HHS Public Access

Author manuscript

Gene Ther. Author manuscript; available in PMC 2016 May 18.

Published in final edited form as:

Gene Ther. 2015 December ; 22(12): 984–992. doi:10.1038/gt.2015.69.

Prediction of Adeno-Associated Virus Neutralizing Antibody Activity for Clinical Application

Mei Wang[1,2], **Ashley Crosby**[2], **Eric Hastie**[2], **Jade J. Samulski**[5], **Scott McPhee**[5], **Grieger Joshua**[2], **R. Jude Samulski**[2,3,*], and **Chengwen Li**[2,4,*]

[1]Institute of Hematology, Chinese Academy of Medical sciences, Tianjin, China

[2]Gene Therapy Center, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599

[3]Department of Pharmacology, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599

[4]Department of Pediatrics, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599

[5]Asklepios BioPharmaceutical Inc., Chapel Hill, NC 27517

Abstract

Patients with neutralizing antibodies (Nab) against adeno-associated virus (AAV) are usually excluded from treatment with AAV vectors. To develop a standard assay for detecting Nab inhibition activity, we systematically studied current AAV Nab assays *in vitro* and *in vivo*. Several factors were found that influence the Nab titers based on the *in vitro* assay, including: sera volume, AAV dose/cell, cell number and choice of transgenes. When the Nab titer assay was performed *in vivo* via intramuscular (IM) or systemic administration, a 4-fold increase in sensitivity for measurement of Nab titers was observed compared to an identical *in vitro* test. To better mimic the clinical setting, after passively transferring human Nabs into mice, blood was collected before systemic injection of AAV vector and used for Nab titer analysis *in vitro* or via IM injection. The results showed that AAV delivered via IM injection had a similar inhibition pattern to systemic administration. These studies indicate critical parameters necessary for optimizing Nab sensitivity and that an *in vivo* Nab assay is more sensitive than an *in vitro* assay for inclusion/exclusion criteria. The variables identified by this study may explain some of the compounding clinical data seen to date with respect to efficiency of AAV transduction in various Phase I clinical trials.

Keywords

AAV; gene therapy; neutralizing antibody; muscular injection; systemic administration

*To whom correspondence should be addressed: Gene Therapy Center, 7119 Thurston-Bowles, CB 7352, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, Phone: (919)-962-3285, Fax: (919)-966-0907, ; Email: chengwen@med.unc.edu, ; Email: rjs@med.unc.edu

Conflict of Interest:
The authors declare no conflict of interest.

Introduction

Gene therapy using adeno-associated virus (AAV) as a delivery vehicle has been successful in Phase I clinical trials in patients with blood diseases and blindness([1–8]). A major restriction for systemic AAV vector application is the high prevalence of AAV-specific neutralizing antibodies (Nabs) in the human population. More than 90% of the population is naturally infected with AAV, with approximately 50% having Nabs against the virus([9–21]). AAV Nabs inhibit AAV transduction not only following systemic administration, but also intramuscular (IM) injections. In one clinical trial of hemophilia B patients, the same dose of AAV2 induced detectable factor IX (F.IX) expression in one patient without AAV Nabs while no F.IX expression was observed in a patient with detectable AAV Nabs([4]). In agreement, muscle biopsies of patients enrolled in a clinical trial for Duchenne Muscular Dystrophy (DMD), showed that Nab-naïve patients had measurable IM AAV genomes([22]) while AAV genomes were virtually undetectable in patients with measurable AAV Nabs prior to the trial.

In an early report, the prevalence of Nabs against AAV2 was about 40%([9]). More recently, several studies have investigated Nabs against AAV2 and other serotypes. Although assays for measuring Nabs are not standardized, the prevalence of AAV Nabs is dramatically different in the human population, reportedly ranging from 22% to about 90% for AAV2([6, 7, 10–12, 16, 19]). This wide difference may result from various conditions used for Nab assays among different labs([23]). Factors that impacted assay results include cut offs (2, 3, 10, 20 and even 100-fold dilution of serum), cell lines (293, IB3, HeLa, CHO, B16F10, U87, Huh7, etc…), addition of Adenovirus (Ad) or chemicals that enhance AAV transduction, varying doses of AAV vector/cell (10^3 to 10^5), temperature or length of time for incubation of virus with human sera, volume of serum used to interact with AAV, transgenes (luciferase, GFP, LacZ, etc.), culture duration after AAV transduction, and heat inactivation of complement proteins([6, 7, 10–12, 16, 19, 20, 24–41]). It has been reported that no transgene expression can be observed *in vivo* in instances where the *in vitro* Nab titer is more than 1:3([17]). This highlights the significant point that the accuracy of a Nab assay is crucial for the purposes of excluding patients from receiving AAV gene therapy in clinical trials. In this study, we systematically performed a series of experiments to standardize the approach for Nab analysis *in vitro* and *in vivo*. We demonstrated that the Nab titer to AAV *in vitro* was independent of cell lines, time and temperatures of AAV incubation with Nabs, addition of Ad or heat inactivation of serum. However, certain factors influenced the sensitivity of the Nab assay, including: serum volume, AAV particles/cell, cell number, and transgene. Upon carrying out an *in vivo* Nab assay, we demonstrated that the *in vivo* Nab assay was more sensitive than an *in vitro* protocol using the same Nab concentrations. This increased sensitivity *in vivo* over *in vitro* was true for both IM and systemic application as long as the same ratio of AAV to Nab dose was used. To determine which assay would better predict the Nab activity in humans, we mimicked the human setting in mice by injecting either human intravenous immunoglobulin (IVIG) or human serum into mice, followed by measurement of Nab activity *in vitro* (through blood draw) and via IM administration. We found that similar inhibition of transgene expression was achieved in mice with systemic administration as well as in mice receiving IM injection of AAV vector, supporting the *in vivo* assay as far more sensitive than the *in vitro* assay.

Results

Factors not affecting Nab titer *in vitro*

To study Nab activity *in vitro*, we first screened which cell lines could be transduced efficiently with three serotypes of AAV. AAV2, 8, and 9 encoding firefly luciferase were used to transduce 7 cell lines: HEK293, C2C12, RC32, HeLa, Huh7, HepG2 and U87, at a dose of 1000 particles/cell in the presence of Ad dl309. Luciferase activity from transduced cells was analyzed 24 hours (hrs) later. With the exception of RC32 cells, which contain AAV rep gene integration for AAV vector replication in the presence of Ad, the highest transduction was consistently observed in Huh7 cells, for all three AAV serotypes (Figure S1). We therefore chose Huh7 cells for *in vitro* Nab assay in all successive experiments.

AAV8 has been successfully applied in multiple clinical trials for hemophilia B patients([5], [6]). We used AAV8 and human IVIG to study the different factors *in vitro* that influence measuring of Nab titers. To determine whether there was a difference in Nab titers across different cell lines, after incubation with different amounts of human IVIG, AAV8/luc vector was used to infect 7 cell lines (293, C2C12, RC32, HeLa, Huh7, HepG2 and U87). As shown in Figure S1B, the Nab titer from these cell lines was the same at 1 mg/ml of IVIG (Table 1). This result suggests that cell type is an independent factor for measuring the Nab titer.

We further examined whether the incubation temperature of AAV8 virus with IVIG impacted Nab titer. AAV8 vector was delivered to Huh7 cells after incubation with IVIG for 2 hrs at either 4°C or 37°C; we found no temperature-based difference in transduction (Figure S2). We next incubated AAV8 virus with IVIG at 4°C for 1, 2, 3, or 4 hrs, then added the virus to Huh7 cells. Again, the transduction level was the same regardless of incubation time (Nab titers at 1mg/mL). Previous studies have reported that addition of Ad enhances AAV transduction. To determine whether the addition of Ad also influences measurement of Nab titer, we compared AAV8 vector transduction with or without Ad. Nab titer was not affected by the addition of Ad. Additionally, after AAV transduction, the duration of AAV8 infection in cells did not impact measurement of Nab titer. The Nab titer was similar regardless of serum inactivation with heat (Figure S2).

