High-Density Recombinant Adeno-Associated Viral Particles are Competent Vectors for *In Vivo* Transduction

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Recombinant adeno-associated viral (rAAV) vectors have recently achieved clinical successes in human gene therapy. However, the commonly observed, heavier particles found in rAAV preparations have traditionally been ignored due to their reported low *in vitro* transduction efficiency. In this study, the biological properties of regular and high-density rAAV serotype 8 vectors, rAAVRD and rAAV^{HD}, were systemically compared. Results demonstrated that both rAAVRD and rAAV^{HD} exhibited similar DNA packaging profiles, while rAAV^{HD} capsids contained fewer VP1 and VP2 proteins, indicating that the rAAV^{HD} particles contained a higher DNA/protein ratio than that of rAAVRD particles. Dynamic light scattering and transmission electron microscopy data revealed that the diameter of rAAV^{HD} was smaller than that of rAAVRD. *In vitro*, rAAV^{HD} was two- to fourfold less efficient in transduction compared with rAAVRD. However, the transduction performance of rAAV^{HD} and rAAVRD was similar *in vivo*. No significant difference in neutralizing antibody formation against rAAVRD and rAAV^{HD} was observed, suggesting that the surface epitopes of rAAVRD and rAAV^{HD} are congruent. In summary, the results of this study demonstrate that rAAVRD and rAAV^{HD} are equally competent for *in vivo* transduction, despite their difference *in vitro*. Therefore, the use of rAAV^{HD} vectors in human gene therapy should be further evaluated.

Keywords: AAV, density, gene therapy, capsid

INTRODUCTION

ADENO-ASSOCIATED VIRUS (AAV) is a non-pathogenic parvovirus. The viral genome consists of a small (~4.7 kb), single-stranded DNA that contains two large open-reading frames (ORFs) flanked by inverted terminal repeats (ITRs). One ORF encodes the non-structural proteins, Rep78, Rep68, Rep52, and Rep40, and the other ORF encodes the structural proteins, VP1, VP2, and VP3. The genome is packed into a viral capsid that is non-enveloped icosahedron formed by 60 proteins consisting of VP1, VP2, and VP3 at an approximate ratio of 1:1:10. To generate recombinant AAV (rAAV) vectors, the two ORFs are replaced with DNA sequence encoding the therapeutic protein.

Recently, there have been a number of advancements in rAAV gene therapy for genetic diseases such as congenital blindness,^{1,2} choroideremia,³ $\alpha(1)$ -antitrypsin deficiency,⁴ lipoprotein lipase deficiency,^{5,6} and hemophilia.⁷ Encouraging preliminary results are moving rAAV gene therapy closer to a routine clinical application. Although there have been continuous and significant progressions toward flexible and scalable rAAV production in recent years,⁸⁻¹³ a high demand still exists for more efficient vector production. This is partially due to the overwhelming amount of quality vectors needed to support preclinical research using large animal models and clinical trials.^{14,15} Furthermore, low transgene expression in clinical trials has increased

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the demand for quality rAAV vectors in order to administer high-dose injections.¹⁶

Naturally occurring AAV produced using adenovirus (Ad) as a helper can be fractionated by isopycnic centrifugation in cesium chloride (CsCl) into three predominant classes: defective interfering (DI)-AAV, regular AAV, and heavy AAV.¹⁷ The most prominent visible band in the CsCl gradient contains DI-AAV, with a density of 1.32–1.35 g/cm³. These particles are referred to as defective interfering particles because they can interfere with the replication of regular and heavy AAV. They appear “empty” when examined using an electron microscope, and have been reported to contain either no DNA¹⁸ or DNA of a truncated/incomplete genome.¹⁹ Truncated/incomplete genomes will have deleted internal regions but retain the genome termini.^{20–22} The band containing regular AAV2 has a density of 1.41 g/cm³, whereas the band containing heavy AAV2 is 1.45 g/cm³.¹⁷ It has been previously reported that heavy AAV particles were unstable in CsCl and present in a greater proportion early after infection.¹⁷ In these studies, the ratio of regular and heavy AAV ranged from 1:1 to 1:3 at different time post infection.¹⁷ The heavy AAV particles have also been identified in preparations of AAV1,²³ AAV3,²⁴ AAV4,¹⁹ avian AAV,²⁵ and H-1 parvovirus.²⁶

The physical and biological properties of regular and heavy AAV particles have been previously investigated using different methods. Electron microscopy revealed that the heavy AAV2 was 10–20% smaller in diameter than the regular AAV2.²⁷ De la Maza and Carter found that both regular and heavy bands had the same DNA/protein ratio and the same density in metrizamide gradients.¹⁷ Spectrophotometric analysis and velocity sedimentation of regular and heavy AAV2 particles failed to demonstrate any significant difference, and the DNA extracted from both regular and heavy AAV2 appeared to be similar.¹⁷ However, contradictory reports have been published that reveal that heavy AAV2 has a higher DNA/protein ratio than regular AAV2 has.^{24,27} It was also determined that the ratio of infectious units to physical particles of regular AAV2 is 16–300-fold higher than that of heavy AAV2 particles.^{17,28} Other reports have found no significant differences in the infectivity between the regular and heavy particles of AAV4, the minute virus of mice (MVM), or the H1 parvovirus.^{19,26,29} However, the AAV particles used in the aforementioned studies were all wild-type AAV, and did not address particles from rAAV preparations.

