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Efficient Serotype-Dependent Release of Functional Vector into the Culture Medium During Adeno-Associated Virus Manufacturing

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Abstract

Vectors based on adeno-associated virus (AAV) are the subject of increasing interest as research tools and agents for *in vivo* gene therapy. A current limitation on the technology is the versatile and scalable manufacturing of vector. On the basis of experience with AAV2-based vectors, which remain strongly cell associated, AAV vector particles are commonly harvested from cell lysates, and must be extensively purified for use. We report here that vectors based on other AAV serotypes, including AAV1, AAV8, and AAV9, are found in abundance in, and can be harvested from, the medium of production cultures carried out with or without serum. For AAV2, this difference in compartmentalization is largely due to the affinity of the AAV2 particle for heparin, because an AAV2 variant in which the heparin-binding motif has been ablated gives higher yields and is efficiently released from cells. Vector particles isolated from the culture medium appear to be functionally equivalent to those purified from cell lysates in terms of transduction efficiency *in vitro* and *in vivo*, immunogenicity, and tissue tropism. Our findings will directly lead to methods for increasing vector yields and simplifying production processes for AAV vectors, which should facilitate laboratory-scale preparation and large-scale manufacture.

Introduction

Adeno-associated viruses (AAVs) and vectors based on them are icosahedral 20-nm capsids containing a single-stranded DNA genome. Virus replication is characterized by a latent phase from which the virus can escape only after co-infection with a helper adenovirus or herpesvirus (Berns and Parrish, 2007). Like most other nonenveloped viruses, AAVs are not known to have an active egress pathway from infected cells (Smith and Enquist, 2002). In addition, they produce no cytopathogenic effects (CPEs) (Berns and Parrish, 2007). Therefore, release of virus from the cell is thought to be dependent on the CPE resulting from helper virus infection (Smith and Enquist, 2002).

Virtually all of the early work with AAV vectors used capsids derived from AAV serotype 2 (AAV2). These studies demonstrated the potential of this vector platform and provided the first convincing clinical success for *in vivo* gene therapy, in patients with Leber's congenital amaurosis (Bainbridge *et al.*, 2008; Hauswirth *et al.*, 2008; Maguire *et al.*, 2008). Vectors based on other natural AAV serotypes have shown different tissue tropisms *in vivo*, improved performance in terms of transduction efficiency, and fewer problems associated with humoral and/or cellular immunity (Vandenberghe *et al.*, 2006, 2009; Calcedo *et al.*, 2009).

Several methods of large-scale manufacturing of AAV have been optimized to yield high titer and quality vector. However, further improvements to the efficiency and simplicity of the process remain important to address future needs for clinical applications. Many protocols have vector particles purified from a cell lysate, necessitating extensive downstream purification. These methods were developed using AAV2-based vectors. Here, we report that vectors based on serotypes other than AAV2 are efficiently released into the culture medium after transient transfection of human embryonic kidney 293 (HEK293) cells. The option of harvesting from supernatants rather than cell lysates should greatly simplify downstream processing of AAV vectors for research and clinical applications.

Materials and Methods

Animals

Six- to 8-week-old male C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) were injected via the tail vein with 10^{11} genome copy (GC) particles in a volume of 200 μl or

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intramuscularly, for the immunology studies, with 3×10^{10} GC particles in a volume of 50 μl. Serum samples were obtained by retro-orbital venipuncture. All experimental protocols were approved by the Institutional Animal Care and Use Committee, and use of the vectors in the protocols was approved by the Office of Environmental Health and Radiation Safety and the Institutional Biosafety Committees of the University of Pennsylvania (Philadelphia, PA).