Factors impacting Nab titers *in vitro*

When AAV8 virus was incubated with different volumes of IVIG, higher IVIG volumes induced stronger inhibition on transduction. Generally, increased doses of AAV8 vector decreased the sensitivity of titer measurement. Specifically, Nab activity was detected when 10^5 particles of AAV8 vector/cell were used in the presence of IVIG at 8mg/ml. However, when 10 to 1000 particles of AAV8 vector/cell were used, Nab titers were similar at 1mg/ml. Also, cell density was important; fewer cells seeded in a 48-well plate decreased Nab sensitivity. Finally, different transgene cassettes have been used to evaluate Nab titers. We compared Nab titers using GFP as a second transgene; we transduced Huh7 cells with AAV8 vector encoding the GFP transgene, and GFP expression in cells was detected by flow cytometry. As shown in Figure S3, the IVIG Nab titer against AAV8 using a GFP transgene was 4mg/ml. Sensitivity of the Nab assay with a GFP transgene was thereby lower than that with the luciferase transgene, with which the IVIG Nab titer against AAV8 was 1mg/ml.

Nab analysis *in vivo*

To determine the *in vivo* sensitivity of the Nab assay in the context of IM administration, we first incubated human IVIG with 1×10^9 particles of AAV8/luc vector for 2 hrs at 4°C. Subsequently, the AAV8 vector was directly injected into mouse hind limb muscles. Three weeks later, imaging was performed and photon intensities were calculated. Transgene expression was 50% lower in animals injected with AAV8/luc that had first been incubated with 2.5 mg/ml of human IVIG (Figure S4).

To examine the Nab titer after systemic administration of vector, we first incubated 1×10^{10} particles of AAV8/luc with PBS or human IVIG*,* followed by retro-orbital injection of the Nab/vector mix. At day 7 after AAV8 injection, intravital imaging was performed and photons to the general liver area were measured. As shown in Fig. 1, when 25mg/ml of IVIG was incubated with AAV8 vector, transgene expression was inhibited by more than 50%.

Based on the observation above, the titer of IVIG to AAV8 was 1mg, 2.5mg, and 25mg when 1×10^8, 1×10^9, and 1×10^{10} particles of AAV were used for Huh7 transduction, IM administration, and systemic administration, respectively. After adjusting to the amount of IVIG per 1×10^8 particles of AAV8, the IVIG Nab titer was 1mg, 0.25mg, and 0.25mg for Huh7 transduction, IM administration, and systemic administration, respectively. The same Nab titer was detected with systemic and IM injection, which was 4-fold more sensitive than that with *in vitro* analysis. To confirm whether IM administration increases the Nab sensitivity from human sera, we screened sera from 10 subjects in Huh7 cells and found no Nabs against AAV8 in 7 patients (Fig. 2a). Next, 1×10^9 particles of AAV8/luc vector was incubated with serum from 8 different individuals from the population above at the same ratio of AAV vector dose to serum volume as in Huh7 cells. After 2 hrs incubation at 4 _C, AAV8 vector was administered via IM injection. For easy comparison, one leg was injected with AAV8 incubated with PBS, and the other with vector incubated with human serum. As shown in Fig. 2b and 2c, two more subjects (No. 1 and No. 7) manifested Nab activity.

Passive IVIG administration mimics Nab contribution to gene transfer in clinical settings

AAV Nabs are continually circulating within seroconverted patients. To mimic this clinical condition and to gauge the stability of human IVIG *in vivo*, we adoptively transferred commercially available human IVIG into the circulatory systems of mice. The circulating concentration of human IVIG in mouse blood was measured at various time points. As shown in Figure S5, the concentration of human IVIG was stable between 1 hr to 24 hrs after IVIG passive transfer. Therefore, we administered AAV vector 3 hrs post-human Nab transfer for successive experiments.

In current clinical trials for patients with hemophilia B, AAV vector doses have been proposed at 2×10^{12} particles/kg([5], [6]). The identical dose of AAV vector per kilogram of body weight was chosen to determine IVIG inhibition ability in mouse studies; all mice in our studies had an average body weight of 20 grams. Based on our *in vitro* Nab titer findings as well as the kinetics of IVIG maintenance in mouse blood, various amounts of human IVIG were administered into C57BL/6 mice via retro-orbital injection. Three hours later, mouse blood was collected for Nab activity analysis via IM injection or in cell lines *in vitro*, then 4×10^{10} particles of AAV8/luc were injected through the retro-orbital route. Three days after AAV8 injection, intravital imaging of the mice was performed (Fig. 3a). In mice receiving 500 μg of human IVIG, the luciferase expression was more than 50% lower than that in control mice (Fig. 3b).

We also studied the inhibition efficiency of AAV8 transduction in muscles or Huh7 cells by incubating the AAV8 vector with serum from mice having human IVIG passive transfer at the same ratio of AAV dose to total plasma volume in mice with human IVIG administration. Based on a plasma volume of 4% of body weight in a 20 gram mouse, the plasma volume was determined as 800 μl, so the concentration of AAV vector in the blood was 5×10^7 particles/μl of plasma when 4×10^{10} particles of AAV were injected. To determine the inhibition efficiency of serum from mice with human IVIG, we incubated 1×10^9 particles of AAV8/luc *in vitro* with 20 μl serum from IVIG-transferred mice for 2 hrs at 4°C; the concentration of vector was 5×10^7/μl serum (same as in mice having systemic administration of AAV8). Then AAV8/luc vector was injected into mouse muscles. One week later, the luciferase expression was detected (Fig. 3c). As shown in Fig. 3d, the serum from mice with administration of 500 μg human IVIG inhibited luciferase expression over 50%, compared to the control group given PBS. This data was consistent with our observations with systemic administration of AAV vector in human IVIG passive transferred mice (Fig. 3b). Finally, we performed an analysis of transgene expression inhibition *in vitro* using the same concentration of AAV vector/μl of plasma. 1×10^8 particles of AAV8/luc vector in 12.5 μl were incubated with 12.5 μl PBS containing 2 μl plasma from IVIG-transferred mice for 2 hrs at 4°C; AAV8 vector was used to transduce 1×10^5 Huh7 cells in a 48 well plate in the presence of Ad dl309 at an MOI of 5. The cells were harvested for luciferase activity analysis at 24 hrs post-transduction. When AAV8 vector was incubated with the serum from mice receiving 2 mg or more human IVIG, more than 50% suppression of transgene expression was observed (Fig. 3e).

Prediction of Nab activity in the clinical setting by evaluation of human serum

We have screened for AAV8 Nab presence in 10 samples from normal subjects by incubating 1×10^8 particles of AAV8/luc in 12.5 μl with 12.5 μl of a serial diluted serum. We found that one subject had a high AAV8 Nab titer of 1:640. Based on the Nab titer and body weight of mice, we injected different volumes of human serum into mice. Three hours later, blood was collected and 4×10^{10} particles of AAV8/luc vector were administered via retro-orbital injection. At day 3 after AAV8 administration, bioluminescent imaging was performed (Fig. 4a). When 1.25 μl or more of human serum was injected, more than 50% of AAV8 transduction was inhibited (Fig. 4b). To analyze the inhibition ability of mouse serum with adoptive transfer of human serum on AAV transduction via IM or in Huh7 cells, mouse sera were incubated with AAV8/luc using the same ratio of AAV8 vector to plasma volume in mice that received systemic administration of human serum as described above. In both the IM and *in vitro* assays, more than 50% inhibition of transgene expression was observed using sera from mice transferred with 1.25 µl or 5ul of human serum, respectively (Fig. 4c–e). It has been shown that incubation of AAV vectors with increased volumes of Nab can enhance the sensitivity of a Nab assay. We wondered whether an increased volume of sera from mice with human serum transfer could result in Nab activity close to that seen with IM or systemic administration. To address this question, we incubated 1×10^8 particles of AAV8/luc vector with 10-fold more serum (20 µl) from mice, then transduced Huh7 cells. The inhibition of transgene expression was still lower than *in vivo* analysis, although the Nab titers were 2-fold higher than when using previous volumetric ratios of Nab to virus in an *in vitro* assay (Fig. 4f).

To determine whether Nab activity can be precisely predicted for other serotypes *in vivo*, we compared the inhibition effect of human serum on AAV transduction from serotypes 2 and 9 with the same approaches as described above for AAV8 (Fig. 4). We have found that the serum from the same subject with a high titer of Nab to AAV8 also possessed high Nab titer to AAV2 and 9. Following systemic administration of serum in mice, mouse sera were harvested just before retro-orbital injection of AAV2 or 9. Mouse imaging was performed to determine the human serum Nab activity after AAV systemic injection (Fig. 5a, 5b and 6a, 6b). Additionally, mouse sera were incubated with AAV2 or 9 for Nab activity assays *in vitro* and via IM administration as described above. Similar to AAV8, an inhibition pattern with 50% suppression of transgene expression was observed in systemic administration and IM injection of AAV2 (with injection of 1.25 µl human serum, Fig. 5b and 5b) and AAV9 (2.5 µl, Fig. 6b and 6d), stronger suppression of transgene expression than that with *in vitro* analysis (5 µl and 10 µl for AAV2 and 9, respectively, Fig. 5e and 6e).