For the first time, the physical and biological properties of regular and high-density rAAV

(rAAVRD and rAAV^{HD}) vectors were characterized. This was accomplished through a comparison of genome content, capsid size and composition, *in vitro* and *in vivo* transduction efficacy, and potential antigenicity. Moreover, studies analyzing regular and high-density rAAV have not included the novel isolated serotypes, for example AAV8,³⁰ which has been widely used for liver and heart gene delivery.^{31–33} Defining the properties of rAAVRD and rAAV^{HD} particles using AAV serotype 8, both *in vitro* and *in vivo*, will not only provide a unique insight into gene transduction, but also demonstrate their potential for use in gene therapy.

MATERIALS AND METHODS

Construction of rAAV vector plasmids

The pAAV-hHC_{HL} plasmid consisting of a 3.7 kb AAV genome carrying the heavy chain (hHC) of the human factor VIII (*FVIII*) gene was described previously.³⁴ The pdsAAV-Gluc (1.8 kb) and pdsAAV-EGFP (2.1 kb) plasmids were designed to carry either the Gaussia Luciferase gene (*Gluc*) or enhanced green fluorescent protein (*EGFP*) gene, driven by a human β -actin promoter with a CMV enhancer (CB). These two plasmids contained one full ITR and one mutant ITR, and were used to generate self-complementary AAV vectors (scAAV-EGFP and scAAV-Gluc).

Production and purification of rAAV vectors

All rAAV vectors were generated utilizing the triple plasmid, co-transfection method as previously described.³⁵ Briefly, pAAV-Rep&Cap (serotype 8), pAd helper, and the transgene plasmids were co-transfected into HEK 293 cells cultured in roller bottles at a ratio of 1:1:1. Vectors from both the transfected cells and medium were harvested 72 h post transfection, and purified by two rounds of CsCl gradient ultracentrifuge. The rAAVRD and rAAV^{HD} particles were collected and exchanged extensively against phosphate-buffered saline (PBS) with 5% D-sorbitol. The density of the rAAVRD and rAAV^{HD} particles was determined by collecting the vectors dropwise following the second round of CsCl gradient ultracentrifuge. The density of the fractions (refractive index: 1.380–1.362) was measured, and the capsid of the vectors were characterized by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and quantitative polymerase chain reaction (qPCR) analysis.

Characterization of rAAV capsid composition

The capsid composition of the rAAV vectors was determined by both silver staining and Western blot analysis. Briefly, rAAV vectors ($\sim 1 \times 10^{10}$ particles)

were electrophoresed using a 10% SDS-PAGE gel. Following separation, the gel was stained using a commercially available silver staining kit (Pierce® Silver Stain Kit; Thermo Scientific, Rockford, IL). Western blot analysis was also performed as previously reported.³⁶ In short, an anti-AAV capsid antibody (B1; American Research Products, Belmont, MA) was diluted to a 1:500 ratio, and applied to a membrane containing the rAAV vectors. Next, a fluorescently labeled, secondary antibody (IRDye® 800CW Goat Anti-Mouse IgG [H + L]; LI-COR, Lincoln, NE) was diluted at a ratio of 1:1,000, and the membrane was applied. The resulting membrane was scanned using an Odyssey imaging system.

qPCR and Southern blot analysis of rAAV genomes

For titration of the rAAV vectors, a 10 μ L aliquot of each vector was treated with Benzonase and proteinase K, and diluted to different ratios. To elucidate the genome copy number in the liver of injected mice, the total DNA was extracted from the liver cells using a GeneJET genomic DNA purification Kit (K0721; Thermo Scientific). All qPCR analysis was performed using the Fast SYBR Green Master Mix, which has been previously described,^{35,37} using the following primers: hHC forward primer, 5'-CTGAAATGGATGTGGTCAGG-3'; hHC reverse primer, 5'-AGTCCCAGTCCTCCTCTCA-3'; Gluc forward primer, 5'-CCACCGAGAAC AACGAAGA-3'; Gluc reverse primer, 5'-CATCTC TTTGAGCACCTCCA-3'; EGFP forward primer, 5'-TGACCCTGAAGTTCATCTGC-3'; EGFP reverse primer, 5'-GAAGTCGTGCTGCTTCATGT-3'. The genome copy number of vector found in each sample was determined by comparison with a standard curve made from serial dilutions of plasmid containing the corresponding AAV genomes. A Southern blot analysis of genomes extracted from the vectors was performed using a ³²P-labeled hHC probe made from a fragment of the plasmid pAAV-hHC_{HL} digested using the enzymes *AccI* and *KpnI*.