Vector preparation

Small-scale vector preparations were made in 6-well plates by cotransfection of HEK293 cells with plasmids carrying the vector genome, AAV packaging functions, and adenovirus helper genes as previously described (Vandenberghe *et al.*, 2009). Transfected cultures were maintained for 24 hr at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), after which the medium was replaced with DMEM without serum or with 10% FBS, and incubation was continued. Two days later, transfected cultures were harvested by scraping the cells into the medium. Cells were collected by centrifuging the suspension at 1000×*g* for 5 min. The supernatant ("medium" fraction) was transferred to a separate tube for assay. For some experiments in which a wash fraction was collected, the cell pellet was gently resuspended with 500 μl of phosphate-buffered saline (PBS), and then precipitated by centrifugation at 1000×*g* for 5 min. The PBS supernatant ("wash" fraction) was transferred to a new tube for assay. The cell pellet was resuspended with 500 μl of RSBI (50 m*M* Tris-HCl [pH 8], 200 m*M* NaCl, 2 m*M* $MgCl_2$) as the "lysate" fraction. Medium, wash, and lysate fractions were subjected to three freeze–thaw cycles (dry ice, 37°C), and then digested with 40 units of Benzonase (EMD Chemicals, Gibbstown, NJ) at 37°C for 30 min before genome titration.

For large-scale preparations, cell lysates were prepared from forty 15-cm plates 72 hr after transfection, of which the final 48 hr was in DMEM in the absence of serum. After three freeze–thaw cycles (–80°C, 37°C), particles were purified from lysates by two rounds of cesium chloride centrifugation. Gradient fractions containing pure vector were concentrated and desalted, using Amicon Ultra-15 centrifugal filtration devices (Millipore, Bedford, MA). Glycerol was added to the concentrate to a final concentration of 5% (v/v), and the preparations were aliquotted and stored at –80°C. Culture medium from the same forty 15-cm plates (800 ml) 72 hr posttransfection were pooled and clarified through a 0.5-μm (pore size) Mini Profile II depth filter (Pall, Port Washington, NY). The clarified pool was concentrated to 20 ml by tangential flow filtration (TFF), using an LV Centramate system with two 0.2-cm^2 100-kDa molecular weight cutoff Omega ultrafiltration membranes (Pall), maintaining a transmembrane pressure of 25 psi, according to the manufacturer's recommended procedures. The concentrate was subjected directly to two rounds of cesium chloride gradient centrifugation and fractions containing pure vector were processed as described previously. TFF filters were cleaned and sanitized with 0.2 *N* NaOH between runs, and then rinsed copiously with water such that the normalized water recovery (NWP) was greater than 80%. All vector preparations were evaluated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) for purity. The purity of preparations used in studies were 70% or higher as determined by band intensity on the SDS–polyacrylamide gel.

Vectors

For *in vitro* studies, vector DNA encoding the firefly luciferase gene driven by the cytomegalovirus (CMV) early promoter was packaged in various serotypes. Infectivity assays and *in vivo* studies were performed with a bicistronic construct expressing human α_1-antitrypsin (AAT) and green fluorescent protein (GFP) separated by a T2A self-cleaving site from a CMV-enhanced chicken β-actin promoter and intron. In the immunization studies, vectors encoded a shortened version of HIV-1 *gag* as described previously (J. Lin *et al.*, 2007).

Quantitative polymerase chain reaction

Quantitative polymerase chain reaction (qPCR) targeting vector DNA polyadenylation signals was performed on an 7500 fast real-time PCR instrument (Applied Biosystems, Foster City, CA), using the recommended thermocycling profile. TaqMan universal master mix (part no. 4326614), custom primers, and custom probes were purchased from Applied Biosystems. Amplification results were analyzed with the auto-C_t set function of the 7500 system software. Assay parameters with threshold cycle (C_t) values and calculated quantities for each reaction were exported for further analysis. Samples were treated with DNase before qPCR in order to eliminate nonencapsidated DNA. DNA plasmid standards were used to determine absolute titers.

