Journal of Immunological Methods 387 (2013) 114–120

Contents lists available at SciVerse ScienceDirect

Journal of Immunological Methods

journal homepage: www.elsevier.com/locate/jim

Research paper

Assessment of a passive immunity mouse model to quantitatively analyze the impact of neutralizing antibodies on adeno-associated virus-mediated gene transfer

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ARTICLE INFO

Article history:
Received 9 September 2012
Received in revised form 4 October 2012
Accepted 5 October 2012
Available online 11 October 2012

Keywords:
Adeno-associated virus
Neutralizing antibodies
Passive immunity

ABSTRACT

Adeno-associated viruses (AAVs) are common infective agents of primates. As such, healthy primates carry a large pool of AAV-specific neutralizing antibodies (NAbs), which inhibit AAV-mediated gene transfer therapeutic strategies. Thus, a clinical method to screen patient candidates for AAV-specific NAbs prior to treatment, especially with the frequently used AAV8 capsid component, will facilitate individualized treatment design and enhance therapeutic efficacy. In this study, we evaluated the efficacy and sensitivity of a passive immunity mouse model to quantitatively assess anti-AAV8 NAb titers, as compared to an *in vitro* immunoassay. The passive transfer model was established in C57BL/6 mice by tail vein injection of pre-defined sera from 23 male rhesus monkeys. The mice were then administered low dose (3e10 GC/mouse) self-complementary (sc) AAV8. The *in vitro* NAb assay indicated that 69.57% of the rhesus donors had pre-existing anti-AAV8 NAb. The *in vivo* NAb assay, however, was better able to detect low NAb titer ($\leq$1:5), which can mediate neutralization *in vivo*. Indeed, 17 rhesus donors (74.0%) had pre-existing anti-AAV8 neutralization by *in vivo* NAb assay. Our findings indicated that the *in vivo* NAb assay is superior to the *in vitro* assay for detecting low NAb titers.

1. Introduction

Adeno-associated virus (AAV) vectors are promising delivery vehicles for gene therapy. AAV-mediated gene transfer can achieve sustained transgene expression and has an excellent safety profile in human patients (Manno et al., 2006; Kaplitt et al., 2007; Bainbridge et al., 2008; Eberling et al., 2008; Li et al., 2010). The traditional AAV vectors are single-stranded (ssAAV) which require a process of second strand synthesis and conversion into double strand genome *in vivo*. To overcome these limitations, self-complementary (sc) AAV vectors were developed. The scAAV genomes form double-stranded intermediates independent of second-strand synthesis and bypass the need for nucleus synthesis and the *in vivo* procedure (Gao et al., 2006).

Various serotypes of AAV exist and feature distinctive cell type specificities, genomic integration capacity, and immunogenicities. AAV2, for example, can be stably transduced without toxicity in animal models (Snyder et al., 1997). Although AAV2 is the most widely used serotype for gene therapy strategies to date, AAV8 produces similarly stable transduction and has been successfully applied to treatment

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http://dx.doi.org/10.1016/j.jim.2012.10.003

of many diseases in various organs (Davidoff et al., 2004; Evans et al., 2010). We constructed a hybrid scAAV2/8 (also known as scAAV8) by using a double-stranded AAV2-based genome and the AAV8 capsid with the aim of improving gene delivery and therapeutic efficacy.

Unfortunately, since the AAVs are common natural infective agents, most primates harbor a broad profile of pre-existing AAV-specific neutralizing antibodies (NAbs). This represents a major challenge to AAV-mediated gene therapy (Gao et al., 2009). A previous study detected anti-AAV8 NAb in 38% of the humans examined (Boutin et al., 2010). However, even though non-human primates are common models of disease therapy research, very little information on the presence and prevalence of anti-AAV8 NAbs is available. Thus, we aimed to determine the anti-AAV8 NAb profile in a representative rhesus monkey population from a commonly used research facility in China. The current NAb detection methods are immunoassays. However, recent studies have shown that this *in vitro*-based method lacks accuracy and sensitivity (Scallan et al., 2006). Therefore, we designed our study of rhesus monkey anti-AAV8 NAbs to also investigate the efficacy of a novel *in vivo*-based neutralizing capacity assay and determine if this *in vivo* approach may be more accurate and sensitive than the traditional *in vitro* method for quantitatively detecting pre-existing immunity and its effects on scAAV8-mediated gene transfer.