Particle size measurement by dynamic light scattering and transmission electron microscopy

The diameter of the rAAV particles was measured using a particle analyzer (Zetasizer Nano ZS; Malvern Instruments, Westborough, MA). For measurement, a 735 μ L ($\sim 1 \times 10^{12}$ vg) aliquot of vector in PBS solution was loaded into the disposable, low-volume cuvette and mounted in the dynamic light scattering (DLS) chamber. Each sample was measured three times, with 12 runs in each measure-

ment. The average size of the particles was calculated from these multiple measurements in order to estimate the predominant viral particle size in the population. For transmission electron microscopy (TEM) analysis, formvar was coated on a microscope slide and floated onto a water bath. Cleaned 200 mesh grids were placed on this film and collected using parafilm. Five microliters of purified AAV ($\sim 1 \times 10^{13}$ vg/mL) was placed on the grid and allowed to dry, then 8 μ L of 1% phosphotungstic acid (Electron Microscopy Science, Hatfield, PA) was applied and drawn off. After drying, the grids were observed using a Philips Transmission Electron Microscope CM 12 (Philips, Eindhoven, Netherlands) with an accelerating voltage of 100 KV and imaged with a DVC detector controlled by AMT software (Danvers, MA). The diameter of the rAAV particles was measured using Image J software.

rAAV transduction *in vitro*

The cell lines used in this study—Hela, CV1, BHK, HEK 293, GM16095, and Cos-1—were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT), penicillin (100 IU/mL), and streptomycin (100 μ g/mL) at 37°C in a moisturized environment supplied with 5% CO₂. For each transduction experiment, 50,000 viable cells were seeded in a 24-well plate 24 h prior to transduction. Regular and heavy AAV particles of scAAV8-EGFP or scAAV8-Gluc, with or without Ad5, were added directly to each well. EGFP expression was observed 24 h post infection using a fluorescent microscope. Gaussia luciferase (Gluc) expression from the media was determined by adding 5 μ L of coelenterazine substrate (CLZ, 200 μ g/mL) in 0.1 M of Tris buffer (pH 7.5) containing 0.5 M of NaCl. Bioluminescence was measured with a POLARstar Omega plate reader (BMG Labtech, Ortenberg, Germany). All experiments were performed in triplicate, and the results are presented as averages of the experimental data.

rAAV transduction *in vivo*

In vivo transduction experiments were carried out using 6–8-week-old BALB/c male and C57BL6/Svj129S female hemophilia A (HA) mice. All mice were housed in a specific pathogen-free environment, supplied a normal diet, and treated in accordance with National Institutes of Health guidelines and as approved by IACUC at Temple University (ACUP 4142). During all procedures, the mice were anesthetized using isoflurane (induction 3–5%,

maintenance 1–3%) and monitored every 5 min for absence of limb withdrawal from toe pinch, absence of the eye-blink reflex, lowering of the heart or respiration rate, and absence of muscle tone. Post procedure, the animals were monitored every 15 min until ambulatory. Multiple doses of scAAV8-Gluc vector were injected into mice via the tail vein as previously described.³⁵ For these injections, the high dose was 6×10^{11} vg/mouse and the low dose was 2×10^{11} vg/mouse. Mouse plasma was harvested post vector administration by retro orbital eye bleeding at regular intervals as described. Plasma was diluted in 0.1 M of Tris buffer (pH 7.5) containing 0.5 M of NaCl, and Gluc expression was measured following the *in vitro* protocol. To terminate the experiment, mice were sacrificed by asphyxiation from inhalation of carbon dioxide followed by cervical dislocation. DNA from the mouse liver was extracted using a GeneJET genomic DNA extraction Kit for qPCR analysis.

rAAV neutralization assay

To determine the neutralizing antibody (NAb) response from mice injected with rAAVRD and rAAV^{HD} vectors, the NAb-mediated inhibition of rAAV transduction was measured *in vitro*. Briefly, the sera of mice injected with either rAAV8-GlucRD or rAAV8-Gluc^{HD} were collected 6 months post injection. BHK cells were seeded in a 96-well plate at a density of 5×10^4 cells/well in 100 μ L of DMEM, supplemented with 10% FBS, 4 h before inoculation. The inoculum contained rAAV-GlucRD or rAAV-Gluc^{HD} (2×10^8 particles) preincubated with 1:100 diluted mouse anti-serum for 2 h at 4°C in a total volume of 100 μ L. Twenty-four hours after inoculation, Gluc expression was measured following the *in vitro* Gluc assay. A total of 50 human serum samples (Division of Human Genetics, Cincinnati Children's Hospital Medical Center, Cincinnati, OH) were included to study the effects of NAb against rAAV8-GlucRD and rAAV8-Gluc^{HD} further. Serum samples at 1:100 dilution that inhibited rAAV transduction by $\geq 50\%$ compared with normal mouse serum were considered as positive.

Statistical analyses

Both the two-tailed Student's *t*-test and one-way analysis of variance with Bonferroni multiple comparisons post test were performed. The differences were considered significant when the *p*-value was < 0.05 . The analysis was performed using SPSS v11.0 (SPSS, Inc., Chicago, IL).