Infectivity titration

B50 cells (Gao *et al.*, 1998) were cultured in DMEM supplemented with 10% FBS and G418 (0.5 g/liter). For vector titration, 96-well tissue culture plates were plated with 4×10^4 cells per well in DMEM–10% FBS without G418 and incubated at 37°C in a 5% CO_2 atmosphere for 16 to 20 hr to allow cell attachment. Immediately before infection, 10-fold serial dilutions of vector samples were prepared in serum-free DMEM containing 3.2×10^8 wild-type adenoviral particles/ml. For infection, culture medium was removed from wells to be inoculated and replaced with 50 μl of diluted vector. Five dilutions were inoculated per vector, eight wells per dilution. Two hours postinfection, 50 μl of DMEM–10% FBS without G418 was added to each well, giving a final volume of 100 μl containing 5% FBS. Plates were sealed with AirPore tape sheets (Qiagen, Hilden, Germany) and incubated at 37°C in a 5% CO_2 atmosphere. Three days later, cells were lysed by addition of 85.1 μl of lysis solution per well, giving final concentrations of 0.25% deoxycholate, 0.45% Tween 20, 1 m*M* Tris-HCl (pH 8), 1 m*M* EDTA, 0.1% SDS, and proteinase K (0.3 mg/ml). DNA was released by incubation at 37°C for 1 hr, 56°C for 2 hr, and then 95°C for 30 min. Vector copy number in each lysate was analyzed by qPCR as described previously. Wells containing at least 10 copies of vector DNA after input subtraction were considered positive. Titer, expressed as 50% tissue culture infective dose ($TCID_{50}$) per unit volume, was calculated from the number of vector-positive replicates at each dilution level, using the Spearman–Karber method (Finney, 1964). $TCID_{50}$ and qPCR were

Table 1. Relative *In Vitro* Infectivity of AAV1, AAV8, and AAV9

	Physical titer (GC/ml)	*Infectious titer (IU/ml)*	*P/I ratio*
AAV1			
Lysate	7.38×10^{12}	2.00×10^{11}	36.90
Medium	9.65×10^{12}	1.58×10^{11}	46.75
AAV8			
Lysate	3.92×10^{12}	8.29×10^{9}	472.62
Medium	7.38×10^{12}	1.77×10^{10}	416.69
AAV9			
Lysate	1.13×10^{13}	1.67×10^{10}	678.00
Medium	1.12×10^{12}	7.44×10^{8}	1506.27

Abbreviations: AAV, adeno-associated virus; GC, genome copies; P/I ratio, particle-to-infectivity ratio.

performed three times independently on a single vector lot for consistency. Averages of all measurements are presented in Table 1.

Transgene expression

AAT levels were determined from serum samples by AAT-specific enzyme-linked immunosorbent assay (Vandenberghe *et al.*, 2006). For heart and liver histology, tissues were fixed overnight in formalin, washed in PBS for 30 min, and frozen in O.C.T. compound (Sakura Finetek USA, Torrance, CA). Cryosections were prepared at a thickness of 8 μm and examined by direct fluorescence for GFP.

MHC class I tetramer staining and phenotypic analysis

Phycoerythrin (PE)-conjugated MHC class I H2-Kd-AMQMLKETI (HIV-1 Gag p24, amino acids 199–207) tetramer was obtained from Beckman Coulter (Fullerton, CA). Tetramer staining was performed as described previously (J. Lin *et al.*, 2007). Briefly, lymphocytes isolated from whole blood were stained for 30 min at room temperature with PE-conjugated tetramer, fluorescein isothiocyanate (FITC)-conjugated anti-CD8a antibody (Ly-2; BD Biosciences, San Jose, CA), PE/Cy5-conjugated anti-CD127 antibody, and PE/Cy7-conjugated anti-CD62L antibody (eBioscience, San Diego, CA). After staining, red blood cells were lysed and cells were fixed with iTAg MHC lysing solution mixed with fix solution (Beckman Coulter) for 10 min at room temperature. Data were acquired with an FC500 flow cytometer (Beckman Coulter) and analyzed with FlowJo software (Tree Star, San Carlos, CA). Tetramer-positive $CD8^+$ T cells were phenotyped on the basis of CD127 and CD62L antibody staining (i.e., effector T cells, $CD127^-CD62L^-$; effector memory T cells [TEM], $CD127^+CD62L^-$; and central memory T cells [TCM], $CD127^+CD62L^+$).

