Near-perfect infectivity of wild-type AAV as benchmark for infectivity of recombinant AAV vectors

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Abstract

Viral vectors derived from adeno-associated viruses (AAV) are widely used for gene transfer both in vitro and in vivo. The increasing use of AAV as a gene transfer vector, as well as recently demonstrated immunological complications in clinical trials, highlight the necessity to define the specific activity of vector preparations beyond current standards. In this report, we determined the infectious, physical and genome-containing particle titers of several wild-type AAV type 2 (wtAAV2) and recombinant AAV type 2 (rAAV2) preparations that were produced and purified by standard methods. We found that the infectivity of wtAAV2 approaches a physical-to-infectious particle ratio of one. This near-perfect physical-to-infectious particle ratio defines a “ceiling” for the theoretically achievable quality of recombinant AAV vectors. In comparison, for rAAV2, only approximately 50 out of 100 viral particles contained a genome and more strikingly only approximately one of the 100 viral particles was infectious. Our findings suggest that current strategies for rAAV vector design, production and/or purification should be amenable to improvements. Ultimately, this could result in the generation of near-perfect vector particles, a prospect with significant implications for gene therapy.

Keywords

wtAAV; rAAV; particle-to-infectivity ratio; specific activity
Introduction

Successful viral gene therapy relies on the efficient production of genetically engineered viruses of high quality. Recently, viral vectors based on adeno-associated virus (AAV) have shown great promise as gene delivery vehicles due to their long-term gene expression and relative safety as demonstrated by exciting successes in human clinical trials. Although great advances have been made in large-scale AAV vector production, the criteria for assessing specific recombinant AAV (rAAV) vector infectivity, defined as the number of infectious viral particles normalized to the total viral particle number, remain vague and not standardized. In this report we propose to define the benchmark for the ideal rAAV as the specific infectivity of wild-type AAV (wtAAV).

Despite the emerging clinical success in using AAV vectors, some vector related complications have surfaced in recent clinical trials that highlight the importance of a renewed assessment of the specific vector infectivity. For instance, in an otherwise promising clinical trial aimed at treating factor-IX-deficiency-induced hemophilia, the destruction of rAAV-transduced hepatocytes by cytotoxic T-cells was observed. It was later suggested that pre-formed capsid protein in the virus preparation might have been the major source of epitopes that triggered the observed immune response. These results illustrate the importance of producing vectors with a near-perfect specific infectivity (i.e. lacking defective viral particles), which would allow the reduction of the vector dose required to achieve therapeutic efficacy, thereby minimizing the risk of an adverse immune response.

The specific infectivity of viral preparations is defined by the ratio of physical viral particles (vp) to infectious units (iu). Thus, the optimal vector preparation reaches the theoretical limit of specific infectivity, i.e. a ratio of one; put differently, every viral particle in such a preparation is functional. Several investigators have reported the specific infectivity of rAAV or wtAAV preparations produced and purified by varying methods. However, non-standardized production, purification and titration methods render cross-laboratory comparison difficult. Furthermore, the specific infectivities of the viral preparations are not always reported using the same units. Significantly, the specific infectivity of both rAAV and wtAAV-produced and purified identically–has not been reported rendering the direct a comparison between wtAAV and rAAV impossible. For rAAV, for example, Salvetti et al., reported an impressive genome-containing particle (gcp)-to-iu ratio of 1.4. However, this publication (as well as several others) did not report how many total viral particles the preparation contained, an important measure with respect to gene therapy efficacy and immune response. On the other hand, rAAV preparations with a vp-to-gcp ratio of 1 were reported showing that it is possible to remove all empty particles. However, these reports did not mention how many of the viral particles were infectious, a crucial measurement to determine the specific infectivity. The Kleinschmidt group was the first to report extensive analysis of both rAAV and wtAAV preparations using all three measurements (i.e. vp, iu, gcp) to determine the specific infectivity. They reported a vp-to-iu ratio for rAAV preparations of 1800 and for wtAAV preparations a ratio of 42. However, these viruses were not purified, thus their vp-to-gcp ratios of 8 and 21 for rAAV and wtAAV, respectively show that the preparations contained empty viral particles, which as
reported here and elsewhere15, can largely be removed. Overall, the literature referenced highlights the importance of reporting all three infectivity-defining measures (i.e. vp, gcp and iu) in order to characterize fully the relevant properties of an AAV vector preparation, especially with respect to their use in gene therapy approaches. To our knowledge, this report is the first to systematically compare rAAV2 and wtAAV2 preparations—produced and purified identically—with respect to the specific infectivity defined by these three measurements. For our study, we reasoned that wtAAV is likely to have evolved an optimal strategy to replicate and package infectious viral genomes and thus would define the theoretical limit for rAAV’s specific infectivity. Surprisingly, our results indicate that near-perfect vp-to-iu ratios can be achieved for wtAAV2. In contrast, this is not the case for rAAV2. Comparison of wild-type and rAAV stocks should then establish a basis for dissecting the molecular mechanisms that are responsible for the differences observed between wtAAV and rAAV. Based on our findings, we propose that it might be possible in the future to develop rAAV2 production and purification methods that yield rAAV2 stocks with vp-to-iu ratios approaching the ones of wtAAV2. Such improvements would have important implications for the use of rAAV vectors in basic research and especially in clinical studies and applications.