2. Materials and methods

2.1. AAV2/8 vectors

The scAAV2/8.CB.eGFP and scAAV2/8.CB.hAAT vectors were kindly provided by Prof. Guangping Gao from the University of Massachusetts Medical School (Worcester, MA, USA). In brief, the vectors had been created with AAV8 capsid using a double-stranded AAV2-based genome and expressed enhanced green fluorescent protein (eGFP) or human α-1 anti-trypsin (hAAT) transgene, respectively, from the constitutively-active chicken β-actin promoter (CB), which avoids eliciting cytotoxic T lymphocyte (CTL) responses (Franco et al., 2005; Gao et al., 2009).

2.2. In vitro neutralizing antibody assay

Twenty-three adult male rhesus macaques were screened for the presence of NAbs against AAV8, as described previously (Gao et al., 2011). All macaque-related procedures were carried out at the Chengdu National Center for Safety Evaluation of Drugs (Chengdu, China). Anti-AAV8 NAb titer was determined by incubating twofold serial dilutions of serum with HEK293 cultured cells and observing the dilution at which the number of GFP-positive cells was reduced by 50%, as compared with control wells. Briefly, two 96-well plates were seeded with either 293 cells (100 μL of 7×10^5/μL) and wild-type ADV (20 μL of 5×10^6/μL), or sera sample (60 μL) and AAV (60 μL). After incubation at 37 °C for 2 h, 60 μL of solution was removed from the 293 + ADV wells to confirm that the rate of 50 ADV particles/cell had been achieved. Then, 100 μL was removed from the sera sample + AAV well and added to the remaining 60 μL sample in the 293 + ADV wells. The sera samples + AAV plates were then discarded and the 293 + ADV combined with prepared sera + AAV plates were incubated at 37 °C for 1 h. Then, 100 μL of 20% FBS was added to each well and the plates were incubated at 37 °C overnight. The next day, the number of GFP expressing cells was recorded and used to calculate NAb titer using the following equation: $\%\text{Negative} = [\text{GFP}_{\text{negative}} - \text{GFP}_{\text{sample}}]/\text{GFP}_{\text{negative}} \times 100\%$, where NAb titer was the dilution at which the number of GFP expressing cells is equal to one-half of the negative control GFP cells.

2.3. Generation of a passive immunity mouse model to quantitatively assess anti-AAV8 NAb titers

Fifty male C57BL/6 mice (6–8 weeks old) were purchased from the animal breeding facility of Sichuan University (Chengdu, China) and housed in a pathogen-free environment with unrestricted access to water and standard rodent diet. All mice-related procedures were carried out in accordance with protocols approved by the Institutional Animal Care and Use Committee at the University of Pennsylvania (Wang et al., 2010).

To establish the passive immune model, mice were administered with a tail vein injection of pre-defined sera from the rhesus monkeys. One group of three mice received 200 μL of serum sample from an individual rhesus monkey. A negative control group of three mice received an intravenous injection of 200 μL of mouse serum (Sigma-Aldrich, USA). Two hours after the passive transfer, the mice were then administered a low dose (3e10 GC/mouse) of scAAV2/8.CB.hAAT by intravenous injection. The 3e10 dose was selected as optimal based on a previous screening test of our experimental system using 3e8, 3e9, and 3e10 (data not shown). A blank control group of three mice was set aside after the passive transfer and received no scAAV2/8.CB.hAAT vector.