Results

We evaluated vector particle distribution in cultures of HEK293 cells transfected to package AAV2 inverted terminal repeat (ITR)-flanked genomes in AAV1, 2, 5, 6, 7, 8, and 9, and rh32.33 capsids. The cell culture medium was collected separately from the cellular fraction 72 hr after transfection. At the time of harvest, cells were incubated either in the presence or absence of 10% fetal calf serum. Total vector yields, measured as DNase-resistant vector genome copies detected by quantitative PCR (qPCR), varied as a function of the vector capsid serotype and culture conditions, by as much as 10-fold (Fig. 1). AAV2 and AAV6 gave the lowest total particle yields, particularly in the absence of serum. AAV7, AAV8, AAV9, and rh32.33 gave the highest particle yields in the presence and absence of serum. Interestingly, AAV5 gave high yields only in the absence of serum.

Substantial numbers of complete particles of all vector serotypes were found in the culture medium, although relative proportions varied (Fig. 1). AAV2 consistently gave the fewest particles in medium, both in absolute numbers and in proportion to total yield. Production of AAV2 vectors in the absence of serum reduced overall particle yields by 61% and decreased the proportion of particles in the supernatant from 20% in the presence of serum to 9% in the absence of serum. AAV6 performed similarly, but with relatively more vector particles released into the supernatant. Total AAV6 particle yield was 80% lower in the absence of serum; 88% of particles were found in the culture medium in the presence of serum, 68% without serum. The majority of vector particles from all serotypes except AAV2 and AAV5 was found in the supernatant. Proportions ranged from 51% for AAV9 cultured in

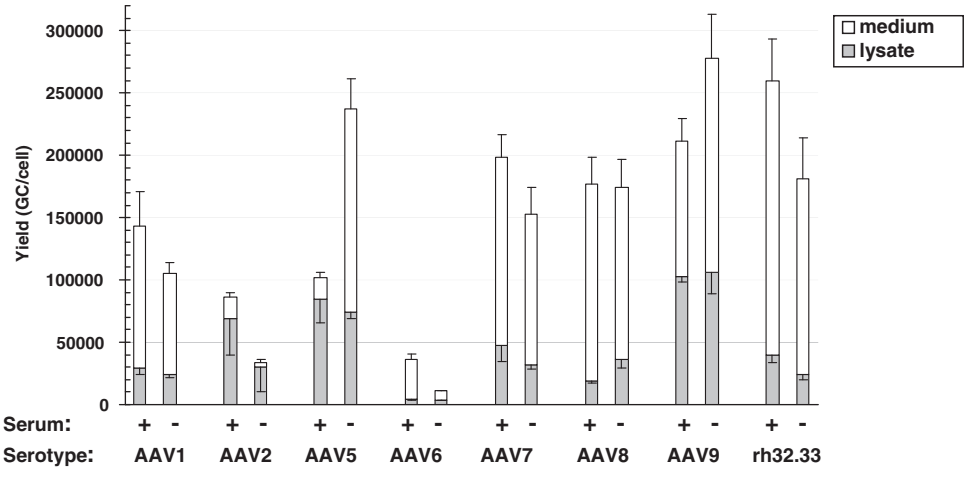

FIG. 1. Serotype-dependent particle distribution in vector production cultures. Vector particle distribution was evaluated by quantitative PCR for DNase-resistant vector genomes in "cell" and "medium" fractions from small-scale production cultures 72 hr after transfection by the $CaCl_2$ method, followed by incubation in the absence or presence of fetal bovine serum. Columns represent total yield of vector from $n = 3$ preparations, and columns are subdivided between the proportions of vector retrieved from the medium versus the cellular fraction. GC, genome copies.

