+	−	+
Liver	+++	+++	−	+
Gallbladder	+	+	−	+++
Pancreas	+	+	−	−
Spleen	++++	++++	++	+++
Esophagus	+	−	−	−
Stomach	−	−	−	−
Jejunum	−	−	−	−
Ileum	−	−	−	−
Colon	+	−	−	−
Kidney	+	−	+	+
Adrenal gland	+	−	−	++
Bladder	+	−	−	−
Tonsil	++	+	+++	++
Thymus	−	−	−	−
Parotid gland	−	−	−	++
Submandibular gland	−	−	−	−
Thyroid gland	−	−	−	−
Axillary lymph node	++	++	+	++
Hilar lymph node	++	++++	++	−
Mesenteric lymph node	++	+	+	−
Iliac lymph node	++	+++	++	−
Inguinal lymph node	+	++	+++	−
Testis/Ovary	−	−	−	−
Epididymis/Uterus	−	−	−	−

(−), <10^2 gc/0.5 μgDNA; (+), 10^2-10^3 gc/0.5 μgDNA; (++), 10^3-10^4 gc/0.5 μgDNA; (+++), 10^4-10^5 gc/0.5 μgDNA; (++++), >10^5 gc/0.5 μgDNA.

In Experiment III 1.0×10^{11} gc of AAV2(EGFPtub) (Fig. 1) was administered to four monkeys (#10, 11, 12, 13), as was done in Experiment II, and the vector DNA in various tissues was examined at 5 months pi (Table 4). The vector distribution was similar to that observed at 3 months pi, indicating the vector DNA was stably maintained in various tissues. The levels of vector DNA in the tissues were similar to those observed at 3 months pi (Experiment II).

Distribution of AAV2/10(EGFPatub) and AAV2/11 (EGFPbtub) in cynomolgus monkeys: Since the data in the several recent studies indicated that the AAVs of different serotypes have different tissue tropism, in Experiment IV, the AAV2/10 and AAV2/11 pseudotype vectors (Fig. 1) and the AAV2 vector were compared as to the vector DNA distribution pattern in monkeys (Table 5). The entire coding regions of

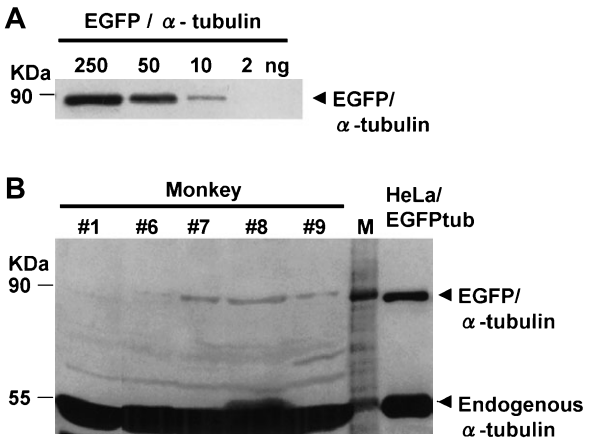

Fig. 3. Detection of transgene expression in monkey tissues. (A) Sensitivity of immunoblotting for fusion protein of EGFP and alpha-tubulin (EGFP/alpha-tubulin). The complex of endogenous tubulin and EGFP/alpha-tubulin was extracted from HeLa cells expressing EGFP/alpha-tubulin. The extracts were electrophoresed in a SDS-polyacrylamide gel and transferred to a nylon membrane. The EGFP/alpha-tubulin was detected with a mixture of anti-EGFP and anti-alpha-tubulin antibodies. The amount of EGFP/alpha-tubulin was estimated by the comparison with known amount of commercially available purified tubulin. (B) Expression of EGFP/alpha-tubulin in the lymph node of monkeys. The complex of endogenous tubulin and EGFP/alpha-tubulin was extracted from the axillary lymph node of monkeys, injected with mock inoculate (monkey #1) or AAV2(EGFPtub) (monkeys #6, 7, 8, and 9). The EGFP/alpha-tubulin was detected as described above.