RESULTS

Comparison of the physical properties of rAAVRD and rAAV^{HD}

It has been previously demonstrated that both regular and heavy AAV particles are present in wild-type AAV preparations when Adenovirus is used as a helper.¹⁷ Accordingly, both particles are also found in rAAV vector preparations, as are DI particles. Fig. 1 demonstrates the presence of these three particles in a preparation of rAAV8 vector containing a 3.7 kb AAV genome. Fig. 1A illustrates the three particles with different buoyant densities. Using silver staining analysis of each fraction, the presence of three peaks corresponding to the three bands observed in the CsCl gradient was confirmed. They were identified as defective interfering rAAV (rAAV^{DI}), rAAVRD, and rAAV^{HD}, having buoyant densities of 1.33, 1.39, and 1.43 g/cm³, respectively (Fig. 1B).

In order to explain the density discrepancy between the rAAVRD and rAAV^{HD} vectors, the size of the packaged genomes was evaluated. All of the vectors were treated with benzonase to remove any unprotected nucleic acid before proteinase K treatment was applied to release the genomes. Fig. 1C demonstrates the major DNA band, 3.7 kb in length, for both the rAAVRD and rAAV^{HD} vectors. This band represents the product of annealed, plus and minus genome strands, which corresponds to the size of the AAV genome of the pAAV-hHC_{HL} plasmid. The Southern blot analysis confirmed that the packaged DNA for both the rAAVRD and rAAV^{HD} vectors was similar (Fig. 1D). Analysis of DNA from self-complementary vectors (scAAV8-Gluc, 1.8 kb) also supported that the regular and heavy particles packaged the same size genomes (data not shown).

Gel electrophoresis and Southern blot analyses indicated that the packaged DNA genome did not significantly contribute to the difference in density between the regular and heavy particles. Next, the capsid composition of the two vectors was examined in order to delineate the different physical properties of the two particles. Fig. 1E provides the results of SDS-PAGE analysis of the protein components for rAAVRD and rAAV^{HD}. All three structural capsid proteins were present in both rAAVRD and rAAV^{HD} particles. Furthermore, there is evidence of the presence of another protein (VP1.5) in addition to VP1, VP2, and VP3 in both vectors. The VP1.5 protein may be the result of a VP2 variant that occurred due to a G/C change at position 219 introducing a novel CTG start codon between VP1 and VP2.³⁸ Although the rAAV^{HD} particles

Figure 1. Physical properties of recombinant adeno-associated viral (rAAV) particles in cesium chloride (CsCl) gradient. **(A)** 3.7 kb rAAV8-hHC_{HL} vectors were produced and purified by two rounds of CsCl gradient ultracentrifugation. The three rAAV bands were observed after CsCl gradient. Defective interfering rAAV (rAAV^{DI}, 1.33 g/cm³), regular density rAAV (rAAVRD, 1.39 g/cm³), and high-density rAAV (rAAV^{HD}, 1.43 g/cm³) particles were identified. **(B)** Silver staining of CsCl gradient rAAV fractions. Fractions with density ranging from 1.48 to 1.33 g/cm³ were selected for analysis. A total of 2 μ L of each fraction was electrophoresed on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel. The density of each fraction was shown on top. Peak fractions for rAAV^{DI}, rAAVRD, and rAAV^{HD} were identified. **(C)** Genome size of rAAVRD and rAAV^{HD} vectors with 3.7 kb genome. A total of 10 ng of DNA from each vector was extracted, electrophoresed in 1% agarose gel, and stained with SYBR Gold. **(D)** Vector DNA from rAAVRD and rAAV^{HD} analyzed by Southern blot. DNA extracted from 1×10^{10} particles of rAAVRD or rAAV^{HD} was hybridized with a hHC-specific probe. **(E)** Capsid composition rAAVRD or rAAV^{HD} particles. About 1×10^{10} of purified viral particles was denatured, electrophoresed on 10% SDS PAGE gel, and visualized by silver staining. VP1, VP2, VP3, and an additional band VP1.5 was identified in the figure. **(F)** Western blot analysis of capsid from rAAVRD and rAAV^{HD} vectors. Anti-AAV capsid B1 antibody was used at a dilution of 1:500. The final individual capsid bands were identified by a fluorescent dye labeled secondary antibody (RDye[®] 800CW Goat Anti-Mouse IgG [H + L] at a dilution of 1:1,000. Color images available online at www.liebertpub.com/hum

contained all the same classes of VP proteins, when compared, the relative amounts of VP1, VP1.5, and VP2 were less than rAAVRD. The ratio of VP1:VP1.5:VP2:VP3 was approximately 1:1.5:1:4.5 and 1:1:2:8 in rAAVRD and rAAV^{HD}, respectively. This result was confirmed by Western blot analysis using the B1 antibody that specifically targets the AAV VP polypeptides (Fig. 1F). The phenomenon that rAAV^{HD} had less VP1, VP1.5, and VP2 was also observed with other heavy particles containing different transgenes (data not shown).