Material and Methods

Cell culture

HEK 293T cells were cultured in Dulbecco’s modified Eagle medium (DMEM; Cellgro, Herndon, VA) supplemented with 10% fetal bovine serum (FBS, Gemini, Woodland, CA). HeLa and HeLa C1215 cells were grown in minimum essential medium eagle (MEM, Cellgro), supplemented with MEM nonessential amino acids (1X, Cellgro), Sodium Pyruvate (1mM, Cellgro), 10% FBS and G418 Sulfate (50μg/ml, Cellgro) for C12 cells. All cells were grown at 37°C and 5% CO2.

Virus production

2.5×10^7 cells were seeded in a triple-layer flask. 24 hours later, the cells were double transfected using the calcium phosphate method and incubated in DMEM low glucose (1g/l, Cellgro, Manassas, VA) supplemented with 2% FBS and 25mM HEPES. Plasmids used for wtAAV: pAV2, containing the wtAAV2 genome and pDG13, containing the rep, cap genes and the Adenovirus helper functions. Plasmids used for rAAV2-GFP: pDG and pTRUF11, containing the CMV enhancer/chicken β-actin promoter, the humanized GFP transgene and the SV40 poly-A signal and a neo expression cassette flanked by the AAV2 ITRs. Plasmids used for rAAV2-mCherry: pDG and pTW149, which was cloned from pTRUF11 by replacing the GFP with the mCherry transgene (kindly provided by Dr. Roger Tsien, University of California, San Diego27). Plasmids were transfected at the molar ratio of 1:1. Plasmid DNA for transfection was produced using the endofree DNA maxi-prep kit as recommended by the supplier's instructions (Qiagen, Valencia, CA). 72 hours post transfection the cells were harvested by centrifugation for 10 minutes at 1100 rpm (Sorvall RC3C), washed in PBS, resuspended into 9ml lysis buffer (150mM sodium chloride, 50mM Tris pH 8.5) and lysed by three freeze/thaw cycles (alternating dry ice/ethanol and 37°C water bath). 1mM MgCl2 and 150units/ml Benzonase (Sigma, St.Louis, MO) were added.
and the lysate was incubated one hour at 37°C. The cell lysate was clarified by centrifugation at 3400g for 20 minutes and the supernatant was stored at −80°C.

**Virus purification**

The virus was purified by iodixanol step gradient. The gradient was formed in Optiseal polyallomer centrifuge tubes (Beckman Coulter, Palo Alto, CA) by first adding 7.3 ml of 15% iodixanol (Axis shield PoC AS, Oslo, Norway) in 1M sodium chloride, 1X TD-buffer (1X PBS, 1mM MgCl₂, 2.5mM KCl) and then underlining, in succession, 4.9 ml 25% iodixanol in 1X TD buffer containing 12.5μg/ml Phenol Red (Gibco, Grand Island, NY), 4 ml 40% iodixanol in 1X TD buffer and 4 ml 60% iodixanol containing 12.5μg/ml Phenol Red. The cell lysate was then applied on top of the gradient, which was centrifuged at 69000rpm at 18°C for one hour in a 70Ti rotor. The virus was extracted with an 18-gauge needle from the 40%-60% iodixanol interphase as well as the majority of the 40% iodixanol phase. A vivaspin20, 100kDa cut off concentrator (Sartorius Stedim, Goettingen, Germany) was used to concentrate the virus as well as to exchange the buffer to Lactated Ringer’s solution (Baxter, Deerfield, IL). The concentrator was spun at 1200g and the virus concentrated to a final volume of 1 ml. Finally, the virus was filtered through a 0.22 μm filter and stored at −80°C.