AAV10 and AAV11 were recently isolated from cynomolgus monkeys, and the pseudotyped vectors were produced as previously described (12). To compare the distribution of the three vectors in the same monkey, AAV2(EGFPtub), AAV2/10(EGFPatub), and AAV2/11(EGFPbtub) were mixed together, and a mixture containing 1.0×10^{10} gc of each vector was administered intravenously to three monkeys (#15, 16, 17). One monkey (#14) received saline as a negative control. The PCR DNA derived from three vector genomes are distinguishable by measuring the sizes of DNA fragments produced by the digestion of the PCR DNA with *Hin*dIII, because the EGFP genes of AAV2/10(EGFPatub) and AAV2/11(EGFPbtub) have the *Hin*dIII site at different positions (Fig. 1). The vector inoculations did not induce any clinical symptoms, thus indicating that the AAV10 and AAV11 capsids exerted no acute toxicity. Serum samples were collected 1 week before and 1, 5, 9, and 11 weeks pi. Three monkeys (#14, 15, 16) were sacrificed at 3 months pi, and one monkey (#17) was sacrificed at 7 months pi.

The AAV2/10(EGFPatub) DNA was found primarily in lymphoid tissues such as the spleen and lymph nodes (Table 5). The levels of the AAV2/10(EGFPatub) DNA in the lymphoid tissues at 3 months pi (monkeys #15 and 16) were similar to those at 7 months pi (monkey #17), suggesting that the vector genome was stably maintained. The AAV2/11(EGFPbtub) DNA was similarly found in the lymphoid tissues of monkeys #15 and 17, but was not found in any tissues from monkey #16 (Table 5). Consistent with our previous observation that the vector DNA was not found in the liver of mice intravenously injected with the AAV2/11 pseudotyped vector (12), no AAV2/11(EGFPbtub) DNA was found in the livers of monkeys #15 and 17.

Table 4. Experiment III: Detection of the vector genomes in the various tissues. Monkeys injected with AAV2(EGFPtub) (1.0×10^{11} gc) were sacrificed at 5 months after the injection

Tissue	Monkey			
	#10 (5m)	#11 (5m)	#12 (5m)	#13 (5m)
Cerebrum	−	−	−	−
Cerebellum	−	−	−	−
Bone marrow	−	−	−	+
Skin	−	−	−	−
Muscle	−	+	−	+
Trachea	−	−	−	−
Lung	+	+	−	+
Heart	+	+	−	−
Liver	++	+++	−	+++
Gallbladder	−	++	−	−
Pancreas	++	−	+	−
Spleen	++	++++	+++	+++
Esophagus	−	−	−	−
Stomach	−	−	−	−
Jejunum	−	−	−	−
Ileum	−	+	−	−
Colon	−	+	−	+
Kidney	−	+	+	+
Adrenal gland	−	+	−	+
Bladder	−	+	−	−
Tonsil	+	+	++	−
Thymus	−	+	−	+
Parotid gland	−	−	−	−
Submandibular gland	−	−	+	−
Thyroid gland	−	+++	−	−
Axillary lymph node	−	++	++++	+
Mesenteric lymph node	−	+	+	−
Iliac lymph node	−	+++	−	−
Inguinal lymph node	+	+++	++	−
Testis/Ovary	−	−	−	−
Epididymis/Uterus	−	+	−	−

(–), $<10^2$ gc/0.5 μgDNA; (+), 10^2-10^3 gc/0.5 μgDNA; (++), 10^3-10^4 gc/0.5 μgDNA; (+++), 10^4-10^5 gc/0.5 μgDNA; (++++), $>10^5$ gc/0.5 μgDNA.

