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.