Discussion

In this study, we have demonstrated that the measurement of titer of Nabs against AAV *in vitro* was influenced by serum volume, dose of AAV vector, cell density, and transgene. However, many factors did not play a critical role in predicting/measuring Nab titers. These included cell lines, temperature for incubation of AAV virus with serum, addition of adenovirus to enhance AAV transduction, culture period after AAV delivery to cells, heat inactivation of serum, and the length of incubation of AAV vector with serum. *In vivo* Nab analysis increased assay sensitivity with similarly increased efficiency between intramuscular injection and systemic administration. When we tried mimicking the Nab inhibition effect observed in the clinical setting in previously identified Nab negative patients based on *in vitro* assay, intramuscular administration of AAV vector (incubated with patient serum at the same ratio of vector dose to total plasma volume) accurately predicted Nab activity.

Several clinical trials have applied AAV vectors to target hepatocytes in patients with hemophilia B([4–6]). Successful targeting of the liver requires systemic delivery of AAV vector. As Nabs block effective AAV transduction, the existence of AAV Nabs is a major criterion for exclusion of patients participating in clinical trials. *In vitro* AAV Nab assays have been widely used for this purpose. It has been demonstrated that even low titers of Nabs in the blood can fully suppresses AAV transduction *in vivo*([17]). This discordant observation indicates that Nab titers from an *in vitro* assay are not able to predict the inhibition efficiency of Nabs *in vivo*. Therefore, it is crucial to develop a more sensitive assay to detect AAV Nabs and predict clinical results after systemic AAV administration. More importantly, the approaches for Nab assays vary among laboratories and there is currently no standard method available to compare the sensitivity of different protocols. In this study, we found that the ratio of vector dose per cell to serum volume is the most important factor influencing AAV Nab titer predictions. This finding is supported in our experiments using different volumes of human IVIG, different doses of AAV vector, and different cell densities. When more serum is incubated with AAV vector, the sensitivity of the Nab assay increases. Another factor that influences Nab titer prediction is the use of alternative transgenes (GFP vs luciferase), specifically because GFP assays are less quantitative than luciferase assays. Thus, proteins that are functional or directly measureable might prove more favorable than less directly measurable ones in predicting the truest Nab levels. In this study, human IVIG was used and had similar neutralizing activity in different cell lines. It should be noted that IVIG is comprised of a pool of serum from thousands of human subjects. Because of the large amount of participant serum, this pool could perhaps contain every antibody in existence that can interfere with AAV infectious pathways. This is likely somewhat different from an individual's serum. It is possible that AAV transduction in different cell lines may utilize different mechanisms such as different receptors or different endocytosis routes([42], [43]). Thus, when performing Nab assays with serum from a single patient over different cell lines, Nab titers may not be identically matched.

Animal models have been used to study Nab activity against AAV in prior studies. When human IVIG was adoptively transferred into mice, despite having *in vitro* Nab titers of 1:3, transgene expression was completely inhibited([17]). This observation strongly suggests that analysis of Nabs *in vitro* cannot predict the neutralizing activity of human IVIG *in vivo*. In another study to survey Nab against AAV8 in primate samples, about 70% of samples had Nabs against AAV8 based on an *in vitro* assay. When 100 μl of sera from these primates was passively transferred into mice followed by delivery of 3×10^{10} particles of AAV8 via tail vein injection, one of the samples showing no *in vitro* Nab activity displayed transgene expression suppression *in vivo*([44]). This result in fact underestimated the sensitivity of the *in vivo* Nab assay because the ratio of AAV particles to Nab amount in the study was much higher *in vitro* as compared to the *in vivo* assay. We have performed the *in vivo* Nab assay via muscular administration and observed 4 fold more sensitive than that *in vitro* analysis. Consistent with these observations, similar Nab titer is obtained between IM and IV. When AAV vectors were incubated with human IVIG at the ratio of AAV vector to serum amount showing no Nab activity *in vitro* (for example at 1×10^8 particles of AAV with 0.5mg/ml of IVIG) and administered into mice via IM or systemic injection, transgene expression was decreased by more than 90% compared to control group. From these results, we conclude that Nab assays performed *in vivo* are far more sensitive than those performed *in vitro.*

No current *in vitro* Nab assay considers parameters such as patient body weight with respect to the dose of AAV vector proposed. As our *in vitro* assay has demonstrated, this neglected factor is one of the most crucial determinants that can impact Nab activity (i.e. the ratio of AAV vector to Nab dose). Higher amounts of Nabs will increase Nab assay sensitivity, but when more AAV vector is used, transgene expression is less inhibited by Nabs. The same phenomenon can be applied to human trials. If low AAV doses are proposed, the inhibition of AAV transduction should be more directly related to Nab titers. However, when higher doses of AAV vectors are used, transgene expression may still be obtained despite a higher Nab titer *in vitro*. To address this concern and develop a more precise approach to predict Nab activity in a clinical setting, we injected different amounts of human IVIG or serum into the mouse, and blood was drawn for Nab determination before systemic AAV administration and used for subsequent Nab inhibition assays *in vitro* and *in vivo*. When the same ratio of AAV vector to the amount of human Nabs was used as systemic administration of AAV vector in mice receiving human IVIG or serum, IM injection of AAV vector induced precisely the same transgene expression efficiency as with systemic administration, both of which were more sensitive than the *in vitro* assay despite having used 10-fold more serum in the *in vitro* protocol. This result suggests that *in vitro* Nab assays cannot predict the inhibition efficiency of Nabs in a clinical setting, and that IM administration is a simple and reliable approach for Nab assays to predict Nab activity in clinical trials. Several factors may help explain why *in vivo* Nab assays are more sensitive than *in vitro* ones. It has been reported that the transgene expression was not significantly reduced in SCID mice with deficiency of NK cells even after passive transfer of human IVIG([17]). This indicates that NK cells may play a role in Nab sensitivity. Also, when AAV is used as an immunogen to induce a humoral immune response, some antibodies can bind to functional domains on the AAV virion surface to influence the ability of the AAV vector to attach to cells or influence intracellular trafficking in target cells. Other antibodies can also bind to AAV virion but do not impact AAV transduction *in vitro*. However, the binding of antibodies on non-functional motifs of the AAV virion surface would still activate the complement system, which enhances phagocytes or other immune cells to uptake the AAV virion and induce lower circulating AAV vector, reducing transduction of target tissues *in vivo*.

It is interesting to note that incubation with human IVIG or serum enhanced AAV transduction when very low amounts of human IVIG or serum were used. This result suggests that the interaction of AAV vector with serum proteins can interfere with AAV transduction efficiency. Several studies have found that certain serum proteins can bind to the AAV surface to influence AAV transduction. Galectin 3 binding protein (G3BP), a soluble scavenger receptor, can interact with the AAV vector to form aggregates and inhibit AAV transduction([45]). AAV vector also interacts with C-reactive protein (CRP), which results in lower AAV transduction([46]). The interaction of AAV vector with serum proteins is both serotype and species-dependent. Both AAV1 and AAV6 interact with G3BP from human and dog but not from primate and mouse. Additionally, these two serotypes also interact with mouse CRP but not human. AAV8 and AAV9 do not interact with G3BP or CRP from human([46]). It is unknown which proteins in human sera contribute to enhanced AAV transduction. Proteomics analysis of human serum proteins purified from interaction with the AAV vector can be used to identify proteins responsible for enhanced AAV transduction.

In summary, through comprehensive evaluation of the Nab titers performed both *in vitro* and *in vivo*, we conclude that *in vivo* Nab assays are a more sensitive approach than those conducted *in vitro*, and that IM administration of AAV vectors incubated with patients serum in the same ratio of AAV dose to total plasma volume of clinical patients will accurately predict the Nab inhibition activity. The *in vivo* approach should be used to exclude patients who need to receive systemic administration for AAV gene therapy. We suggest that the Nab assay should be first performed *in vitro*, and if the Nab titer is greater than or equal to 1:2, the patient should be excluded from clinical trials. When the Nab titer is less than 1:2, a more sensitive *in vivo* assay with IM injection of AAV vector incubated with serum/plasma *in vitro* at the same ratio of total AAV vector dose to total volume of plasma in patient should be carried out. If transgene expression is more than 50% of that observed in a control group, the patient should qualify for systemic administration of AAV vector for gene therapy.