**Purity assessment of the virus preparations**

The maximum volume (1.22×10₁¹ vp and 7.76×10⁹ vp of wtAAV2 and rAAV2 preparations, respectively) of the purified virus sample was mixed with 5x protein loading buffer (Fermentas, Glen Burnie, MD) and 20x reducing agent (Fermentas) and boiled for 5 minutes at 95°C. The samples were loaded and run on a 10% SDS polyacrylamide protein gel, which was fixed 2x for 20 min in 50% methanol, washed for 30 minutes in water and stained for 15 minutes in 47.1mM silver nitrate, 0.076% sodium hydroxide, 1.4% ammonium hydroxide. The gel was then washed 3× 5 minutes in water and developed to satisfaction in 0.005% citric acid, 0.019% formaldehyde. The developing reaction was stopped with 10% methanol and the gel was scanned wet (Figure S1).

**Infectious center assay**

HeLa or C1215 cells were infected in serial dilution with wtAAV2 or rAAV2, respectively and co-infected with Ad5 at an MOI of 25 pfu/cell. 40 hours post-infection, the cells were spread on 0.45μm nylon (Millipore, Ireland) membranes. The membranes were denatured for 5 minutes in 5M sodium chloride, 0.5M sodium hydroxide, neutralized for 5 minutes in 1M Tris pH7.4, 1.5M sodium chloride, UV cross-linked and hybridized to a rep-specific probe for wtAAV or a CMV-promoter-specific probe for rAAV2 over night in PerfectHyb Plus hybridization buffer (Sigma, St. Louis, MO). Primers used to amplify the rep probe fragment: 5′-GTTCCTGAGTCAGATTCGCG and 5′-AAAAAGTCTTTTGACTTCGCTT. Primers used to amplify the CMV probe fragment: 5′-TCAATTACGGGTCATTAGTTC and 5′-AATACATCGTAGAGATGACTGCC. The fragment was labeled by random prime DNA labeling (Roche, Indianapolis, IN). The membranes were washed at high stringency (according to the manufacturer’s instructions) and exposed to a phosphoimager screen for 14-16 hours. The Storm 860 phosphoimager and
the Image Quant™ software were used to analyze the membranes. For the titer calculation, each positive signal, corresponding to one infected cell and therefore to one infectious unit, was counted and the titer was calculated according to the volume and dilution of virus used for the infection. The experiment has been done three times in triplicate for wtAAV and rAAV2-GFP and once in triplicate for all other viruses.

**Transducing units assay**

1×10⁵ C1215 cells were seeded and infected 24 hours later with rAAV2-GFP in 10-fold serial dilutions. The assay was done both with and without co-infection with Ad5 at an MOI of 20 pfu/cell. 40 hours post-infection, the cells were harvested and analyzed by flow cytometry for GFP expressing cells. To determine the transducing unit titer the percentage of GFP positive cells, the total number of infected cells and the amount of virus used for the infection were used. The titer was calculated using the average of the two dilutions that showed a linear correlation of GFP expression and amount of virus used.

**Real-time quantitative PCR**

The genome-containing particle titer was determined by quantitative real-time PCR 18, using SYBR Green Jump Start Taq ready mix without MgCl₂ (Sigma-Aldrich, St. Louis, MO) according to the manufacturers instructions on a Light Cycler (Roche, Indianapolis, IN). The MgCl₂ concentration was optimized to 4mM. Linearized plasmid DNA was used to set up the standard curve, using 2ng (=2.53×10⁸ double stranded (ds) DNA molecules) to 0.002ng (=2.53×10⁵ ds DNA molecules) plasmid. For wtAAV2 viruses pAV2 and primers in the cap gene were used (5′-TTCTAGATGCTGCGTACCGGAAA and 5′-TCTGCCATTTGAGGTGGTACTTGGT). For rAAV2-GFP viruses pTRUF11 and primers in the GFP gene (5′-GCTCCTCCCAGACAACCATTACC and 5′-ATCCCAGCAGCGTGTCACAAAC) were used. For AAV2-mCherry pTREK-3, containing mCherry, and primers in the mCherry gene (5′-CTGAAGGGCGAGATCAAGCAGAG and 5′-GATGGTGTAGTCTCGTGTTGGG) were used. The experiment has been done three times in triplicate for wtAAV2 and rAAV2-GFP and once in triplicate for all other viruses.