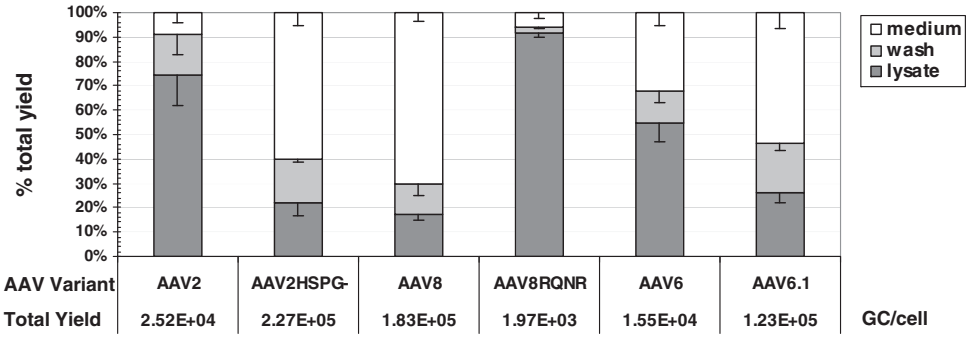

FIG. 2. Dependence of AAV vector yield and distribution on capsid heparin affinity. Physical vector particles were measured in "cell," "wash," and "medium" fractions from small-scale production cultures using AAV2, AAV8, AAV6, and variants by quantitative PCR for DNase-resistant vector genomes. Relative particle content in each of the three fractions was normalized to total yield, the sum of total particles in all fractions, which was set at 100% for the analysis. Total yields of infectious particles were calculated by addition of infectious particles detected by end-point dilution titration in each of the three fractions. Averages of three productions are represented with the standard deviation. GC, genome copies.

the presence of serum to more than 85% for AAV8 and rh32.33 in the presence or absence of serum. Inclusion of serum in the culture medium during vector production did not substantially affect relative particle distribution except for AAV5.

The lower yields of AAV2 and AAV6, in addition to their reduced yield and supernatant release in the absence of serum, prompted us to pursue the hypothesis that the heparin affinity of the capsid may impact on its release into the supernatant. AAV2 and AAV6 particles are know to have an affinity for heparin, although this interaction is defined by different structural determinants and mechanisms (Summerford and Samulski, 1998; Halbert *et al.*, 2001). We used variants of AAV2, AAV8, and AAV6 to investigate further the influence of heparin affinity on vector yields and distribution in the AAV production culture (Fig. 2). Ablation of the heparin-binding motif of AAV2 585RGNR to SGNT (AAV2HSPG-) indeed led to a significant increase in overall yield, which was largely attributed to release of particles into the medium (Vandenberghe *et al.*, 2006). Conversely, addition of the arginine-rich motif onto AAV8 (AAV8RQNR) (Vandenberghe *et al.*, 2006) led to the creation of a heparin-binding AAV8 particle that is retained in the cellular fraction almost exclusively and reduces particle yields dramatically. The correlate of reduction in titer with heparin affinity of the particle was also observed in the AAV2 situation, although not as substantially as observed with AAV8, for which the addition of the arginine-rich heparin-binding motif reduced overall yield almost 100-fold. The fact that heparin-binding AAVs that are retained in the cell are also lower yielding may be due to intracellular saturating effects that vector release into the culture medium prevents from occurring. The effect of heparin binding on particle release into the medium was supported in part by similar results for vectors packaged with wild-type AAV6, the capsid of which binds heparin, and mutant AAV6.1, which binds heparin less well because of single amino acid change K531E on the VP3 protein (Wu *et al.*, 2006; Vandenberghe *et al.*, 2009). Particle yields with the mutant were approximately 10-fold higher than yields with wild-type AAV6, and the proportion of particles in the culture medium increased significantly, albeit not as dramatically as we saw for AAV2HSPG- (Fig. 2).

Equivalence of bioactivity across vector preparations used for gene therapy or vaccine experiments and trials is critical. We conducted further experiments to compare the biological activity of AAV vector particles isolated from cell lysates, as per standard AAV manufacturing procedures, with that of particles purified from culture fluids. We used purified preparations of AAV1-, AAV8-, and AAV9-based vectors, three serotypes found predominantly in culture medium, in studies of *in vitro* and *in vivo* transduction (Table 1 and Figs. 3 and 4). Aside from a tangential flow filtration step for reduction of culture medium harvest volume before CsCl density gradient centrifugation, the purification processes applied to the two culture fractions were analogous. Purity of vector preparations from supernatant and pellet was at minimum 70% for all studies.