Materials and Methods

Cell lines

HEK293, C2C12, HeLa, RC32 (HeLa cells with stable AAV2 rep expression), Huh7, HepG2 and U87 cells were maintained at 37°C in 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin.

AAV virus production

A standard approach with three-plasmid transfection was used to produce AAV vector. Briefly, AAV transgene plasmid pTR/CBA-luc was co-transfected with AAV helper plasmid (pXR2, pXR8 and pXR9) and adenovirus helper plasmid pXX6-80 into HEK293 cells. 60 hours (hrs) later, cells were harvested and lysed, and supernatant was ultracentrifuged against a CsCl gradient. AAV virions were collected and titered by dot-blot.

Human IVIG and plasma

10% human IVIG (Gamunex$^{@}$-c) was purchased from Grifols Therapeutics Inc. (Research Triangle Park, NC). Individual human serum was purchased from Valley Biomedical (Minchester, VA). Human IVIG and serum were aliquoted and stored at −80 °C for future use.

Neutralizing antibody analysis

Nab analyses were performed as described previously with slight modification([20]). Human IVIG or serum was serially diluted 2 fold. Cells were seeded on a 48-well plate in 300 µl DMEM containing 10% FBS. Cells were cultured for 3–4 hrs at 37 °C and allowed to adhere to the well. AAV/luc or AAV/GFP was incubated with either human IVIG or serum. The mixture was added to the indicated cells. Cells were lysed with passive lysis buffer (Promega) and luciferase activity was measured with a Wallac1420 Victor 2 automated plate reader. Nab titers were defined as the highest dilution for which luciferase activity or the number of GFP positive cells was 50% lower than serum-free controls.

Neutralizing antibody analysis with AAV intramuscular administration

1×10^9 particles of AAV/luc vector were incubated with human Nabs for 2 hr at 4°C. Then the mixture was directly injected into the hind leg muscles of 6–8 week old C57BL/6 mice or BALB/c mice. At the indicated time points, imaging was performed using a Xenogen

IVIS Lumina imaging system (Caliper Lifesciences, Hopkinton, MA) following intraperitoneal injection of D-luciferin substrate at 120 mg/kg (Nanolight, Pinetop, AZ). Bioluminescent images were analyzed using Living Image software. Housing and handling of mice were approved by the Institutional Animal Care and Use Committee at The University of North Carolina at Chapel Hill, and carried out in compliance with the National Institutes of Health guidelines.

Neutralizing antibody analysis with AAV systemic administration

1×10^{10} particles of AAV/luc vector were incubated with human Nabs for 2 hrs at 4°C followed by retro-orbital administration of the mixture of AAV with human Nabs into C57BL/6 mice. At the indicated time points, imaging was performed and bioluminescent images in the liver area were analyzed.

Establishment of mouse model with human Nab

Different amounts of human IVIG or serum containing AAV Nabs were injected into the retro-orbital vein of C57BL/6 mice. Three hrs later, blood was collected for Nab activity assay via intramuscular injection or in cell lines *in vitro*, then followed by systemic injection of 2×10^{12} particles/kg of AAV/luc vector. Imaging was performed 1 week after AAV administration. For Nab activity analysis via intramuscular administration, 1×10^{9} particles of AAV/luc vector were incubated with mouse serum which contained passive transferred human Nabs for 2 hrs at 4 °C. The ratio of the AAV vector dose to the volume of mouse serum for IM was equal to that in mice receiving systemic application to human Nabs and AAV. The mixture was then injected into the muscle of C57BL/6 mice. Imaging was carried 3 weeks after IM. For Nab activity analysis *in vitro*, 1×10^{8} particles of AAV/luc vector were incubated with mouse serum at the same ratio of the AAV vector dose to serum for IM for 2 hrs at 4 °C. The mixtures were then used to infect 1×10^{5} Huh7 cells in a 48 well plate in the presence of Ad dl 309 at MOI of 5. After 24 hrs, cells were lysed and luciferase activity was analyzed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are grateful to Xiaojing Chen and Karen Hogan for their excellent technical assistance, as well as Marc Weinberg for his critical reading of the manuscript. The authors acknowledge the UNC Biomedical Research Imaging Center (BRIC) Small Animal Imaging (SAI) facility for assistance of mouse imaging. This work was supported by National Institutes of Health Grants R01DK084033 (to C.L. and R.J.S.), P01HL112761, R01AI072176, R01AR064369 (to R.J.S.), P30-CA016086-35-37, U54-CA151652-01-04 (to the BRIC SAI facility) and a research grant from Asklepios BioPharmaceutical (to C.L.).

References

1. Bennett J, Ashtari M, Wellman J, Marshall KA, Cyckowski LL, Chung DC, et al. AAV2 gene therapy readministration in three adults with congenital blindness. Science translational medicine. 2012; 4(120):120ra15. Epub 2012/02/11.

2. Maguire AM, High KA, Auricchio A, Wright JF, Pierce EA, Testa F, et al. Age-dependent effects of RPE65 gene therapy for Leber's congenital amaurosis: a phase 1 dose-escalation trial. Lancet. 2009; 374(9701):1597–605. Epub 2009/10/27. [PubMed: 19854499]

3. Maguire AM, Simonelli F, Pierce EA, Pugh EN Jr, Mingozzi F, Bennicelli J, et al. Safety and efficacy of gene transfer for Leber's congenital amaurosis. The New England journal of medicine. 2008; 358(21):2240–8. Epub 2008/04/29. [PubMed: 18441370]

4. Manno CS, Pierce GF, Arruda VR, Glader B, Ragni M, Rasko JJ, et al. Successful transduction of liver in hemophilia by AAV-Factor IX and limitations imposed by the host immune response. Nature medicine. 2006; 12(3):342–7. Epub 2006/02/14.

5. Nathwani AC, Reiss UM, Tuddenham EG, Rosales C, Chowdary P, McIntosh J, et al. Long-term safety and efficacy of factor IX gene therapy in hemophilia B. The New England journal of medicine. 2014; 371(21):1994–2004. Epub 2014/11/20. [PubMed: 25409372]

6. Nathwani AC, Tuddenham EG, Rangarajan S, Rosales C, McIntosh J, Linch DC, et al. Adenovirus-associated virus vector-mediated gene transfer in hemophilia B. The New England journal of medicine. 2011; 365(25):2357–65. Epub 2011/12/14. [PubMed: 22149959]

7. Simonelli F, Maguire AM, Testa F, Pierce EA, Mingozzi F, Bennicelli JL, et al. Gene therapy for Leber's congenital amaurosis is safe and effective through 1.5 years after vector administration. Molecular therapy : the journal of the American Society of Gene Therapy. 2010; 18(3):643–50. Epub 2009/12/03. [PubMed: 19953081]

8. Testa F, Maguire AM, Rossi S, Pierce EA, Melillo P, Marshall K, et al. Three-year follow-up after unilateral subretinal delivery of adeno-associated virus in patients with Leber congenital Amaurosis type 2. Ophthalmology. 2013; 120(6):1283–91. Epub 2013/03/12. [PubMed: 23474247]

9. Blacklow NR, Hoggan MD, Kapikian AZ, Austin JB, Rowe WP. Epidemiology of adenovirus-associated virus infection in a nursery population. American journal of epidemiology. 1968; 88(3): 368–78. Epub 1968/11/01. [PubMed: 4301609]

10. Blacklow NR, Hoggan MD, Rowe WP. Serologic evidence for human infection with adenovirus-associated viruses. Journal of the National Cancer Institute. 1968; 40(2):319–27. Epub 1968/02/01. [PubMed: 4295610]

11. Georg-Fries B, Biederlack S, Wolf J, zur Hausen H. Analysis of proteins, helper dependence, and seroepidemiology of a new human parvovirus. Virology. 1984; 134(1):64–71. Epub 1984/04/15. [PubMed: 6200995]

12. Xiao W, Chirmule N, Berta SC, McCullough B, Gao G, Wilson JM. Gene therapy vectors based on adeno-associated virus type 1. Journal of virology. 1999; 73(5):3994–4003. Epub 1999/04/10. [PubMed: 10196295]