**Dot blot analysis**

DNA dot blot analysis was used for the determination of the genome-containing particles of the virus preparations. A series of 12 two-fold dilutions of linearized plasmid DNA (the same plasmids were used as in real-time PCR), ranging from 50ng (=6.3×10⁹ ds DNA molecules) to 0.024ng (=3×10⁶ ds DNA molecules) was used for the standard curve. 400μl of denaturation solution (0.4N sodium hydroxide, 10mM EDTA) was added to each standard and to 1μl and 0.1μl of the virus sample. The samples were boiled for 10 minutes and transferred on a Hybond-XL membrane (Amersham Biosciences, Buckinghamshire, UK) onto a dot blot apparatus. Each slot was washed with 400μl denaturation solution and the membrane was UV-cross-linked. The membrane was hybridized to a rep-specific probe for wtAAV2 or a CMV-primer-specific probe for rAAV2 and washed as described for the infectious center assay. The membrane was exposed to a phosphoimager screen for 2 hours and analyzed using a Storm 860 phosphoimager. The signal intensity was recorded using the
Image Quant™ software and the titer was calculated using linear regression. The experiment has been done three times in triplicate.

**Amino acid analysis**

Determination of the protein content by amino acid analysis for the calculation of the viral particle titer was conducted at the W.M. Keck Foundation Biotechnology Resource Laboratory at Yale University. Briefly, the virus sample was hydrolyzed for 16 hours at 115°C and analyzed by ion-exchange HPLC. The particle titer was calculated from the nmoles of each individual amino acid, the amino acid composition (Gen-Bank accession numbers AAC03780, AAC03778, AAC03779) of the wtAAV2 capsid, the 1:1:10 ratio of VP1:VP2:VP3 and the amount of virus analyzed. The average amount from the most reliably measurable 10 amino acids was used to determine the final particle titer. The experiment has been repeated twice for wtAAV2a, which yielded a ratio between the two measurements of 1.07 (3.47×10^{12} vp/ml and 3.71×10^{12} vp/ml) and demonstrating that the amino acid analysis is reproducible and accurate. Therefore the titer measurements for all other virus preparations were done once.

**Electron microscopy**

5μl of virus were applied to 300 mesh formvar/carbon copper grids (Electron Microscopy Sciences, Hatfield PA). After letting the sample dry the grid was washed with several drops of water and the excess water wicked off with a filter paper. 5-10 μl of 2% (w/v) uranyl acetate was applied to the grid. After 5 min. the excess uranyl acetate was wicked off and the grid was let dry. Grids were analyzed at 50'000-fold magnification with a Hitachi 7650 transmission electron microscope. For each sample three fields of approximately 200 particles were counted, the percentage is given in mean ± SEM.

**Statistical analysis**

All titer comparisons were done using the two-tailed student's T-test for paired samples or two samples with equal variance. All error bars represent the SEM, the means are drawn from three experiments, each done in triplicates or one experiment done in triplicate as specified.