To determine the potency of AAV isolated from the two distinct sources *in vitro*, particle-to-infectivity (P/I) ratios were determined on the basis of qPCR measurements of both physical and infectious titer (Table 1). Infectivity assays were performed *in vitro* on the HeLa-derived B50 cell line by $TCID_{50}$ determination. For each serotype, particles purified from cell lysate and culture medium gave comparable P/I values, with the exception of AAV9, which was found to be 2-fold less infectious *in vitro* when purified from medium (Table 1). Yields obtained from AAV9 in culture medium were considerably lower for this lot as anticipated on the basis of prior data obtained from material from small-scale productions before purification (Fig. 1), likely due to differential technical loss during purification of cellular versus supernatant vector.

We injected male C57BL/6 mice intravenously with purified particles encoding a bicistronic expression cassette including the human serum protein α_1-antitrypsin (AAT) gene and a green fluorescent protein (GFP) gene. Quantification of AAT protein levels in serum by ELISA at multiple time points after treatment gave similar results for all animals injected with each vector regardless of source, that is, culture medium or cell lysate (Fig. 3A). Preparations from culture medium and cell lysates were equally effective. Also, reported differences in transgene expression across serotypes (Vandenberghe *et al.*, 2009) were replicated in our experi-

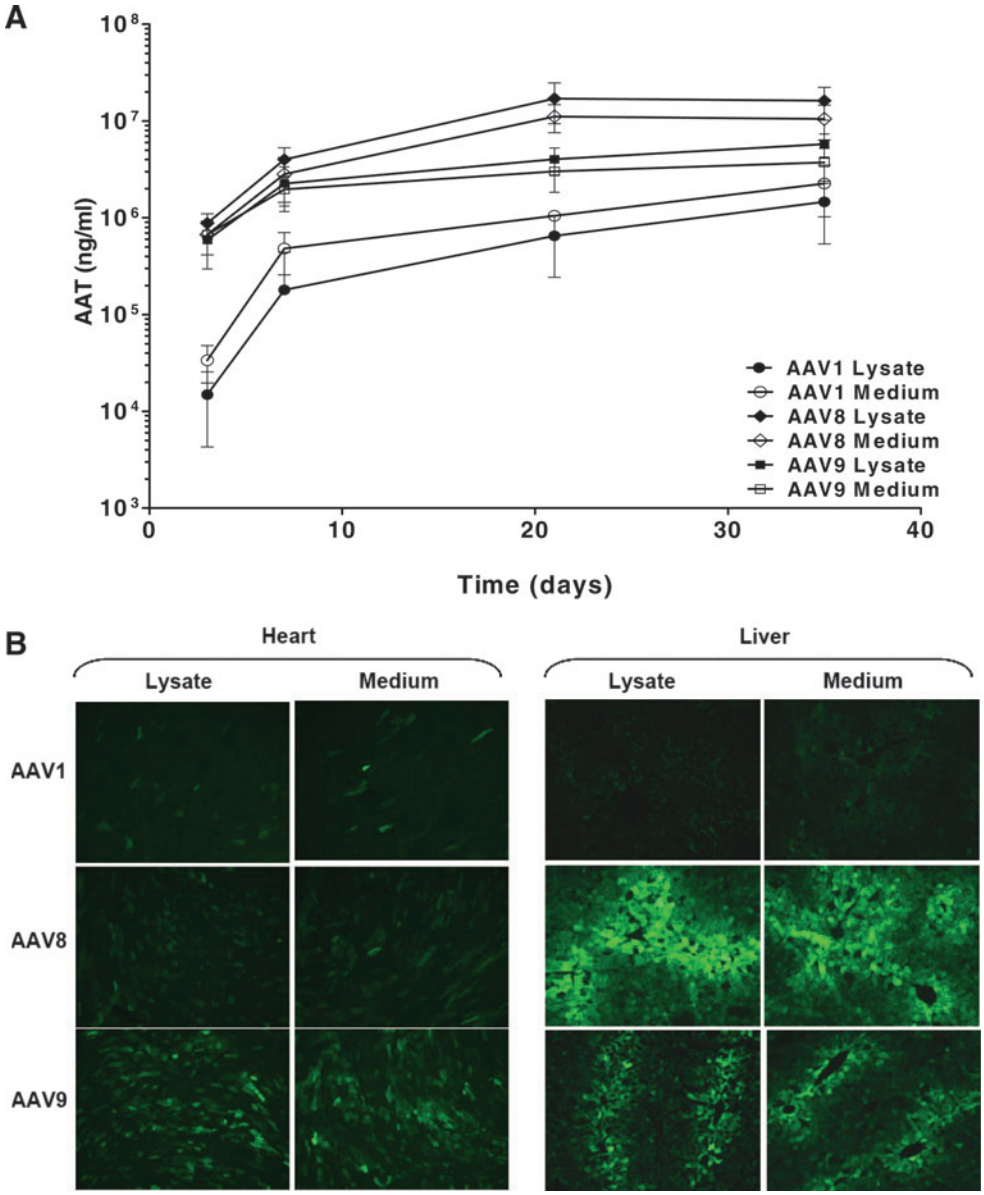

FIG. 3. *In vivo* transgene expression by AAV vectors purified from cell lysates and culture fluids. **(A)** α_1-Antitrypsin (AAT) transgene product concentrations in serum over time after intravenous injection of C57BL/6 mice ($n = 5$) with 10^{11} AAV1, AAV8, and AAV9 vector particles purified from cells or culture fluids; **(B)** expression of GFP in liver and heart tissues 35 days after vector injection.