rAAV^{HD} particles are smaller than rAAVRD particles

As rAAV^{HD} particles contain less VP1, VP1.5, and VP2, it is reasonable to suspect that the size of the heavy particles is smaller than the regular particles. Therefore, a DLS experiment was performed to investigate the size differences of the regular and heavy particles. Results of this experiment determined that rAAV^{HD} had a size distribution ranging from 18.2 to 91.3 nm, whereas the size of rAAVRD ranged from 15.9 to 105.7 nm (Fig. 2A). The rAAV^{HD} peaked at 35.2 ± 0.4 nm,

which is less than the rAAVRD, which peaked at 37.8 ± 0.7 nm (Fig. 2C). Similarly, the average size of rAAV^{HD} was 38.2 ± 0.6 nm, whereas the average size of rAAVRD was 41.2 ± 0.9 nm. Interestingly, a ~ 700 nm peak was observed with the rAAV^{HD} vectors. It likely represents large capsid aggregates, and was excluded during the average size calculation. Both rAAVRD and rAAV^{HD} were slightly elliptic particles, close to round, when they were detected by TEM, with the mean length of the major axes being 26.1 ± 3.0 nm and 24.8 ± 3.9 nm, respectively (Fig. 2B, C, and D). Therefore, the rAAV^{HD} was approximately 5–7% smaller in diameter than the rAAVRD, in agreement with what was observed using AAV2.^{17,28} Equal amounts of AAV particles (quantified based on qPCR results) were analyzed by silver staining after separation using a SDS-PAGE gel. The results demonstrated that rAAVRD and rAAV^{HD} had a similar amount of VP3 protein but that rAAV^{HD} particles had less VP1, VP1.5, and VP2. Therefore, it was concluded that the DNA/protein ratio of heavy particles was higher than that of regular particles, and the total mass (DNA + Capsid) of rAAV^{HD} was less than

Figure 2. Size distribution of rAAVRD and rAAV^{HD} particles. **(A)** Size distribution of rAAV particles determined by dynamic light scattering (DLS). About 1×10^{12} rAAV particles in phosphate-buffered saline were examined using a particle analyzer. **(B)** Size distribution of rAAV particles determined by transmission electron microscopy (TEM). **(C)** Key properties of rAAVRD and rAAV^{HD} summarized from **(A)** and **(B)**. **(D)** TEM image of rAAVRD and rAAV^{HD} particles. Color images available online at www.liebertpub.com/hum

rAAVRD. As the rAAV^{HD} was more compact than rAAVRD (1.43 g/cm^3 and 1.39 g/cm^3), it can also be speculated that rAAV^{HD} is smaller than rAAVRD. This observation is supported by size measurement using DLS and TEM technology. However, due to the limitations of DLS and TEM, the actual physical size of the rAAV^{HD} particles will need to be further quantified in the future.

Different *in vitro* transduction efficiency between rAAVRD and rAAV^{HD}

It is well known that the N-termini of VP1 contains a phospholipase A2 domain (PLA2), nuclear localization signals (NLSs), and several protein sequence motifs (PSMs) that are necessary for efficient infection *in vitro*.^{39–44} Therefore, since it was determined that the rAAV8^{HD} particles contain less VP1, VP1.5, and VP2, it is logical to hypothesize that they will also have a lower transduction efficiency compared with rAAV8RD. Using the viral preparations described above, the transduction efficiency of rAAVRD and rAAV^{HD} vectors was characterized in the different cell lines HEK 293, BHK, GM16095, HeLa, CV1, and Cos-1. The transduction efficiency of the two vectors was measured using EGFP (scAAV8-EGFP) and Gluc (scAAV8-Gluc) as reporter genes. The EGFP and Gluc expression patterns were examined 24–72 h post infection. As illustrated in Fig. 3A, the EGFP

positive cells were significantly lower in cultures infected with scAAV8-EGFP^{HD} than those infected with scAAV8-EGFPRD. Among the cell lines tested, BHK produced the highest EGFP expression. Similar results were observed when Gluc was used as reporter; AAV8^{HD} transduction was fourfold less efficient than rAAV8RD in 16095 cells (Fig. 3B). In other cell lines, there was a calculated twofold difference between the rAAV8RD and rAAV8^{HD} transduction. These results indicate that the difference in transduction efficiency between rAAVRD and rAAV^{HD} is less than what was previously reported using an AAV2 infection, which demonstrated a 10–100-fold difference.¹⁷ Previous reports included the co-infection of Ad. Therefore, the effect of Ad on rAAVRD and rAAV^{HD} transduction was examined. It was demonstrated that the co-infection of Ad dramatically potentiated transduction of both rAAV vectors (Fig. 3C and D). However, Ad increased the transduction of the rAAV^{HD} and rAAVRD by the same degree. Together, these results suggest that rAAVRD and rAAV^{HD} particles only produce a moderate difference in transduction activity *in vitro*.