2.4. Quantitative analysis of AAV transduction efficiency in mice

Transduction efficiency was evaluated at days 7, 14, 21, 28 after the scAAV2/8.CB.hAAT gene delivery by measuring hAAT expression in the sera by enzyme-linked immunosorbent assay (ELISA), as previously described (Wang et al., 2005).

2.5. In vivo neutralizing capacity assay

At days 7, 14, 21, 28 after AAV injection, blood samples were collected from each group of mice by the retro-orbital bleeding method. Sera were separated from the blood samples for detection of transgene hAAT expression using ELISA. Transgene expression was calculated as: $\%\text{Negative} = [\text{hAAT}_{\text{negative}} - \text{hAAT}_{\text{sample}}]/\text{hAAT}_{\text{negative}} \times 100\%$. If the %Negative value was ≤50%, the sample was considered as non-NAb for AAV.

2.6. Statistical analysis

Multiple comparisons of intergroup differences were conducted by ANOVA. Data are presented as mean ± SD. A significant difference was defined by p-value less than 0.05.

3. Results

3.1. Pre-existing anti-AAV8 NAb in rhesus monkeys

Among the 23 rhesus monkeys tested, 69.57% were positive for anti-AAV8 NAb, as defined by titers of at least 1:5. However, 13.04% had titers that were ≤1:20 (Table 1). One of the negative samples, RS11, showed a high level of GFP, as compared to the negative control, suggesting that this monkey had enhanced transgene expression (Fig. 1).

3.2. scAAV8 transduction achieved high and durable levels of expression in mice

The hAAT expression from scAAV2/8.CB.hAAT increased remarkably at day 14 after delivery ($1.4 \pm 0.81 \times 10^6$ ng/mL). Thereafter, the expression only slightly increased (28 days: $1.55 \pm 0.90 \times 10^6$ ng/mL) and remained stable (60 days: $1.38 \pm 1.11 \times 10^6$ ng/mL) (Fig. 2).

3.3. Anti-AAV8 NAb inhibits scAAV8 transduction in passive transfer models

The passive transfer model was successfully established in C57BL/6 mice with rhesus serum. All mice tolerated the rhesus sera well. No mortality was associated with the AAV injection procedure. The anti-AAV8 NAb of rhesus sera detected by the *in vivo* assay is shown in Fig. 3. Highly consistent results were obtained for sera with high titer (>1:5) by this method. Furthermore, high titer of the neutralizing antibodies corresponded to nearly complete inhibition of the transduced AAV8.

3.4. NAb in vivo assay is superior to the in vitro assay for detecting low NAb titer

For low titer samples (≤1:5), the *in vitro* and *in vivo* assays produced dissimilar results. Fig. 4 shows the hAAT levels of samples (4a) and % negative samples (4b) detected at days 14 and 28 post-AAV8 delivery. Six of the rhesus sera (RS) samples that were negative by the *in vitro* assay (RS2, 7, 11, 12, 15 and 20) were also negative by the *in vivo* assay. The RS11 sample was negative by both the *in vitro* and *in vivo* assay, but was higher than the negative control in the *in vivo* assay ($p<0.05$). In addition, RS14 and RS23 (NAb *in vitro* = 1:5) showed considerable neutralizing effects *in vivo*. RS14 inhibited AAV8 by 33.90% and 27.34% at weeks 2 and 4 post-delivery, respectively. Similarly, RS23 inhibited AAV8 by 10.74% and 16.38%. RS6 (NAb *in vitro* = 1:5) showed a partial neutralizing effect *in vivo* (39.69%, 2w; 34.69%, 4w). Another sample, RS18 (*in vitro* = NAb negative), showed a partial neutralizing effect that was even less extensive (42.93%, 2w; 49.86%, 4w).

Table 1
AAV8 pre-existing antibodies in the rhesus population.

Neutralizing antibody titer range*	Sera-positive, %†
	Anti-AAV8
<1:5	30.43 (7/23)
1:5–1:20	13.04 (3/23)
1:20–1:80	17.39 (4/23)
1:80–1:320	17.39 (4/23)
≥1:320	21.73 (5/23)

* Determined by *in vitro* neutralizing titer assay.
† The Asian rhesus population was divided according to the percentage of individuals with AAV neutralizing titers at the ranges shown above.