ment by vector preparations from both sources. AAV8 was more potent than AAV9; AAV1 was considerably less potent than either AAV8 or AAV9 (Fig. 3A).

Because serum AAT levels are not indicative of the tissue tropism of vector transduction, particularly when the vector is administered systemically via the intravenous route, we extended our experiment to assess the *in vivo* transduction pattern in two important therapeutic tissue targets for gene therapy. Tissue sections of heart and liver from treated mice were examined by direct fluorescence for expression of the second transgene, GFP. Observed relative levels and tissue distribution of GFP fluorescence conformed to expectations for all three serotypes, and were equivalent after administration of vector preparations from cell lysates and culture fluids (Fig. 3B). Both AAV8 preparations targeted mouse liver preferentially, but gave moderate levels of transduction in cardiac myocytes as well. Both AAV9 preparations demonstrated high level of cardiac transduction as is anticipated based on previous findings (Inagaki *et al.*, 2006; Pacak *et al.*, 2006; Vandendriessche *et al.*, 2007). Limited GFP fluorescence was observed in tissues from mice injected with either preparation of AAV1, a serotype that does not efficiently transduce either the heart or the liver (Fig. 3B).

Adaptive immune responses to vector capsid and transgene product might also be affected by modification of vector production and purification processes. We evaluated these parameters using vectors based on rh32.33 and AAV8 carrying an HIV-1 *gag* transgene, purified from cell lysates and culture fluids from transfected cultures. AAV.rh32.33-based vectors have been shown to elicit functional $CD8^+$ T cell responses to encoded transgene products whereas vectors based on AAV8 actively suppress or anergize transgene-specific T cell responses (J. Lin *et al.*, 2007; S.W. Lin *et al.*, 2007; Lin *et al.*, 2009). Intramuscular vaccination of mice with 3×10^{10} GC prepared from either source generated roughly equivalent peak levels of antigen-specific $CD8^+$ T