Similar *in vivo* transduction efficiency of rAAVRD and rAAV^{HD}

In previous studies, the infection activity of regular and heavy AAV particles was only com-

Figure 3. Transduction efficiency of rAAV^{HD} and rAAVRD particles *in vitro*. Different cell lines including HEK-293, HeLa, Cos-1, BHK, GM16095, or CV1-T cells were infected with the rAAV^{HD} or rAAVRD vectors (50,000 vg/cell). **(A)** EGFP expression was observed 48 h post transduction of scAAV8-EGFPRD and scAAV8-EGFP^{HD}. **(B)** Six cell lines were infected with scAAV8-GlucRD or scAAV8-Gluc^{HD} (50,000 vg/cell), and the luciferase activity was determined 24 h post transduction by adding 5 μ L of coelenterazine substrate. **(C)** and **(D)** EGFP expression of transduction showed the propensity of Ad to enhance the transduction of scAAV8-EGFPRD and scAAV8-EGFP^{HD} in GM16095 **(C)** and HeLa **(D)** cells. Ad (5 pfu/cell) was infected concurrently with scAAV8-EGFPRD and scAAV8-EGFP^{HD} for 24 h. * $p < 0.01$.

pared using *in vitro* conditions²³; the relative transduction efficiency of rAAVRD and rAAV^{HD} vectors was never evaluated *in vivo*. In this study, BALB/c male HA mice were injected with 2×10^{11} vg of either scAAV8-Gluc^{HD} or scAAV8-GlucRD vectors via the tail vein ($N=6$). Since heavy particles have two- to fourfold less activity *in vitro*, a

high-dose group (6×10^{11} vg/mouse) of heavy particles was included. Every 2 weeks post injection, the luciferase activity was quantified. The relative luciferase values for both vectors were similar (Fig. 4A). The high-dose group of heavy particles produced a two- to threefold increase in activity compared with the regular and heavy particle low-

Figure 4. rAAVRD and rAAV^{HD} achieved equal transduction *in vivo*. Multiple doses of scAAV8-Gluc vectors (high dose: 6×10^{11} vg/mouse; low dose: 2×10^{11} vg/mouse) were injected into C57BL6/Svj129S female mice via the tail vein. **(A)** Quantitation of the luciferase values after scAAV8-GlucRD or scAAV8-Gluc^{HD} injection ($N=6$) at different times. **(B)** Analysis of AAV genome copy number in mouse liver samples after systemic injection of scAAV8-GlucRD or scAAV8-Gluc^{HD} by quantitative polymerase chain reaction at day 90 post injection. * $p < 0.01$. Color images available online at www.liebertpub.com/hum

Table 1. NAbs reaction difference between rAAVRD and rAAV^{HD} particles in BALB/c male HA and C57BL6/Svj129S female HA mice

Number	Mouse species	Sex	Injected vector	NAbs reaction (relative Gluc activity, %)	
				scAAV8-Gluc RD	scAAV8-Gluc ^{HD}
1	C57BL6/Svj129S	Female	scAAV8-Gluc RD	0.28 ± 0.02	0.19 ± 0.08
2	C57BL6/Svj129S	Female	scAAV8-Gluc RD	0.10 ± 0.01	0.11 ± 0.03
3	C57BL6/Svj129S	Female	scAAV8-Gluc RD	0.78 ± 0.41	0.84 ± 0.14
4	C57BL6/Svj129S	Female	scAAV8-Gluc ^{HD}	0.19 ± 0.02	0.14 ± 0.03
5	C57BL6/Svj129S	Female	scAAV8-Gluc ^{HD}	0.07 ± 0.02	0.04 ± 0.01
6	C57BL6/Svj129S	Female	scAAV8-Gluc ^{HD}	0.10 ± 0.03	0.07 ± 0.01
7	Balb/c	Male	scAAV8-Gluc RD	1.35 ± 0.46	0.84 ± 0.14
8	Balb/c	Male	scAAV8-Gluc RD	2.10 ± 1.11	1.57 ± 0.03
9	Balb/c	Male	scAAV8-Gluc RD	0.48 ± 0.08	0.41 ± 0.05
10	Balb/c	Male	scAAV8-Gluc ^{HD}	10.09 ± 3.14	2.51 ± 0.05
11	Balb/c	Male	scAAV8-Gluc ^{HD}	0.81 ± 0.10	0.28 ± 0.07
12	Balb/c	Male	scAAV8-Gluc ^{HD}	96.89 ± 18.62	82.67 ± 3.88

NAbs, neutralizing antibodies.

dose groups. In order to confirm these results, the same vectors were administered intravenously to 4–8-week-old C57BL6/Svj129S female HA mice ($N=6$). Similar to the previous observation, the scAAV8-GlucRD and scAAV8-Gluc^{HD} produced comparable Gluc expression (data not shown). Taken together, these data suggest that rAAV8^{HD} vectors are as efficient as rAAV8RD vectors *in vivo*, which is opposite of the *in vitro* results.