4. Discussion

Over the past few decades, AAV vectors have been developed as important tools of gene transfer to treat human diseases. The AAV8 serotype has recently emerged as a particularly promising gene delivery vehicle (Nathwani et al., 2011; Tamai et al., 2011). Scallan et al. reported that the AAV8 neutralization rate was lower than that of AAV2 and equivalent to that of AAV6 (Scallan et al., 2006). In our previous study of cardiac gene transfer, we determined that AAV8 was a more efficient delivery vehicle than AAV9 (Gao et al., 2011). In our current study, we evaluated the scAAV8 vector in mice and found that this hybrid vector mediated efficient transduction, as evidenced by stable high-level reporter gene expression for at least two months after delivery.

We were then able to exploit these scAAV8-treated mice to evaluate the impact of NAb pre-existing immunity on the efficacy of scAAV8. An advantage of this *in vivo* model system, as opposed to the traditional *in vitro* immunoassays, was that even low titer NAb was able to be evaluated. Since low titers of NAbs can impair the therapeutic efficacy of an AAV-mediated gene transfer strategy, it is necessary to gain a detailed understanding of the underlying mechanisms to develop more targeted and specific approaches and improve therapeutic efficacy. Non-human primates are important model systems to study human immune function and the therapeutic potential of novel gene therapy methods. Therefore, we investigated the profile of NAbs in a representative sample of rhesus macaques from an experimental facility by first using the traditional *in vitro* NAb assay. For *in vitro* neutralization, humoral immune antibody responses, especially of the immunoglobulin (Ig) G1 subclass (Murphy et al., 2009), play an important role. Previous studies of the NAb neutralization capacity have been based on a 1:20 titer dilution, and probably have underestimated the actual neutralization capacity *in vivo*, which can be below 1:5 (Chirmule et al., 1999; Moskalenko et al., 2000). Consistent with this notion, we attempted to detect the NAb of rhesus sera with an *in vitro* assay at starting dilutions of 1:5. We found that 69.57% of samples were positive for anti-AAV8 NAb in the representative rhesus population, and 13.04% of the samples had anti-AAV8 NAb titers lower than 1:20. We were intrigued, however, to find that a negative serum, RS11, showed enhanced transgene expression in the *in vitro* assay. While this finding may indicate an increased sensitivity of the *in vitro* assay, it may also merely reflect the unexpected influence of some unknown serum factors.

Nevertheless, the *in vitro* NAb assay has been recognized by others as not completely accurate and not absolutely predictive of *in vivo* resistance to therapeutic viral infection (Scallan et al., 2006). The mechanisms of viral neutralization *in vitro* and *in vivo* are quite different. *In vitro*, viral capsids bind directly to the antigen-binding variable region (Fab) of the antibody. *In vivo*, both the Fab and the constant region (Fc) are involved in the antibody interaction and subsequent