**Results**

wtAAV2 and rAAV2 containing the GFP transgene (rAAV2-GFP) were produced by double transfection 13 and purified using the iodixanol gradient centrifugation method 7. This method is known to largely separate empty from full viral particles, which is not the case for all methods described in the literature. For purity assessment the virus preparations were analyzed by SDS-polyacrylamide gel electrophoresis followed by silver-staining for the sensitive detection of all proteins. Mainly the three viral capsid proteins VP1, VP2 and VP3 were visible in the expected ratios, indicating the high purity of the samples (figure S1). To characterize fully our virus preparations, we first aimed to determine the arguably most important measure of specific infectivity of a virus preparation, the infectious unit titer, using the well-established infectious center assay 14. Briefly, HeLa cells were infected in serial dilutions with wtAAV2 and co-infected with wild-type adenovirus type 5 (Ad5). Forty
hours post-infection, the cells were transferred to nylon membranes and replicated viral DNA was detected by hybridization to a $^{32}\text{P}$-labeled rep probe. Each positive signal, representing a single-infected cell and infectious particle, was counted and the titer was calculated according to the volume and dilution of virus used for the infection. The first wtAAV2 preparation (wtAAV2a) yielded $3.3\times10^{12}$ iu/ml with a standard error of the mean (SEM) of $1.1\times10^{12}$ iu/ml (figure 1a and table 1). Our rAAV2 preparation, rAAV2-GFPa, was titrated accordingly using C1215 cells, which contain a cap gene encoding the three AAV capsid proteins as well as the rep gene, whose gene products are required for replication of AAV. To test whether C12 and HeLa cells could be infected equally, we performed an infectious center assay in parallel on the two cell lines using wtAAV2a. We found that the infectious titers for wtAAV2a obtained on HeLa and C12 did not differ significantly (ratio of 0.94, data not shown). The infectious titer of rAAV2-GFPa titer determined on C12 cells was $2.9\times10^9$ iu/ml, SEM $5.7\times10^8$ iu/ml (figure 1b and table 1). As a second measure, we determined the transducing units (tu) titer using a transduction assay on C12 cells, using our rAAV2-GFPa preparation in the absence and presence of Ad5 co-infection. The tu titer of rAAV2-GFPa, determined by measuring the percentage of GFP expressing cells by FACS analysis 40 hours post-transduction, was $2.4\times10^9$ tu/ml, SEM $5.6\times10^8$ tu/ml with Ad5 co-infection and $3\times10^7$ tu/ml, SEM $8.3\times10^6$ tu/ml, without Ad5 co-infection (figure 1c). As reported previously16,17, this assay shows that without replication of the viral genome (facilitated by Ad5) the transgene expression in a target cell can be diminished and therefore the tu-assay can under-represent the infectivity of the virus. However, if replication was supported, by co-infection with Ad5, the tu titer determined by the infectious center assay was confirmed.

In order to determine the vp-to-iu and gcp-to-iu ratios, we next sought to determine the number of physical and genome-containing viral particles in the preparations. We determined the viral genome titer by real-time PCR18 and dot blot analysis14. wtAAV2a yielded $3.5\times10^{12}$ gcp/ml, SEM $4.5\times10^{11}$ gcp/ml from the real-time PCR assay and $4.5\times10^{12}$ gcp/ml, SEM $1.7\times10^{12}$ gcp/ml from the dot blot assay (table 1). The student's t-test revealed that the Q-PCR and dot blot titers for wtAAV2a are not significantly different ($p>0.59$; table 1). rAAV2-GFPa yielded $1.6\times10^{11}$ gcp/ml, SEM $9.1\times10^9$ gcp/ml from the real-time PCR assay and $2.2\times10^{11}$ gcp/ml, SEM $2.3\times10^{10}$ gcp/ml from the dot blot assay.

In order to assess the number of physical particles in the virus preparations we used quantitative amino acid analysis10, an accurate method to determine total protein concentration. Based on a capsid protein ratio of VP1:VP2:VP3=1:1:1019 and the determination of the amounts of the most reliably measurable 10 amino acids (table S1) viral particle titers could be calculated. For wtAAV2a we found the viral particle titer to be $3.5\times10^{12}$ vp/ml, SEM $1.4\times10^{11}$ vp/ml, while the rAAV2-GFPa titer was $3.6\times10^{11}$ vp/ml, SEM $2.4\times10^{10}$ vp/ml (table 1). For wtAAV2a, this analysis was done in duplicate and the duplicate titers were near identical (ratio of 0.94). For wtAAV2a the student's t-test revealed no significant difference between the viral particle (aa-analysis) titer and the genome-containing particle titer (Q-PCR, $p>0.88$) or between the viral particle titer (aa-analysis) and the infectious unit (ICA) titer ($p>0.86$, table 1). These results show that wtAAV2a has a vp-
to-iu ratio of 1.1 (table 1), i.e. nearly every particle is infectious and replication competent, revealing an almost perfect specific infectivity of this virus.

The vp-to-iu ratio of the rAAV2-GFPa was determined to be 124 (table 1), i.e. only one in 124 viral particles is infectious and replication competent. The vp-to-tu ratio for rAAV2-GFPa was 157 and 12,222 with and without Ad5 co-infection, respectively.