Next, the viral genome copy numbers remaining in the tissue samples were compared using qPCR (Fig. 4B). In the low-dose group, the viral genome copy number in the liver was approximately 2×10^5 vg/ μ g. There was no significant difference between mice injected with scAAV8-Gluc^{HD} or scAAV8-GlucRD vectors. The viral genome copy number in the high-dose group of scAAV8-Gluc^{HD} was four times higher than that in the low-dose group, which is consistent with the input amount of vectors. This result further demonstrates that the transduction efficiency and genome incorporations of rAAVRD and rAAV^{HD} vectors are similar.

NAbs responses of rAAVRD and rAAV^{HD} particles

To define the antigenic properties of rAAVRD and rAAV^{HD} vectors, their reactions to anti-AAV sera were tested. For this assay, the sera from scAAV8-GlucRD-injected HA mice was used. BHK cells were plated 24 h before infection with rAAV vectors at a multiplicity of infection of 5,000. The scAAV8-Gluc^{HD} and scAAV8-GlucRD vectors were incubated with anti-AAV sera at a dilution of 1:100 before infection. The control groups were incubated with normal mouse sera at the same dilution. Gluc activity was determined 24 h post infection. The results of this experiments demonstrated that all of the mice developed anti-AAV8 NAbs (Table 1).

NAbs induced by the scAAV8-GlucRD vector dramatically inhibited the transduction capability of both scAAV8-GlucRD and scAAV8-Gluc^{HD} vectors. Similar results were found when sera from mice injected with scAAV8-Gluc^{HD} were tested, with 5/6 mice developing anti-AAV NAbs to both vectors (Table 1). When scAAV8-GlucRD and scAAV8-Gluc^{HD} vectors were incubated with different dilutions of anti-AAV sera, similar inhibition was observed (data not shown). These results indicate that the antigenic properties of rAAV8RD and rAAV8^{HD} are similar. As the NAb reaction may vary between mouse strains, the same experiments were performed using sera of Balb/c mice injected with either scAAV8-GlucRD or scAAV8-Gluc^{HD}. Again, the transduction of both scAAV8-GlucRD and scAAV8-Gluc^{HD} was inhibited by the sera of mice injected with either vector.

To analyze further and to provide a translational implication of the NAb response of rAAVRD and rAAV^{HD}, 50 samples of human sera were collected and incubated with either scAAV8-GlucRD or scAAV8-Gluc^{HD}. The results demonstrated that NAbs for both scAAV8-GlucRD and scAAV8-Gluc^{HD} were present in nine human samples (Table 2), and that the NAbs neutralized both rAAV8RD and rAAV8^{HD} in a similar fashion. This indicates that the rAAV8RD and rAAV8^{HD} vectors are capable of generating cross-reactive immune responses, suggesting that the two types of rAAV8 vectors harbor

Table 2. Prevalence of NAbs against rAAVRD and rAAV^{HD} in human serum samples

AAV vectors	Total samples	Positive samples	Cross-reactive samples
rAAV ^{HD}	50	9	9
rAAV RD	50	9	9

similar capsid proteins on their surface and process similar antigenicity.

DISCUSSION

The results of this study demonstrate that both regular and heavy bands of rAAV8 after CsCl purification contain infectious rAAV particles, which parallels previous results obtained using wild-type AAVs and other parvoviruses.^{17,23,25,26,29,35} The AAV particles purified from these bands were reported to have significantly different infectious properties *in vitro*. De la Maza and Carter found a 16-fold difference in infectivity between the regular and heavy AAV2 particles,¹⁷ whereas Roy *et al.* reported a 300-fold difference in infectivity.²⁸ In contrast, another previous study of AAV4 proposed that the infectivity of heavy AAV4 was almost the same as regular AAV4.¹⁹ In the current study, a two- to fourfold difference in transduction activity was demonstrated between rAAV8RD and rAAV8^{HD} particles *in vitro*. This difference in transduction activity was confirmed in six different cell lines.

Previously, Clinton and Hayashi demonstrated that regular and heavy particles from MVM were equally infectious after maximum adsorption was reached, but that the heavy particles had a slower rate of adsorption.²⁹ Paradiso found that the regular and heavy H1 parvovirus particles had similar particle/infection ratio.²⁶ For MVM and H-1, there is evidence indicating a precursor-product relationship between the regular and heavy density particles. However, a subsequent study did not demonstrate a precursor-product relationship between regular and heavy AAV particles.¹⁷ Therefore, the dissimilarity between AAV and other parvoviruses may explain the activity difference between rAAVRD and rAAV^{HD} particles. It is suggested that the discrepancy between the work presented here and that of others may be due to the contribution of Ad. Previously it had been proposed that Ad exerted multiple effects on AAV transduction.^{45–47} Co-infection of Ad with rAAV^{HD} and rAAVRD in HeLa and GM16095 cells dramatically potentiated transduction (Fig. 3). However, Ad increased the transduction of the rAAVRD and rAAV^{HD} to a similar degree. The decreased transduction activity of rAAV^{HD} *in vitro* is not due to its genome, as agarose gel and Southern blot analysis of DNA from both rAAV^{HD} and rAAVRD did not indicate any genomic differences. The present results are consistent with previous findings showing no difference in genomic DNA using velocity sedimentation and restriction endonuclease digestion analyses.¹⁷