13. Moskalenko M, Chen L, van Roey M, Donahue BA, Snyder RO, McArthur JG, et al. Epitope mapping of human anti-adeno-associated virus type 2 neutralizing antibodies: implications for gene therapy and virus structure. Journal of virology. 2000; 74(4):1761–6. Epub 2000/01/22. [PubMed: 10644347]

14. Erles K, Sebokova P, Schlehofer JR. Update on the prevalence of serum antibodies (IgG and IgM) to adeno-associated virus (AAV). Journal of medical virology. 1999; 59(3):406–11. Epub 1999/09/29. [PubMed: 10502275]

15. Chirmule N, Propert K, Magosin S, Qian Y, Qian R, Wilson J. Immune responses to adenovirus and adeno-associated virus in humans. Gene therapy. 1999; 6(9):1574–83. Epub 1999/09/22. [PubMed: 10490767]

16. Halbert CL, Rutledge EA, Allen JM, Russell DW, Miller AD. Repeat transduction in the mouse lung by using adeno-associated virus vectors with different serotypes. Journal of virology. 2000; 74(3):1524–32. Epub 2000/01/11. [PubMed: 10627564]

17. Scallan CD, Jiang H, Liu T, Patarroyo-White S, Sommer JM, Zhou S, et al. Human immunoglobulin inhibits liver transduction by AAV vectors at low AAV2 neutralizing titers in SCID mice. Blood. 2006; 107(5):1810–7. Epub 2005/10/27. [PubMed: 16249376]

18. Calcedo R, Vandenberghe LH, Gao G, Lin J, Wilson JM. Worldwide epidemiology of neutralizing antibodies to adeno-associated viruses. The Journal of infectious diseases. 2009; 199(3):381–90. Epub 2009/01/13. [PubMed: 19133809]

19. Boutin S, Monteilhet V, Veron P, Leborgne C, Benveniste O, Montus MF, et al. Prevalence of serum IgG and neutralizing factors against adeno-associated virus (AAV) types 1, 2, 5, 6, 8, and 9 in the healthy population: implications for gene therapy using AAV vectors. Human gene therapy. 2010; 21(6):704–12. Epub 2010/01/26. [PubMed: 20095819]

20. Li C, Narkbunnam N, Samulski RJ, Asokan A, Hu G, Jacobson LJ, et al. Neutralizing antibodies against adeno-associated virus examined prospectively in pediatric patients with hemophilia. Gene therapy. 2012; 19(3):288–94. Epub 2011/06/24. [PubMed: 21697954]

21. Liu Q, Huang W, Zhang H, Wang Y, Zhao J, Song A, et al. Neutralizing antibodies against AAV2, AAV5 and AAV8 in healthy and HIV-1-infected subjects in China: implications for gene therapy using AAV vectors. Gene therapy. 2014; 21(8):732–8. Epub 2014/05/23. [PubMed: 24849042]

22. Bowles DE, McPhee SW, Li C, Gray SJ, Samulski JJ, Camp AS, et al. Phase 1 gene therapy for Duchenne muscular dystrophy using a translational optimized AAV vector. Molecular therapy : the journal of the American Society of Gene Therapy. 2012; 20(2):443–55. Epub 2011/11/10. [PubMed: 22068425]

23. Louis Jeune V, Joergensen JA, Hajjar RJ, Weber T. Pre-existing anti-adeno-associated virus antibodies as a challenge in AAV gene therapy. Human gene therapy methods. 2013; 24(2):59–67. Epub 2013/02/28. [PubMed: 23442094]

24. Fisher KJ, Jooss K, Alston J, Yang Y, Haecker SE, High K, et al. Recombinant adeno-associated virus for muscle directed gene therapy. Nature medicine. 1997; 3(3):306–12. Epub 1997/03/01.

25. Halbert CL, Standaert TA, Aitken ML, Alexander IE, Russell DW, Miller AD. Transduction by adeno-associated virus vectors in the rabbit airway: efficiency, persistence, and readministration. Journal of virology. 1997; 71(8):5932–41. Epub 1997/08/01. [PubMed: 9223483]

26. Manning WC, Zhou S, Bland MP, Escobedo JA, Dwarki V. Transient immunosuppression allows transgene expression following readministration of adeno-associated viral vectors. Human gene therapy. 1998; 9(4):477–85. Epub 1998/04/03. [PubMed: 9525309]

27. Halbert CL, Standaert TA, Wilson CB, Miller AD. Successful readministration of adeno-associated virus vectors to the mouse lung requires transient immunosuppression during the initial exposure. Journal of virology. 1998; 72(12):9795–805. Epub 1998/11/13. [PubMed: 9811715]

28. Hernandez YJ, Wang J, Kearns WG, Loiler S, Poirier A, Flotte TR. Latent adeno-associated virus infection elicits humoral but not cell-mediated immune responses in a nonhuman primate model. Journal of virology. 1999; 73(10):8549–58. Epub 1999/09/11. [PubMed: 10482608]

29. Beck SE, Jones LA, Chesnut K, Walsh SM, Reynolds TC, Carter BJ, et al. Repeated delivery of adeno-associated virus vectors to the rabbit airway. Journal of virology. 1999; 73(11):9446–55. Epub 1999/10/09. [PubMed: 10516053]

30. Chao H, Samulski R, Bellinger D, Monahan P, Nichols T, Walsh C. Persistent expression of canine factor IX in hemophilia B canines. Gene therapy. 1999; 6(10):1695–704. Epub 1999/10/12. [PubMed: 10516718]

31. Cottard V, Mulleman D, Bouille P, Mezzina M, Boissier MC, Bessis N. Adeno-associated virus-mediated delivery of IL-4 prevents collagen-induced arthritis. Gene therapy. 2000; 7(22):1930–9. Epub 2000/01/11. [PubMed: 11127581]

32. Chirmule N, Xiao W, Truneh A, Schnell MA, Hughes JV, Zoltick P, et al. Humoral immunity to adeno-associated virus type 2 vectors following administration to murine and nonhuman primate muscle. Journal of virology. 2000; 74(5):2420–5. Epub 2000/02/09. [PubMed: 10666273]

33. Anand V, Chirmule N, Fersh M, Maguire AM, Bennett J. Additional transduction events after subretinal readministration of recombinant adeno-associated virus. Human gene therapy. 2000; 11(3):449–57. Epub 2000/03/04. [PubMed: 10697119]

34. Nathwani AC, Davidoff A, Hanawa H, Zhou JF, Vanin EF, Nienhuis AW. Factors influencing in vivo transduction by recombinant adeno-associated viral vectors expressing the human factor IX cDNA. Blood. 2001; 97(5):1258–65. Epub 2001/02/27. [PubMed: 11222368]

35. Wagner JA, Nepomuceno IB, Messner AH, Moran ML, Batson EP, Dimiceli S, et al. A phase II, double-blind, randomized, placebo-controlled clinical trial of tgAAVCF using maxillary sinus delivery in patients with cystic fibrosis with antrostomies. Human gene therapy. 2002; 13(11): 1349–59. Epub 2002/08/07. [PubMed: 12162817]