Immune responses to the vectors varied from monkey to monkey. Since mouse anti-AAV2, AAV10, and AAV11 VP2 sera neutralized the AAV2, 10, and 11 vectors in a type-specific manner (12), it is very likely that the neutralizing activity of monkey antibody against AAV2, 10, and 11 is type-specific. Since it is possible that the monkeys had been infected with AAVs immunologically cross-reactive to AAV2, 10, and 11, the pre-administration serum obtained 1 week before the inoculation was considered as the baseline antibody titer (Fig. 4). Monkey #15 developed anti-AAV11 and low-level anti-AAV10 neutralizing antibodies, but did not develop anti-AAV2 antibody (Fig. 4), although similar levels of AAV2 (EGFPtub), AAV2/10(EGFPatub), and AAV2/11(EGFPbtub) DNAs were found in various lymphoid tissues (Table 5). The low level of anti-AAV11 neutralizing antibody that was present in the pre-administration serum was not found to inhibit the distribution of AAV2/11(EGFPbtub) (Table 5).

Monkey #16 developed anti-AAV2 and anti-AAV10 neutralizing antibodies, but did not develop anti-AAV11 antibody (Fig. 4). As was the case with monkey #15, the low level neutralizing antibody against AAV10 in the pre-administration serum (Fig. 4) did not inhibit the distribution of AAV2/10(EGFPatub) (Table 5). Since no anti-AAV11 neutralizing antibody was detected in the pre-administration serum of monkey #16, the absence of AAV2/11(EGFPbtub) DNA in monkey #16 (Table 5) could not be explained by the neutralization of the vector.

Monkey #17 responded to the three vector capsids and developed antibodies against AAV2, 10, and 11. It is likely that low doses of the vectors induced various immune responses of the monkeys against the vectors.

DISCUSSION

In this study, we intravenously administered AAV vectors (AAV2, AAV2/10, and AAV2/11) at a dose of 2×10^9 gc to 5×10^{10} gc/kg weight to cynomolgus monkeys, and we examined the behavior of the vectors and the responses of the monkeys. We assumed that intravenous injections at a relatively low dose of vector would mimic systemic conditions caused by targeted high-dose injections. We found that vector DNA persists for a long period of time, probably without replication, in various tissues, and in particular in the lymphatic tissues. We also demonstrated that the transgene is expressed in the axillary lymph nodes. These data are pertinent for the assessment of vector safety, although the status of the persisting vector DNA remains unclear, and the factors that regulate the transgene expression must still be investigated.

Following AAV2 vector inoculation, rapid excretion of the vector in the urine was very limited. By 1 week pi, the AAV2 vector DNA became undetectable in the circulating blood. At 2 days pi, the vector DNA was readily detected in the lymphoid tissues, especially in the spleen (Table 2). The vector DNA was detected at lower levels in the brain, lung,

Table 5. Experiment IV: Serotype-specifc detection of the vector genomes in the various tissues. Monkeys injected with the mixture of AAV2(EGFPtub), AAV2/10(EGFPatub) and AAV2/11(EGFPbtub) (1.0×10^{10} gc each) were sacrificed at 5 or 7 months after the injection