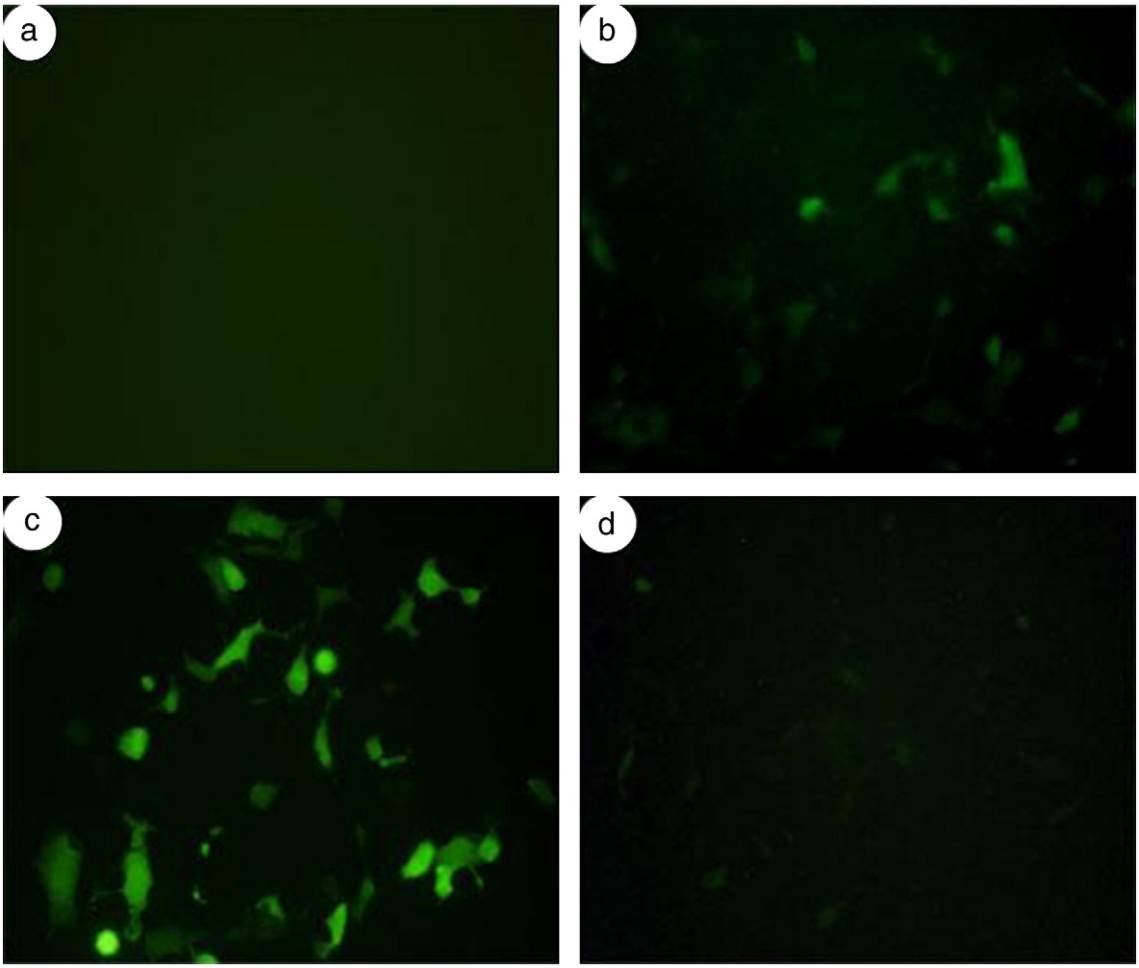

Fig. 1. AAV8-Nab *in vitro* assay. Serial dilutions of rhesus and naive mouse serum were incubated with 1×10^9 genomic copies of scAAV2/8.CB.EGFP. Rhesus serum 11 (R11) enhanced the GFP transgene expression. (a) Blank control; (b) naïve mouse serum (negative control); (c) R11 serum without neutralizing antibodies; (d) rhesus serum with neutralizing antibodies. Magnification 400×.

function (Scallan et al., 2006). Moreover, *in vivo*, humoral and cellular immune components can influence the antibody function (Zaiss and Muruve, 2008) and promote virus clearance, such as through the opsonization process (Crowe et al., 2001; Klasse and Sattentau, 2002). Therefore, *in vivo* methods are necessary to detect the neutralizing capacity in more complex and dynamic environments, such as therapeutic viruses encountered in the host system (Scallan et al., 2006; Wang et al., 2010).