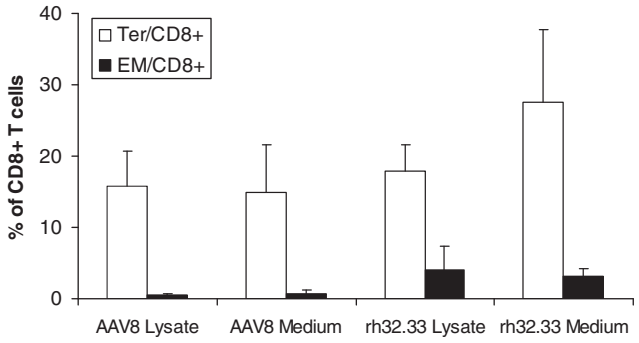

FIG. 4. Immunogenicity of AAV vectors purified from cell lysates and culture medium. Preparations of AAV8- and rh32.33-based vectors encoding HIV-1 *gag*, purified from cell lysates and culture fluids, were administered intramuscularly at 3×10^{10} GC per mouse. Three weeks postvaccination, peripheral blood mononuclear cells were evaluated by flow cytometry for the presence of functional HIV-1 p24-specific CTLs by MHC-I tetramer binding ($Ter/CD8^+$) and expression of CD127 and CD62L. Effector memory (EM) cells were identified as the $CD8^+Tet^+$ $CD127^{hi}CD62L^{lo}$ population.

lymphocytes (CTLs) irrespective of the preparation method (Fig. 4). As expected, both AAVrh32.33-based preparations distinguished themselves by generating an effector memory T cell population that is largely absent in all mice injected with AAV8, further supporting functional equivalence of preparations from cell lysates and culture fluids.

Discussion

AAV has become an attractive gene transfer vector. Initially, AAV was considered solely as a vector for therapeutic gene therapy but recently, its application as a research tool for somatic transgenesis is gaining ground. Unfortunately, production of purified AAV vectors at the scale required for *in vivo* studies relies on cumbersome, highly technical and costly procedures that are generally best completed by contract services in specialized core facilities. Because multiple permutations of a candidate vector and/or transgene cassette must be evaluated in research and preclinical studies, easier and more versatile AAV vector production protocols would benefit both the scientific community and gene therapy researchers. For clinical trials, where high vector titers are required, scalable manufacturing methods are critical.

In the study reported here, we demonstrate that vectors based on many commonly used AAV serotypes are present at high titer in culture medium of transfected cultures, and can be recovered simply by harvesting and concentrating the culture fluids. This finding is supported by similar observations by Okada and colleagues for AAV8 (Okada *et al.*, 2009). Purification from harvested cell lysates, common practice based on experience with AAV2, is not required. We demonstrate further that most of these vectors can be produced to high yields in culture fluids in the absence of serum, further simplifying downstream processing and purification of preparations, decreasing costs, and lessening concerns about possible contamination of AAV clinical products with zoonotic agents such as the prion agent associated with bovine spongiform encephalopathy. In our experiments, critical biological activities, including transduction efficiency *in vitro* and *in vivo*, tissue tropism, and immune response elicited by transgene expression, were equivalent for vector preparations from cell lysates and culture fluids, with one possible exception. A reduced particle-to-infectivity ratio *in vitro* was observed for the AAV9 vector particles in supernatant preparations. This may reflect a novel biological characteristic of AAV9 particles released from the cell. However, *in vivo* we were unable to detect any differences in biological activity between AAV9 vector preparations from cells and culture fluids.

Our results contribute to simplified vector-manufacturing protocols, a benefit for AAV vector applications in both the laboratory and the clinic. The ability to isolate AAV vectors from the culture medium reduces the complexity of the substrate from which particles must be purified and minimizes handling. How AAV, a nonenveloped parvovirus, is released during vector production from the cell in the culture medium in the apparent absence of CPE remains unexplained. This release property, however, is dependent on serotype and specific glycan-binding affinities of the capsid, and is not a function of $CaCl_2$ transfection because other transfection methods produce the same phenomenon (Lock *et al.*, 2010). On the basis of the experiments presented here, we have now developed a GMP-compatible, medium-scale AAV vector-manufacturing process that yields up to 3×10^{14} particles in a single 10-liter run (Lock *et al.*, 2010).

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Author Disclosure Statement

L.H.V. and M.L. are inventors on patents licensed to various biopharmaceutical companies, including ReGenX. J.M.W. is a consultant to ReGenX Holdings, and is a founder of, holds equity in, and receives a grant from affiliates of ReGenX Holdings; in addition, he is an inventor on patents licensed to various biopharmaceutical companies, including affiliates of ReGenX Holdings.

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