It is well known that rAAV preparations contain varying amounts of empty particles, representing a potential explanation for the high vp-to-iu ratios observed in our rAAV2-GFPa preparation. However, the level of empty capsids in a purified AAV preparation depends on the purification method used and the iodixanol purification method used in this study, is known to largely remove empty particles. Nevertheless, in order to exclude the possibility that contaminating empty particles are the main cause of the high vp-to-iu ratios observed, the fraction of such particles in rAAV2-GFPa was determined using transmission electron microscopy. Figure 2 shows that in the control preparation, where empty viral particles were generated using the baculovirus system, 99.2%, SEM 0.4% of the particles appeared indeed empty. In contrast, in the wtAAV2a preparation 94%, SEM 1.6% of the particles were full, confirming our prediction that nearly every particle contained a genome. Consistent with earlier observations, the rAAV2-GFPa preparation consisted of 65.4%, SEM 1.8% full particles, translating into a vp-to-gcp ratio of 1.5. These observations demonstrate clearly that the presence of empty capsids in rAAV preparations alone is not sufficient to account for the decreased infectivity observed in our rAAV2-GFPa stock.

Because we are aware of the unprecedented character of our finding that wtAAV2 can be produced at a near-perfect vp-to-iu ratio, two additional wtAAV2 stocks (wtAAV2b and wtAAV2c) were prepared and characterized independently. Furthermore, in order to show that the ratios observed in our rAAV2 stocks are not mainly a result of the GFP transgene, but rather that the ratios are a more general feature of rAAV2, we prepared, in addition to a second rAAV2-GFP (rAAV2-GFPb), rAAV2-mCherry. The viruses were produced and purified identically to previous preparations and analyzed by the infectious center assay for their iu titer and amino acid analysis for their vp titer. Table 2 shows the titers of these additional AAV preparations and confirms that wtAAV2 preparations have a vp-to-iu ratio of approximately one, while the vp-to-iu ratio of rAAV2 range from 53 to 124 (table 1 and 2).

**Discussion**

In 2006, in an otherwise promising human clinical trial for hemophilia B, the destruction of rAAV transduced hepatocytes by cap-specific CD8+ T-cells was reported. Hauck et al., concluded that the main cause for the observed cytotoxicity was an immune response against the viral capsid proteins. Although potential remedies for this problem are still under debate, it is likely that the strength of a potential immune response is influenced by the amount of viral capsid proteins injected. This highlights the need for using the smallest vector dose possible and, consequently, the use of viruses with the highest achievable specific infectivity.
To date, virus preparations are evaluated by vp-to-iu ratios. Vp to iu ratios define a measure reflective of the amount of active and defective viral particles in a virus preparation and a vp-to-iu ratio of one represents a perfect virus preparation. Many parameters influence the specific infectivity of virus preparations, including the molecular mechanisms underlying replication and packaging and the purification of viral preparations. Traditionally, rAAV was purified using several rounds of cesium chloride (CsCl) gradient centrifugations. While this is a labor-intensive method that often led to varying vector quality and loss of particle infectivity, in some studies very good gcp-to-iu ratios were reported for rAAV that was purified by this method. Subsequently, faster and more reliable purification methods were developed, including the iodixanol gradient purification method that was used in this study. With the establishment of ELISA-based capsid quantifications it became possible to measure not only gcp-to-iu ratios but also vp-to-iu ratios, determining the specific infectivity of the viral preparation. Using this method, Grimm et al. reported vp-to-iu ratios for rAAV to be 1800 and above and for wtAAV to be 42 and above in crude, non-purified virus preparations. These findings implied that viral preparations were contaminated with empty or otherwise defective particles. Therefore, to determine accurately the specific infectivity of a given vector preparation, the physical viral particle, the genome-containing particle and the infectious particle titer of a given vector preparation need to be known.

In this report, we determined these three parameters using well-established AAV titration methods. The resulting vp-to-iu ratio of several wtAAV2 and rAAV2 virus preparations produced and purified with identical, standard methods could be determined. We found that preparations of wtAAV2 can approach not only vp-to-gcp ratios of one but, more importantly, can also approach vp-to-iu ratios of one (table 1 and 2). These results suggest that the “ceiling” for the specific infectivity of AAV-based vectors is close to a vp-to-iu ratio of one, i.e. the ratio of a perfect virus. To our knowledge, to date, no virus has been discovered that has a vp-to-iu ratio approaching the perfect ratio of one. Employing the same methods, we then compared the wtAAV2 preparations to rAAV2 preparations containing either the GFP or the mCherry transgene. In contrast to wtAAV2, rAAV2 preparations showed a vp-to-iu ratio of 53 to 124 (table 1 and 2). The difference between wild-type and recombinant viruses raises the question as to what the underlying mechanisms are, that result in suboptimal rAAV2 preparations. For example, it would be possible that 123 out of 124 viral particles do not contain a genome. However, consistent with earlier observations, our electron microscopy results (figure 2), as well as the Q-PCR and amino acid analysis titers (table 1), show that approximately half of the viral particles contain a genome.