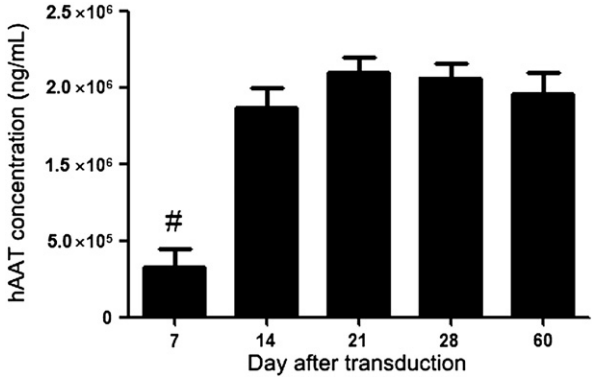

Fig. 2. Efficiency and stability of AAV vectors transduced into C57BL/6 mice. *X*-axis is the day after transduction. Data are presented as mean±SD ($n=25$). $^{\#}p<0.05$ vs. all other time points.

It has been shown that AAV vector fate differs in different species (Hurlbut et al., 2010), and even among different strains of animals, for example in mice (Breous et al., 2010). NAb assay by a passive transfer mouse model is considered to be sufficient to directly evaluate the effect of AAV neutralization by simulating the primate internal response. Scallan et al. were the first to report a neutralizing capacity detection using an *in vivo* passive immunity model using SCID mice; however, they used a commercially-available pool of purified IgG against a single AAV serotype to establish the passive transfer model (Scallan et al., 2006). Due to the complex interactions of the serum proteins, however, it was not suitable for individual patient assessment. Moreover, the *in vivo* assay was not evaluated for its efficacy in comparison to the traditional *in vitro* assay.

Recently, Wang et al. developed a sensitive *in vivo* assay by using individual non-human primate sera passive transfer

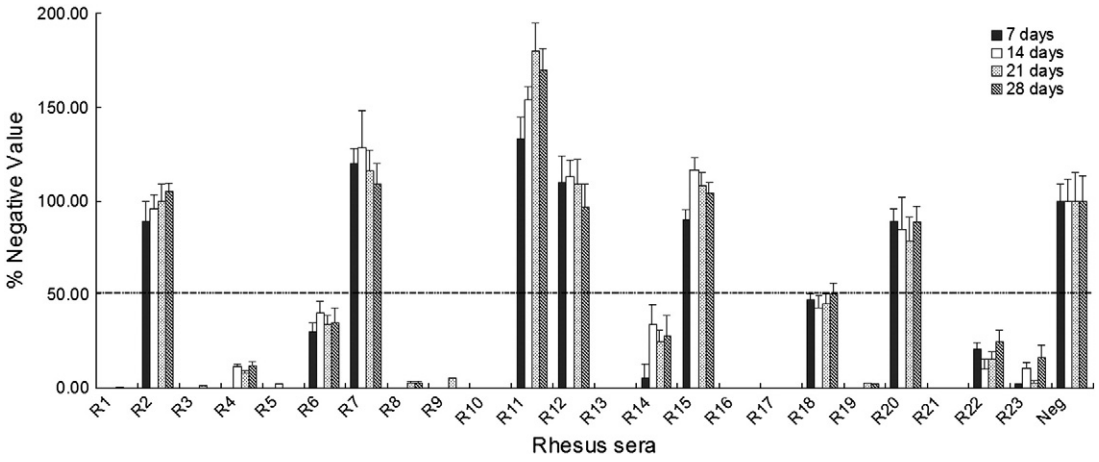

Fig. 3. hAAT expression over time in C57BL/6 mice injected with rhesus sera. Data are presented as percent of each NAb relative to negative control.