36. Huttner NA, Girod A, Perabo L, Edbauer D, Kleinschmidt JA, Buning H, et al. Genetic modifications of the adeno-associated virus type 2 capsid reduce the affinity and the neutralizing effects of human serum antibodies. Gene therapy. 2003; 10(26):2139–47. Epub 2003/11/20. [PubMed: 14625569]
37. Peden CS, Burger C, Muzyczka N, Mandel RJ. Circulating anti-wild-type adeno-associated virus type 2 (AAV2) antibodies inhibit recombinant AAV2 (rAAV2)-mediated, but not rAAV5-mediated, gene transfer in the brain. Journal of virology. 2004; 78(12):6344–59. Epub 2004/05/28. [PubMed: 15163728]
38. Le HT, Yu QC, Wilson JM, Croyle MA. Utility of PEGylated recombinant adeno-associated viruses for gene transfer. Journal of controlled release : official journal of the Controlled Release Society. 2005; 108(1):161–77. Epub 2005/08/30. [PubMed: 16125817]
39. Halbert CL, Miller AD, McNamara S, Emerson J, Gibson RL, Ramsey B, et al. Prevalence of neutralizing antibodies against adeno-associated virus (AAV) types 2, 5, and 6 in cystic fibrosis and normal populations: Implications for gene therapy using AAV vectors. Human gene therapy. 2006; 17(4):440–7. Epub 2006/04/14. [PubMed: 16610931]
40. Jiang H, Couto LB, Patarroyo-White S, Liu T, Nagy D, Vargas JA, et al. Effects of transient immunosuppression on adenoassociated, virus-mediated, liver-directed gene transfer in rhesus macaques and implications for human gene therapy. Blood. 2006; 108(10):3321–8. Epub 2006/07/27. [PubMed: 16868252]
41. Li C, Diprimio N, Bowles DE, Hirsch ML, Monahan PE, Asokan A, et al. Single amino acid modification of adeno-associated virus capsid changes transduction and humoral immune profiles. Journal of virology. 2012; 86(15):7752–9. Epub 2012/05/18. [PubMed: 22593151]
42. Wu Z, Asokan A, Samulski RJ. Adeno-associated virus serotypes: vector toolkit for human gene therapy. Molecular therapy : the journal of the American Society of Gene Therapy. 2006; 14(3): 316–27. Epub 2006/07/11. [PubMed: 16824801]
43. Weinberg MS, Nicolson S, Bhatt AP, McLendon M, Li C, Samulski RJ. Recombinant adeno-associated virus utilizes cell-specific infectious entry mechanisms. Journal of virology. 2014; 88(21):12472–84. Epub 2014/08/22. [PubMed: 25142580]
44. Sun L, Tu L, Gao G, Sun X, Duan J, Lu Y. Assessment of a passive immunity mouse model to quantitatively analyze the impact of neutralizing antibodies on adeno-associated virus-mediated gene transfer. Journal of immunological methods. 2013; 387(1–2):114–20. Epub 2012/10/16. [PubMed: 23063691]
45. Denard J, Beley C, Kotin R, Lai-Kuen R, Blot S, Leh H, et al. Human galectin 3 binding protein interacts with recombinant adeno-associated virus type 6. Journal of virology. 2012; 86(12):6620–31. Epub 2012/04/13. [PubMed: 22496229]
46. Denard J, Marolleau B, Jenny C, Rao TN, Fehling HJ, Voit T, et al. C-reactive protein (CRP) is essential for efficient systemic transduction of recombinant adeno-associated virus vector 1 (rAAV-1) and rAAV-6 in mice. Journal of virology. 2013; 87(19):10784–91. Epub 2013/08/02. [PubMed: 23903832]

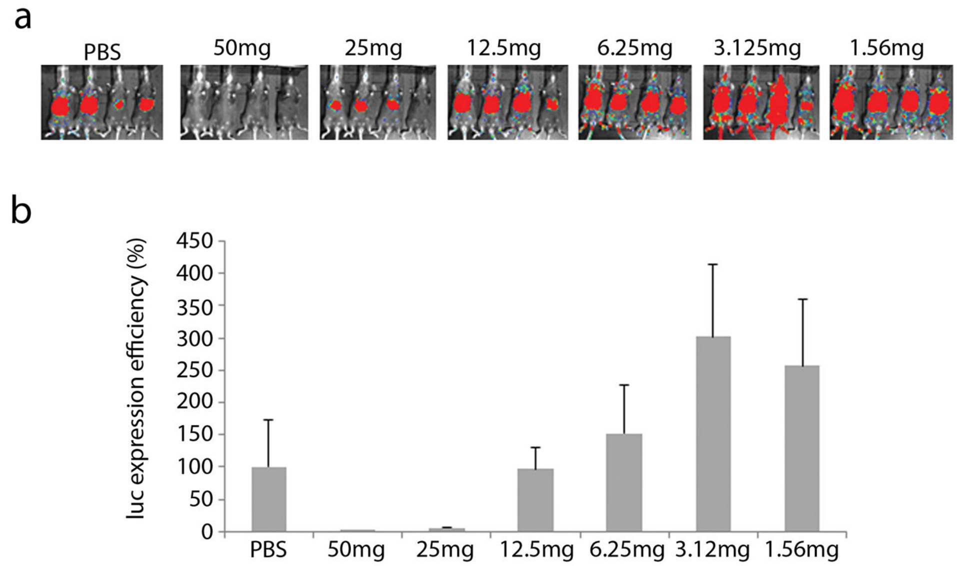

Fig. 1. Nab assay based on systemic injection of human IVIG

1×10^{10} particles of AAV8/luc vectors in 12.5 µl were incubated with equal volumes of different concentration of IVIG or PBS, then administered via retro-orbital injection in C57BL/6 mice. One week later, imaging was performed and analyzed for luciferase expression in liver region. **a.** The imaging of luciferase expression from mice (n=4). **b.** Inhibition of AAV8 systemic transduction using human IVIG. Data represent the average of 4 mice and standard derivation.

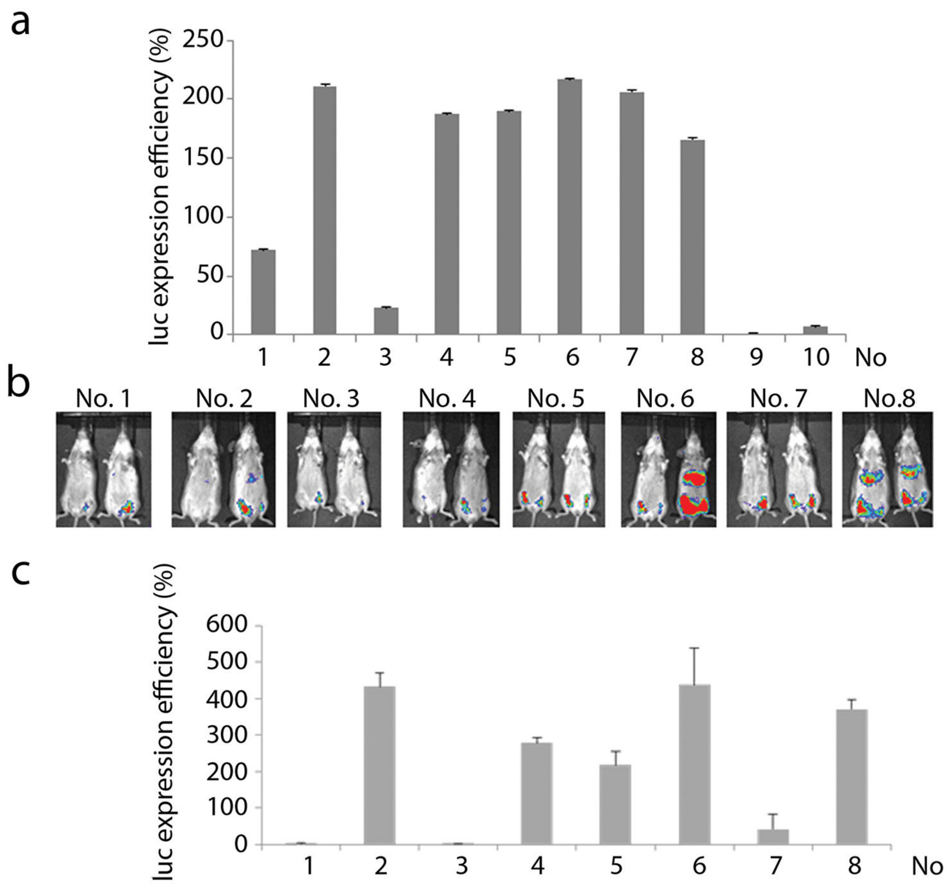

Fig. 2. AAV8 Nab analysis in sera from human subjects

Undiluted sera from 10 human subjects were incubated with AAV8/luc vector for Nab activity analysis *in vitro* and via intramuscular injection. **a**. The effect of sera on transgene expression in Huh7 cells. 1×10^8 particles of AAV8/luc in 12.5 µl were incubated with 12.5 µl undiluted sera for 2hr at 4°C, then the mixture was added to infect 1×10^5 Huh7 cells in 48 well plate in the presence of Ad dl309. 24 hrs later, a luciferase assay was performed. The data indicates the transgene expression efficiency in the presence of sera against control without sera. **b**. The imaging of luciferase expression from mice treated with intramuscular injection of AAV8/serum mix. 1×10^9 particles of AAV8/luc vectors in 100 µl were incubated with equal volumes of human serum, then the mixture was directly injected into the muscles of hind legs in BALB/c mice. Three weeks later, imagies were taken and analyzed for luciferase expression. Face up: left leg-AAV8 with serum, right leg-AAV8 with PBS. **c**. Inhibition of AAV8 transduction via intramuscular injection using human sera. Data represent the average of 2 mice and standard derivation.