In order to reduce the proportion of empty or otherwise defective particles and, hence to improve the infectivity of rAAV preparations, two questions need to be answered: First, what causes the difference in packaging efficiency and specific infectivity of wild-type versus recombinant viruses and second, what can be changed in order to minimize the amount of empty or otherwise defective viral particles in rAAV preparations? The removal of empty capsids from genome-containing vectors has recently been addressed. Qu et al. published an improved and scalable method for the purification of rAAV vectors that can remove empty particles from the vector preparation yielding a vp-to-gcp ratio of 1.2 for rAAV. Unfortunately, it was not reported how many of these full particles were infectious.
A potentially even more promising approach than more advanced purification methods to minimize the fraction of empty or defective particles is to improve the production methods in order to prevent the formation of these particles in the first place. To do this, it is important to understand the reason(s) for the differences in packaging/replication efficiency between wtAAV2 and rAAV2. There are at least four hypotheses: (i) a cis-acting DNA element within the wtAAV2 genome can act as an as yet unidentified replication/packaging signal. Several groups presented evidence for the existence of such an element. In 2000, Tullis and Shenk identified a 1688 base pair cis-acting element that spans a large part of the rep gene and they demonstrated the importance of this element for virus replication. Once this region was deleted from the wtAAV2 genome, its replication efficiency was similar to rAAV2 vectors. Ward et al., confirmed this observation by deleting various parts of the left portion of the wtAAV genome, which lead to a reduction in virus production efficiency. The Salvetti group identified a different cis-acting replication element (CARE) at nucleotide 250-304 in wtAAV2 that is required for rep-dependent replication in the absence of the ITRs. However, in the context of rAAV with ITRs, no effect on replication and encapsidation was reported when this element was deleted. Taken together, a cis-acting replication/packaging element that can improve the overall quality of rAAV preparations—if existent—remains to be identified conclusively. The system provided in this report, i.e. using wtAAV as a benchmark for virus infectivity, could be used to prove the existence of such an element. (ii) rather than a cis-acting element, the complete wtAAV genome might be required to ensure the necessary DNA-capsid interactions. (iii) Expression of the rep or cap gene in trans (rAAV)—as opposed to in cis (wtAAV)—might result in a decrease of replication and/or encapsidation. Ward et al., found a significant reduction in virus production when parts of the cap gene were deleted or a stop mutation was introduced in the cap gene. It was concluded that, as suggested previously, the provision of cap in trans does not sufficiently support the DNA replication-encapsidation link. (iv) The size of the genome to be packaged might have an important impact on the quality of the virus. It was previously shown that genomes smaller than 3500 base pairs are defective in accumulation of single stranded DNA. The genomes of our rAAV2-GFP and rAAV2-mCherry are 4329 and 4323 bases long, respectively. Because these genomes are similar in length to wt-AAV2 (4679 bases), they should allow the synthesis of similar amounts of single stranded DNA when compared to wtAAV.

In conclusion, our data suggest a functional difference between wild-type and recombinant AAV particles. We provide a system, namely the comparison of the specific infectivity of a given rAAV preparation to that of wtAAV2 produced and purified in an identical manner, that in the future could be employed to dissect the molecular differences that underlie the formation of different wtAAV2 and rAAV2 particles. While we propose the use of wtAAV as a benchmark to compare the quality of rAAV vectors, we do not mean to imply that the perfect quality determined in vitro will translate into perfect transduction in vivo. Similarly, comparison of rAAV vectors of alternative serotypes or other AAV isolates as well as vectors purified by different methods will have to be standardized to their respective wtAAV counterpart independently. However, insights gained by such comparison studies could result in improved vector design and lead to the requirement for significantly lower viral
vector doses than are currently used in clinical studies thereby likely not only improving the efficiency of gene transfer but also reducing the risk of a deleterious immune response.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Determination of the infectious unit titer in wtAAV2a and rAAV2-GFPa and the transducing unit titer in rAAV2-GFPa