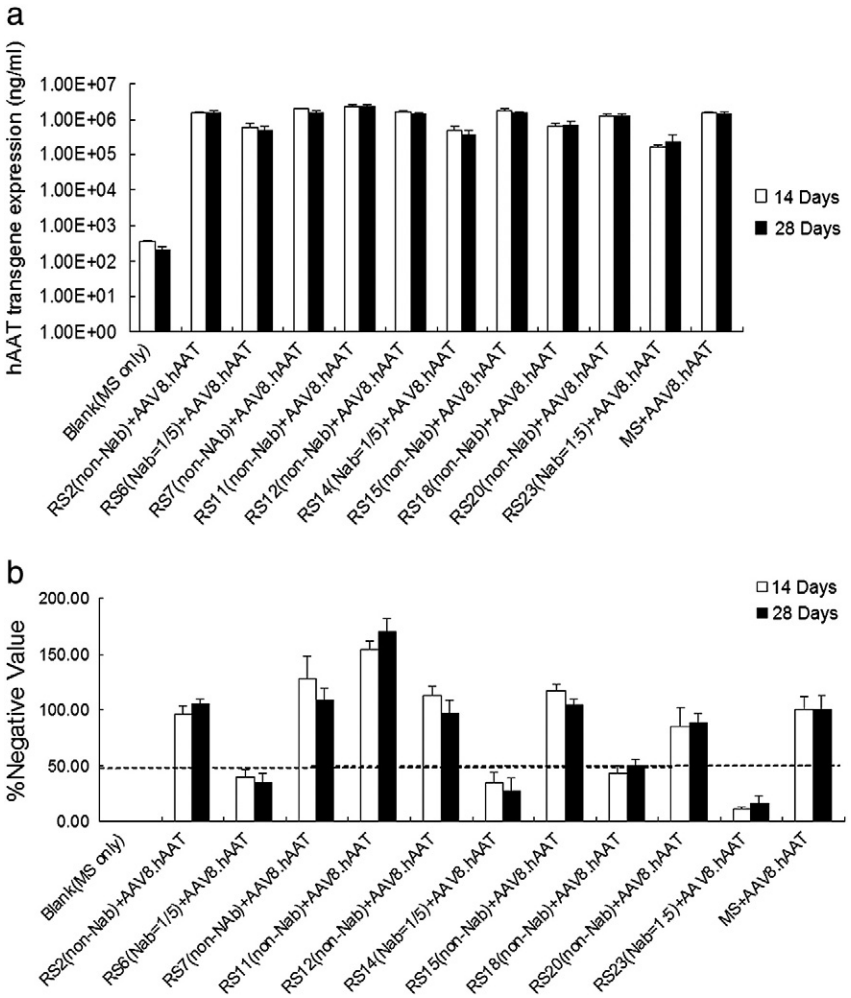

Fig. 4. Anti-AAV8 NAbs in rhesus sera with low NAb titer *in vitro* detected by *in vivo* passive transfer experiment. C57BL/6 mice were infused with (200 μL) serum sample from an individual rhesus (RS) or with naive mouse serum (MS). Two hours after the passive transfer, mice received an intravenous injection of 3e9 viral particles of AAV2/8.CB.hAAT vector. (a) hAAT expression levels in the plasma after vector administration. (b) %Negative value.

to C57BL/6 mice (Wang et al., 2010), similar to that used in our study. Therefore, we carried out such comparisons with our *in vivo* assay, as described herein.

In the study by Wang et al., the difference of transduction *in vivo* was due to the presence of NAb that was not detected by the standard *in vitro* NAb assay. Thus, the NAb *in vivo* assay was conducted at day 7 for confirmation, and rhesus macaques were evaluated for the presence of NAb to the respective AAV capsids before selection. Those that were sero-negative within the assay sensitivity threshold (<1:20) were selected for participation, which was similar to our study approach.

There are, however, some important differences between our study and the one by Wang et al. First, GFP is a morphometric analysis based on a fluorescent test, and it is generally considered not as accurate as the ELISA assay that was used in our study. Second, we choose hAAT as the reporter gene. The animals used by Wang et al. had to be sacrificed to obtain the liver sections for fluorescence testing, which is not preferable from an ethical standpoint. Our approach allowed consecutive testing in a single animal, by detecting hAAT at different time points. However, there were some benefits to the study approach of Wang et al. For one thing, they were able to directly observe the difference between NAb assays *in vivo* and *in vitro*. In addition, they passively transferred the monkeys' sera to mice, which allowed a direct evaluation of concordance. Finally, they delivered the AAV to monkeys and extracted the liver for confirmation evaluation upon sacrifice. In our study, we used *in vitro* defined monkey sera and passive transfer to C57BL/6 mice, and compared these two assays. We did not deliver AAV8 to the monkeys. This precluded our ability to determine the real situation of AAV expression transduction in monkeys. In addition, we extended the assay to 14 and 28 days and found that NAb expression peaked at day 14. Thus, seven days was the shortest time point for the NAb assay *in vivo* used in our study but day 14 was determined to be the optimal assay time point.