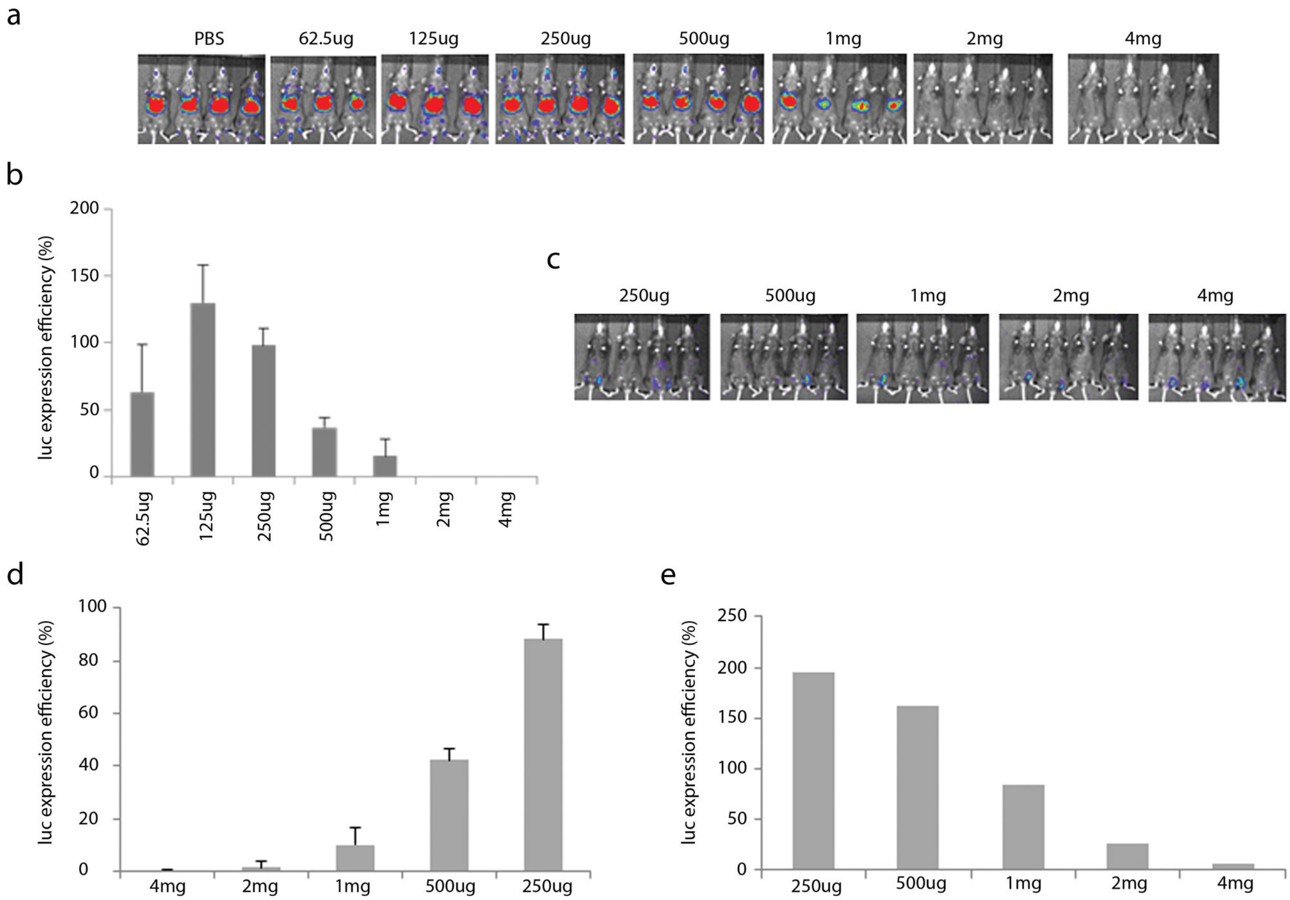

Fig. 3. AAV8 Nab inhibitory effects on AAV transduction in a mouse model using adoptive transfer of human IVIG

a. The imaging of luciferase expression in mice with adoptive transfer of human IVIG followed by systemic injection of AAV8 vector. Different amounts of human IVIG were administered into C57BL/6 mice. Three hours later, blood was harvested followed by administration of AAV8/luc vector at a dose of 2×10^{12} particles/kg via retro-orbital injection. At day 3 post-AAV injection, imaging was carried out and analyzed for luciferase expression in the liver region. **b.** Inhibition of AAV8 systemic transduction using human IVIG. Data represent the average of 4 mice and standard derivation when compared to PBS. **c.** Imaging of luciferase expression in mice after intramuscular injection. 1×10^9 particles of AAV8/luc vectors in 20 µl were incubated with equal volume of serum from mice given adoptive transfer of human IVIG. The mixture was directly injected into the muscles of hind legs in C57BL/6 mice. Left leg (face-up) was injected with AAV8 vector mixed with serum; the right leg was given AAV8 with PBS. Three weeks later, imaging was taken and analyzed for luciferase expression. **d.** Inhibition of AAV8 muscle transduction using human IVIG in mouse serum. Data were the average of 4 mice and standard derivation. **e.** Inhibition of AAV8 transduction in Huh7 cells using human IVIG in mouse serum. 1×10^8 particles of AAV8/luc vectors in 20 µl were incubated with equal volumes of PBS including 2 µl serum from mice with adoptive transfer of human IVIG. The mixture then was used to infect 1×10^5 Huh7 cells in a 48-well plate in the presence of Ad dl308 at an MOI of 5. Luciferase activity was measured 24 hrs later. Inhibition was calculated as 100% minus the ratio of luciferase activity from the serum group to PBS.

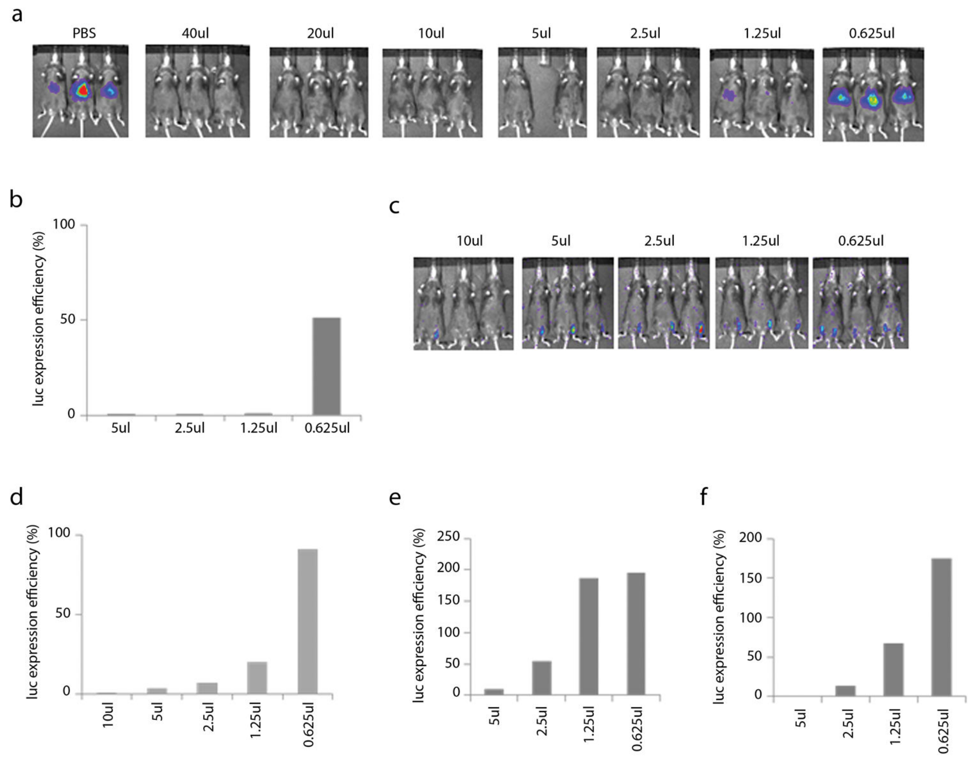

Fig. 4. AAV8 Nab inhibition analysis in a mouse model with adoptive transfer of human serum