(a, b) wtAAV2a and rAAV2-GFPa were titrated by the infectious center assay (ICA). Briefly, HeLa or C1215 cells were infected in serial dilution with wtAAV2a or rAAV2-GFPa, respectively. The cells were co-infected with Adenovirus type 5 (Ad5), harvested 40 hours post-infection and transferred to nylon membranes. The membranes were hybridized to a rep-specific probe for wtAAV2a or a CMV-promoter-specific probe for rAAV2-GFPa. The titer was calculated by multiplying the number of positive cells with the dilution factor and dividing it with the volume of virus (in ml) used for the infection. The dilution factors are as indicated. Cells = HeLa or C12 cells that have not been infected with either AAV or Ad5, Ad5 = cells infected only with Ad5. (c) Determination of the transducing units (tu) titer of rAAV2-GFPa. C12 cells were infected with rAAV2-GFPa in serial dilutions and co-infected with Ad5. 40 hours post-infection the cells were analyzed by flow cytometry for GFP expression. The titer was calculated from the two dilutions within a linear range for the percentage of GFP expressing cells. All experiments were done three times, n=3, each in triplicate. The data is represented as mean±SEM.
Figure 2. Determination of full and empty viral particles by electron microscopy

Virus was applied to formvar/carbon copper grids, dried, washed and stained with 2% uranyl acetate. Grids were analyzed at 50k magnification with a transmission electron microscope. For each sample three fields, n=3, containing approximately 200 particles were counted. Percentages are represented as mean±SEM, scale bar=200nm
Table 1
Comparison of viral genome-containing particle (gcp), physical viral particle (vp) and infectious units (iu) titers by Q-PCR, dot blot, amino acid analysis and infectious center assay, respectively and physical-to-genome-containing particle (vp-to-gcp) ratios as well as physical-to-infectious particle (vp-to-iu) ratios of wtAAV2a and rAAV2-GFPa.

|                     | wtAAV2a<sup>a</sup> | rAAV2-GFPa<sup>a</sup> |
|---------------------|---------------------|-------------------------|
| gcp/ml (Q-PCR)      | 3.5×10<sup>12</sup> ± 4.5×10<sup>11</sup> | 1.6×10<sup>11</sup> ± 9.1×10<sup>9</sup> |
| gcp/ml (dot blot)   | 4.5×10<sup>12</sup> ± 1.7×10<sup>12</sup> | 2.2×10<sup>11</sup> ± 2.3×10<sup>10</sup> |
| vp/ml (AA-analysis) | 3.5×10<sup>12</sup> ± 1.4×10<sup>11</sup> | 3.6×10<sup>11</sup> ± 2.4×10<sup>10</sup> |
| iu/ml (ICA)         | 3.3×10<sup>12</sup> ± 1.1×10<sup>12</sup> | 2.9×10<sup>9</sup> ± 5.7×10<sup>8</sup> |
| ratio vp:gcp        | 1.0                 | 2.3                     |
| ratio vp:iu         | 1.1                 | 124.1                   |

<sup>a</sup>Titers are given in mean±SEM. Q-PCR, dot blot and ICA were done three times n=3, each in triplicate, AA-analysis was done once, mean of 10 individual amino acids, n=10.
Table 2

Determination of physical-to-infectious particle (vp-to-iu) ratio using the physical viral particle titer (AA-analysis) and the infectious units titer (ICA) of wtAAV2 and rAAV2 preparations$^a$.

|                | wtAAV2b                      | wtAAV2c                      | rAAV2-GFPb                  | rAAV2-mCherry                |
|----------------|------------------------------|------------------------------|-----------------------------|------------------------------|
| vp/ml (AA-analysis) | 5.8×10^{12} ± 3.4×10^{11}    | 6×10^{12} ± 3.7×10^{11}      | 2.3×10^{11} ± 3.4×10^{10}   | 2.7×10^{11} ± 3×10^{10}      |
| iu/ml (ICA)     | 6×10^{12} ± 1.3×10^{12}      | 3.9×10^{12} ± 1×10^{12}      | 4.4×10^{9} ± 7.6×10^{8}     | 5×10^{9} ± 4.6×10^{8}        |
| ratio vp:iu     | 0.9                          | 1.5                          | 53.2                        | 55.1                         |

$^a$ Titers are given in mean±SEM. AA-analysis was done once for each virus, the mean was determined from 10 individual amino acids, n=10. ICA was done once in triplicate n=3 for each virus.