The capsid compositions may contribute to the difference in transduction activity between rAAVRD and rAAV^{HD}. Normally, AAV's capsid is composed of the three structural proteins: VP1, VP2, and VP3. For AAV2, VP1 and VP2 differ from VP3 by an N-terminal extension of 65 amino acids,^{39–44} and VP1 contains an additional 137 unique amino acids. The N-termini of VP1 is buried within the capsid interior but becomes externalized during the passage of AAV through the endosomal compartment. It has been previously determined that the N-termini of VP1 contains a PLA2, several NLSs, and PSMs that are necessary for efficient infection *in vitro*. These elements are conserved among all parvoviruses, and mutation of these sequences decrease infectivity *in vitro*.⁴⁰ As presented in Fig. 1, the rAAV8 has an additional capsid protein (VP1.5) as well as VP1, VP2, and VP3 for both regular and heavy particles. VP1.5 is 72 amino acids shorter than VP1.³⁸ The novel capsid composition observed in rAAV8 still retains normal structure and functional activity.⁴⁸ Since results consistently demonstrated that rAAV8^{HD} has less VP1 and VP1.5 than rAAV8RD, it is logical to conclude that the low transduction activity of rAAV^{HD} particles may be due to the presence of VPs that contain less PLA2, NLSs, and/or PSMs. The mutant BR3_K, which contains an alanine-to-lysine substitution in the third basic region of VP1, was 10- to 100-fold-less infectious than rAAV2 in transformed cell lines. In contrast, it produced only a twofold difference in several nontransformed cell lines, such as CV1 and C2C12.⁴⁰ However, in current study, the performance of rAAVRD and rAAV^{HD} in non-transformed cell lines and transformed cell lines is very similar.

Although rAAV^{HD} was less efficient than rAAVRD in transduction *in vitro*, its infectivity was indistinguishable from rAAVRD *in vivo* (Figs. 3 and 4). The underlying cause of infectivity differences between *in vitro* and *in vivo* testing remains unclear. The mutant BR3_K was less infectious than AAV2 *in vitro*, but not *in vivo*.⁴⁰ This is similar to what was previously documented for AAV2 and AAV8. AAV2 can more efficiently transduce cells in tissue culture compared with other serotypes, including AAV8. However, AAV8 consistently achieves a 10- to 100-fold improvement in gene transfer efficiency over AAV2 in the liver.³⁰ Moreover, the preclinical animal studies on rAAV transduction efficiency are not always predictive of the human outcome. It is well known that rAAV8 vectors are very effective in many animal models, including non-human primates. However, human hepatocytes are ~20 times less efficiently transduced compared with mouse hepatocytes.³¹ This phenomenon indicates that

current knowledge of AAV transduction is limited. Therefore, it is important to test AAV-based vector transduction utilizing a variety of targets *in vivo*.

Humoral immunity against AAV vectors represents an important barrier to intravascular gene transfer, resulting in the clearance of the vector before it enters the target cell.⁴⁹ Antibodies directed against the AAV capsid are highly prevalent in humans, a natural host for this virus, and cross-react with a wide range of serotypes because of the degree of homology between the capsid proteins. As a result, even relatively low titers of NAb can block AAV transduction when the vector is introduced into the bloodstream. Studies in humans have found that antibodies to some AAV serotypes are less prevalent than others.⁵⁰ AAV serotype switching and capsid engineering strategies have produced promising results.^{51,52} Because the capsid composition is different between rAAVRD and rAAV^{HD} particles, it can be hypothesized that humoral immunity may also have some difference. Immunoelectron microscopy studies indicated that rAAVRD and rAAV^{HD} may exhibit some antigenic differences.⁵³ This study investigated the humoral immunity of rAAV in two ways. First, HA mice were injected with rAAVRD and rAAV^{HD} vectors. Then, the sera from injected mice were incubated with rAAVRD and rAAV^{HD} vectors.

However, no difference was found between rAAVRD and rAAV^{HD} NAb, confirming that both NAb neutralized the regular and heavy particles equally. Second, NAb against rAAVRD and rAAV^{HD} were screened using healthy subjects. Again, there was no significant difference in the prevalence of NAb against either rAAVRD or rAAV^{HD} particles. Thus, rAAV^{HD} failed to display an altered antigenic profile compared to rAAVRD. This is a reasonable outcome, since the differences between rAAVRD and rAAV^{HD} particles are the amounts of VP1, VP1.5, and VP2, which may not change the outside of the capsid but may change the interior.

Collectively, the work herein is the first report providing evidence that the rAAV^{HD} is as efficient as rAAVRD for *in vivo* gene delivery, and there is no difference in NAb response to these two kinds of rAAV particles.

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AUTHOR DISCLOSURE

No competing financial interests exist.

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