Tissue	Monkey								
	#15 (3m)			#16 (3m)			#17 (3m)		
	2	2/10	2/11	2	2/10	2/11	2	2/10	2/11
Cerebrum	−	−	−	−	−	−	−	−	−
Cerebellum	−	−	−	−	−	−	−	−	−
Bone marrow	−	−	−	−	−	−	−	−	−
Skin	−	−	−	−	−	−	−	+	−
Retina	−	−	−	−	−	−	+	−	−
Muscle	−	−	−	−	−	−	−	−	−
Trachea	−	−	−	−	−	−	−	−	−
Lung	−	−	−	−	−	−	−	−	−
Heart	−	−	−	−	−	−	−	−	−
Liver	+	+	−	−	−	−	−	−	−
Gallbladder	−	−	−	−	−	−	−	−	−
Pancreas	−	−	−	−	−	−	−	−	−
Spleen	++	++	++++	++	++++	−	++	+++	++
Esophagus	−	−	−	−	−	−	−	−	−
Stomach	−	−	−	−	−	−	−	−	−
Jejunum	−	−	−	−	−	−	−	−	−
Ileum	−	−	−	+	+	−	−	−	−
Colon	+	−	−	+	+	−	+	−	−
Kidney	−	−	−	−	−	−	−	−	−
Adrenal gland	−	−	−	−	−	−	−	−	−
Bladder	++	++	+++	−	−	−	−	−	−
Tonsil	+	++	++	+	++	−	−	−	−
Thymus	−	−	−	−	−	−	−	−	−
Parotid gland	−	−	−	−	+	−	−	+	−
Submandibular gland	−	−	−	+	+++	−	−	−	−
Thyroid gland	−	−	−	−	−	−	−	−	−
Axillary lymph node	+	++	++	+	+++	−	+	++	+
Hilar lymph node	−	++	+	+	++	−	+	++	−
Mesenteric lymph node	+	+	+	−	+	−	++	++	−
Iliac lymph node	−	−	−	+	++	−	++	++	+
Inguinal lymph node	+	++	++	+	++	−	−	+	−
Testis/Ovary	−	−	−	−	−	−	−	−	−
Epididymis/Uterus	−	−	−	−	−	−	−	−	−

(−), <10^2 gc/0.5 μgDNA; (+), 10^2-10^3 gc/0.5 μgDNA; (++), 10^3-10^4 gc/0.5 μgDNA; (+++), 10^4-10^5 gc/0.5 μgDNA; (++++), >10^5 gc/0.5 μgDNA.

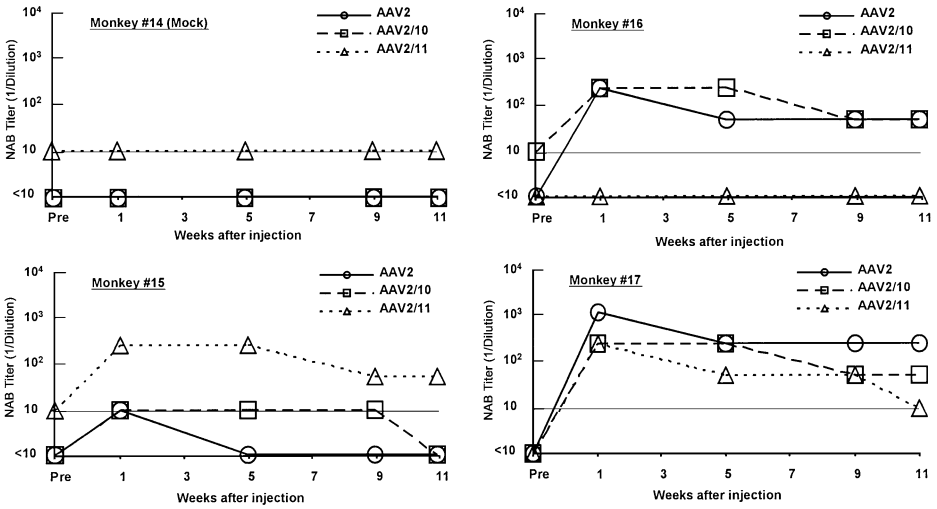

Fig. 4. Induction of anti-AAV neutralizing antibodies in monkeys. The serum from the monkey injected with saline (monkey #14) and the sera from those infected with the mixture of AAV2(EGFPtub), AAV2/10(EGFPatub), and AAV2/11(EGFPbtub) (monkeys #15, 16, and 17) were examined for their neutralizing activities by inhibition of transduction of COS-1 cells by the AAV vectors expressing beta-gal. Neutralizing titer (NAB Titer) of the antiserum was expressed as the reciprocal of the highest dilution that repressed the number of beta-gal positive cells to half of the number obtained with the samples mixed with the similarly diluted serum from a non-immunized mouse.