C57BL/6 mice are important tools for passive transfer experiments since they display long-term expression of the administered non-self transgenes (Chao and Walsh, 2001). In our study, C57BL/6 mice were used to establish the passive transfer model, largely based on their affordability and general acceptance as a suitable animal model for disease studies (Breous et al., 2010). Serum is composed of a broad range of proteins, some of which can enhance AAV transduction and others that can suppress it. Our *in vivo* detection system used a 10-fold higher volume of serum than is used for the traditional *in vitro* system (200 μL vs. 20 μL, respectively); we believe this imparted a higher sensitivity to the *in vivo* assay for assessing the neutralizing capacity. The *in vivo* and *in vitro* assays produced similar results for high titer samples. However, the two assays yielded remarkably different results for low titer samples. This finding indicated the power of the *in vivo* assay for clinical evaluation of an AAV vector (AAV8) for a particular case (RS14 and RS23) that would have been resistant to AAV8 gene therapy. In addition, the *in vivo* assay showed that two low titer samples had partially neutralizing effects, which in clinic would cast suspicion on these and likely prompt a search for an alternative AAV serotype or treatment strategy. To verify our findings, and rule out false negatives/positives, we performed independent duplicate assays of all samples and obtained identical results; thus, the RS18 sample was deemed as a false negative by the *in vitro* assay. We concluded that one of the advantages of the *in vivo* assay is to resolve false negative results from the *in vitro* assay.

We considered reasons for the discordant results obtained for low titer samples with the *in vitro* and *in vivo* assay methods. It is possible that homology of the AAV serotypes used in our study led to cross-reactivity of NAbs (Gao et al., 2005). In fact, we noted in previous investigations that anti-AAV2, 7, 8, and 9 cross-reactivity occurs in human sera samples (data not shown). Therefore, we speculate that the low titer anti-AAV8 NAb combined with pre-existing cross-reactivity of the other AAV serotypes may have impacted the outcome of AAV8 transduction. Even if a single serotype-specific NAb is negative, other positive serotype-specific NAbs may functionally affect the transduction efficiency. A variety of AAVs exist in nature, and we have yet to uncover the interrelationships of them all. Future studies should aim to elucidate the existence and mechanisms of cross-reactivity for the AAVs that are popular tools for gene therapy. Gaining a detailed understanding of the neutralization mechanism will possibly help researchers to manipulate the gene transfer strategy to achieve higher therapeutic efficacy and safety. Regardless, the method of neutralization detection must be highly accurate to lower patient risk and healthcare costs associated with ineffective treatment; the sera passive transfer model system promises to achieve this.

In conclusion, our study showed that the *in vivo* NAb assay has higher sensitivity than the *in vitro* NAb assay. In addition, the representative large primate model of gene therapy and immune response to therapeutically-induced infection, rhesus macaques were shown to harbor a large panel of pre-existing NAbs, including those that could impair hybrid AAV. It would be prudent to establish a benchmark for the NAb baseline to standardize future pre-treatment assessments in both experimental animals and human patients in clinic. We advise an initial screening by *in vitro* NAb assay, followed by more detailed screening of samples with ≤1:5 titer, preferably with the *in vivo* assay. For high titer NAbs, prediction by either method may suffice, but for low titer NAbs, the more sensitive and accurate *in vivo* assay should be considered as a gold standard, especially for patients who are candidates for AAV-assisted gene therapy.

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