a. The imaging of luciferase expression in mice with adoptive transfer of human serum. Different volumes of human serum were administered into C57BL/6 mice. Three hours later, blood was drawn followed by administration of AAV8/luc vector at the dose of 2×10^{12} particles/kg via retro-orbital injection. At day 3 post-AAV injection imaging was performed and analyzed for luciferase expression in liver region. **b.** Inhibition of AAV8 systemic transduction using human sera. Data represent the average of 4 mice and standard derivation as compared to PBS. **c.** Imaging of luciferase expression in mice after intramuscular injection. 1×10^9 particles of AAV8/luc vectors in 20 μl were incubated with equal volumes of serum from mice with adoptive transfer of human serum. The mixture was directly injected into the muscles of hind legs in C57BL/6 mice. The left leg (face-up) was injected with an AAV8 vector mixed with serum; the right one was treated with AAV8 mixed with PBS. Three weeks later imaging was performed to quantify relative luciferase expression. **d.** Inhibition of on AAV8 muscle transduction using mouse serum with human serum adoptive transfer. Data are the average of 4 mice and standard derivation. **e and f.** Inhibition of AAV8 transduction of Huh7 cells using mouse serum with human serum adoptive transfer. 1×10^8 particles of AAV8/luc vectors in 20 μl were incubated with equal volumes of PBS containing 2 μl serum (**e**) or 20 μl serum (**f**) from mice with adoptive transfer of human serum. After transduction in Huh7 cells for 24 hrs luciferase activity was measured and relative inhibition was calculated.

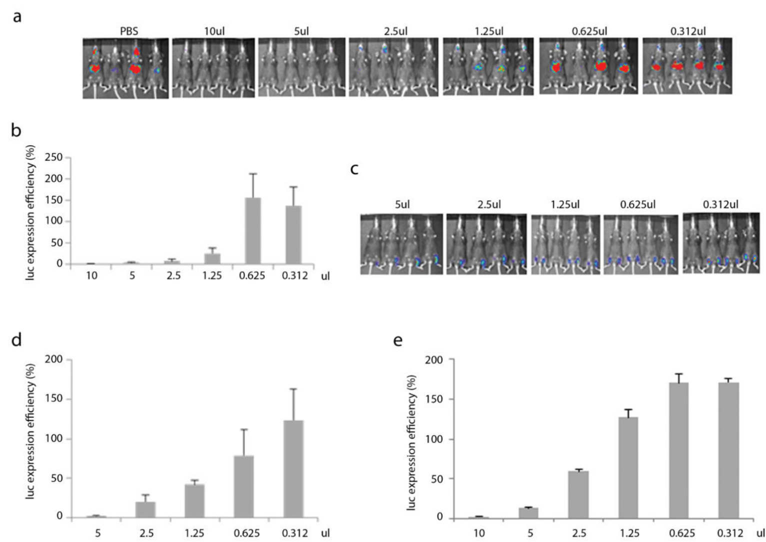

Fig. 5. AAV2 Nab inhibition analysis in a mouse model with adoptive transfer of human serum

a. The imaging of luciferase expression in mice with adoptive transfer of human serum and AAV2 systemic administration. Similar to AAV8 as described in Fig. 5, after administration of human serum followed by systemic administration of AAV2/luc vector at the dose of 2×10^{12} particles/kg, imaging was performed at day 7. **b.** Inhibition of AAV2 systemic transduction using human serum. Data represent the average of 4 mice and standard derivation as compared to PBS. **c.** Imaging of luciferase expression in mice after intramuscle injection. 1×10^{9} particles of AAV2/luc vectors incubated with serum from mice with adoptive transfer of human serum was directly injected into the muscles. Three weeks later imaging was taken to quantify relative luciferase expression. **d.** Inhibition of AAV2 muscle transduction using mouse serum with human serum adoptive transfer. Data are the average of 4 mice and standard derivation. **e.** The inhibition of serum from mice with human serum adoptive transfer on AAV2 transduction in Huh7 cells. 1×10^{8} particles of AAV2/luc vectors in 20 μl were incubated with equal volumes of PBS containing 2 μl serum from mice with adoptive transfer of human serum. Transduction in Huh7 cells for 24 hrs luciferase activity was measured.

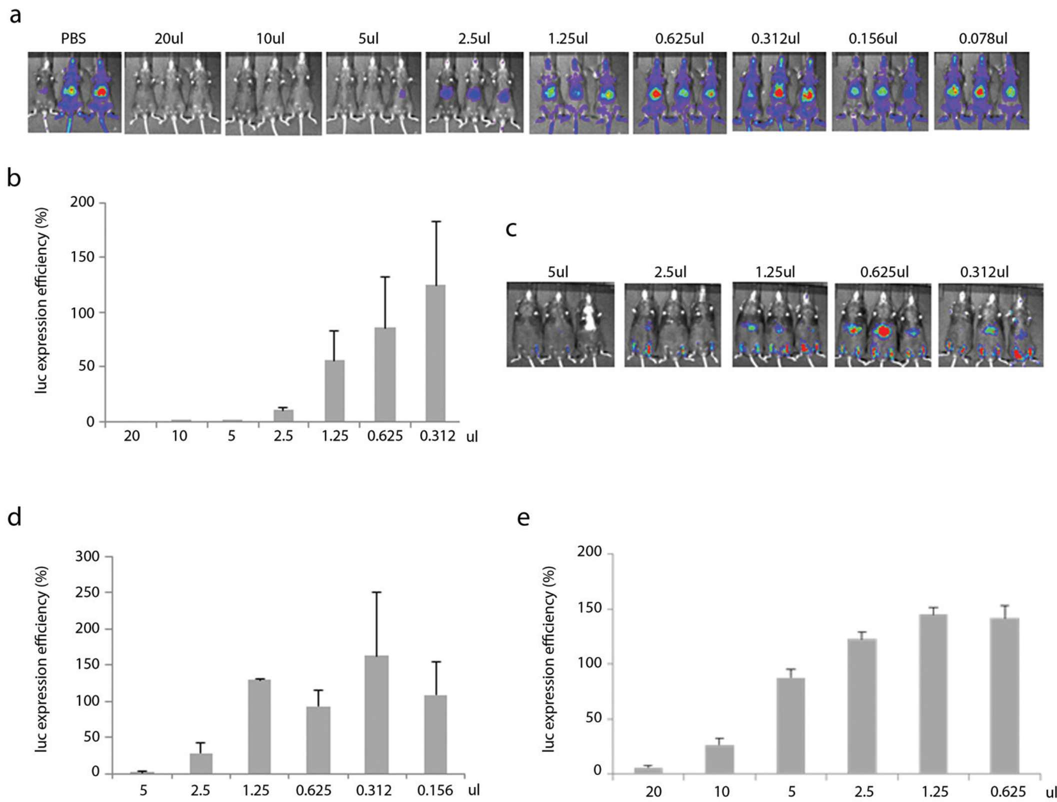

Fig. 6. AAV9 Nab inhibition analysis in a mouse model with adoptive transfer of human serum

a. The imaging of luciferase expression in mice with adoptive transfer of human serum and AAV9 systemic administration. After systemic administration of AAV9 at dose of 2×10^{12} particles/kg into mice receiving human serum, imaging was taken at day 3. **b.** Inhibition of AAV9 systemic transduction using human serum. Data represent the average of 3 or 4 mice and standard derivation as compared to PBS. **c.** Imaging of luciferase expression in mice after intramuscular injection. 1×10^{9} particles of AAV9/luc vectors incubated with serum from mice with adoptive transfer of human serum was directly injected into the muscles. One week later imaging was carried out to quantify relative luciferase expression. **d.** Inhibition of AAV9 muscle transduction using mouse serum with human serum adoptive transfer on. Data are the average of 3 mice and standard derivation. **e. I**nhibition of AAV9 transduction in Huh7 cells using mouse serum with human serum adoptive transfer. 1×10^{8} particles of AAV9/luc vectors incubated with equal volumes of PBS containing 2 µl serum from mice with adoptive transfer of human serum were used to transduce Huh7 cells. Luciferase activity was measured and relative inhibition was calculated.

Table 1

List of factors that impact AAV Nab titers *in vitro*

Factor	Important	Not important
Cell line		X
Serum volume	X	
AAV dose	X	
Temperature		X
Cell number	X	
Adenovirus		X
Culture duration		X
Incubation time		X
Heat inactivation		X
Transgenes (GFP vs luciferase)	X	