liver, gallbladder, pancreas, colon, ovary, uterus, and other organs (Table 2). The transfer of AAV2 vector to the brain via the blood-brain barrier agreed with the results of previous studies; when AAV2 vector has been injected into the liver or lung of non-human primates, vector DNA has been detected in the brain (6,8). No histological abnormalities or inflammatory reactions were observed in the tissues positive for the vector DNA. These results indicated that the vector in the blood was not readily excreted in the urine and rapidly attached to (or trapped by) various tissues, most readily the lymphoid tissues, without inducing any clinical symptoms. The presence of the vector DNA in the lymphoid tissues was consistent with previous findings; when an AAV2 vector containing the EGFP gene was injected into the liver of rhesus monkey fetuses, 0.01 - 0.05% of the monocytes in the spleen and lymph nodes of the infants were EGFP-positive (8). However, such EGFP-positive cells have not yet been characterized in detail.

Although the distribution patterns of the AAV2 vector at 3, 5, and 7 months pi varied from monkey to monkey (Tables 3, 4, and 5), the vector DNA was detected in various tissues of all of the monkeys sacrificed in this study. There was a tendency that from 2 days to 3 months pi, the vector DNA level was lowered to 1/100 in the spleen and to 1/2 in the other tissues. From 3 months pi to 5 months pi, the decrease in the vector DNA in the tissues, including that in the spleen, was marginal. The data strongly suggest that the AAV2 vector DNA would be maintained in various tissues for a long period of time, probably for years, without inducing any clinical symptoms.

The expression of the transgene was observed, at least in the lymph nodes, at 3 months pi. Although this remains to be determined, it is possible that the transgene could be expressed in other tissues harboring the vector DNA at a lower level. Then, the safety of the in vivo administration of AAV vectors would be affected by the properties of the transgene product.

AAV2/10(EGFPatub), AAV2/11(EGFPbtub), and AAV2 (EGFPtub) showed a similar pattern of vector distribution throughout the monkey tissues (Table 5). Previously, AAV2/10 and 2/11 vector DNAs were detected in the muscle tissues of BALB/c mice injected with AAV2/10 and 2/11 vectors (2×10^{11} gc/kg weight) via the tail vein at 6 weeks pi, which suggested that these vectors preferentially enter muscle cells (12). However, no AAV2/10(EGFPatub) and AAV2/11 (EGFPbtub) vector DNAs were detected in the muscle tissues of monkeys #15, 16, and 17 (Table 5). This apparent discrepancy may be ascribed to the difficulty of detecting vector DNA in the muscles of monkeys administered with a low vector dose (2×10^{9} gc/kg weight) such as that used in this study.

The vector distribution obtained in this study is consistent with those reported in previous studies, in which AAV vectors have been instilled in the bronchial epithelium of rhesus monkeys (6) and have been injected into the liver of rhesus fetuses (8) and injected into the muscle of rhesus and cynomolgus monkeys (7). That is, AAV vectors that enter the circulating bloodstream appear to be distributed to various tissues, primarily the lymphoid tissues, and are maintained for a long period of time. Since a small portion of the tissues was subjected to examination for the presence of vector DNA and transgene products, it cannot be ruled out that the vector genome and the transgene products were present in other portions of tissues. Furthermore, extensive histological studies of the tissues and organs will be necessary in order to define the vector DNA-positive cell-species (e.g., liver cells, lung cells, or lymphocytes) and to accurately identify those cells targeted by the vector. In addition, the long-term consequences of vector persistence remain unknown.

The distribution and persistence of the AAV vectors varied from monkey to monkey. This variation is thought to be associated, at least in part, with the genetic heterogeneity of the monkeys. More data with other monkeys should be gathered in order to evaluate safety issues related to gene therapy using AAV vectors.

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