Prediction of adeno-associated virus neutralizing antibody activity for clinical application

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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 ~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, whereas 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 showed that Nab-naive patients had measurable IM AAV genomes,2,5 whereas AAV genomes were virtually undetectable in patients with measurable AAV Nabs before 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 AAV2.6,7,10–21,16,19 This wide difference may result from various conditions used for Nab assays among different labs.23 Factors that impacted assay results include cutoffs (2–3, 10–20 and even 100-fold dilution of serum), cell lines (293, IB3, HeLa, CHO, B16F10, U87, Huh7 and so on), addition of adenovirus (Ad) or chemicals that enhance AAV transduction, varying doses of AAV vector per cell (105–109), temperature or length of time for incubation of virus with human sera, volume of serum used to interact with AAV, transgenes (luciferase, GFP, LacZ and so on), culture duration after AAV transduction and heat inactivation of complement proteins.6,7,10–21,19,20,24–41 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 per 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.

Patients with neutralizing antibodies (Nabs) against adeno-associated virus (AAV) are usually excluded from the 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 per cell, cell number and choice of transgenes. When the Nab titer assay was performed in vivo via intramuscular (IM) or systemic administration, a fourfold increase in sensitivity for measurement of Nab titers was observed compared with 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 complicating clinical data seen to date with respect to efficiency of AAV transduction in various phase I clinical trials.

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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, AAV8 and AAV9 encoding firefly luciferase were used to transduce seven cell lines: HEK293, C2C12, RC32, HeLa, Huh7, HepG2 and U87, at a dose of 1000 particles per cell in the presence of Ad di309. Luciferase activity from transduced cells was analyzed 24 h 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 (Supplementary Figure 1). 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 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 seven cell lines (293, C2C12, RC32, HeLa, Huh7, HepG2 and U87). As shown in Supplementary Figure 1B, the Nab titer from these cell lines was the same at 1 mg ml$^{-1}$ 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 h at either 4 or 37°C; we found no temperature-based difference in transduction (Supplementary Figure 2). We next incubated AAV8 virus with IVIG at 4°C for 1, 2, 3 or 4 h, then added the virus to Huh7 cells. Again, the transduction level was the same regardless of incubation time (Nab titers at 1 mg ml$^{-1}$). 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. In addition, 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 (Supplementary Figure 2).

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 per cell were used in the presence of IVIG at 8 mg ml$^{-1}$. However, when 10–1000 particles of AAV8 vector per cell were used, Nab titers were similar at 1 mg ml$^{-1}$. 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 Supplementary Figure 3, the IVIG Nab titer against AAV8 using a GFP transgene was 4 mg ml$^{-1}$. 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 1 mg ml$^{-1}$.

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 h 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$^{-1}$ of human IVIG (Supplementary Figure 4).

To examine the Nab titer after systemic administration of vector, we first incubated 1 × 10$^{10}$ particles of AAV8/luc with phosphate-buffered saline (PBS) or human IVIG, followed by retro-orbital injection of the Nab/vector mix. At day 7 after AAV8 injection, intravaltral imaging was performed and photons to the general liver area were measured. As shown in Figure 1, when 25 mg ml$^{-1}$ 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 1, 2.5 and 25 mg when 1 × 10$^9$, 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$^9$ particles of AAV8, the IVIG Nab titer was 1, 0.25 and 0.25 mg for Huh7 transduction, IM administration and systemic administration, respectively. The same Nab titer was detected with systemic and IM injection, which was fourfold 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 (Figure 2a). Next, 1 × 10$^9$ particles of AAV8/luc vector was incubated with serum from eight different individuals from the population above at the same ratio of AAV vector dose to serum volume as in Huh7 cells. After 2 h 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 Figures 2b and c, 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 Supplementary Figure 5, the concentration of human IVIG was stable between 1 and 24 h after IVIG passive transfer. Therefore, we administered AAV vector 3 h 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 per kg.5,6 The identical dose of AAV vector per kg 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 g. Based on

Table 1. List of factors that impact AAV Nab titers in vitro

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

Abbreviations: AAV, adeno-associated virus; Nab, neutralizing antibody.
Figure 1. Nab assay based on systemic injection of human IVIG. $1 \times 10^{10}$ 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 the 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 four mice and standard derivation.

Figure 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. $1 \times 10^8$ particles of AAV8/luc in 12.5 μl were incubated with 12.5 μl undiluted sera for 2 h at 4 °C, then the mixture was added to infect $1 \times 10^5$ Huh7 cells in 48-well plate in the presence of Ad dl309. 24 h later, a luciferase assay was performed. The data indicate 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. $1 \times 10^7$ 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, images 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 two mice and standard derivation.
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. Three hours later, blood was collected followed by administration of AAV8/luc vector at a dose of $2 \times 10^{12}$ particles per 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 AAV systemic transduction using human IVIG. Data represent the average of four mice and standard derivation when compared with PBS. (c) Imaging of luciferase expression in mice after intramuscular injection. $1 \times 10^9$ 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 four mice and standard derivation. (e) Inhibition of AAV8 transduction in Huh7 cells using human IVIG in mouse serum. $1 \times 10^8$ 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 was then used to infect $1 \times 10^5$ Huh7 cells in a 48-well plate in the presence of Ad dl308 at an multiplicity of infection of 5. Luciferase activity was measured 24 h later. Inhibition was calculated as 100% minus the ratio of luciferase activity from the serum group to PBS.

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 $1 \times 10^8$ particles of AAV8/luc in vitro with 20 μl plasma from mice having systemic administration of AAV8. Then AAV8/luc vector was injected into mouse muscles. One week later, the luciferase expression was detected (Figure 3c). As shown in Figure 3d, the serum from mice with administration of 500 μg human IVIG inhibited luciferase expression over 50%, compared with the control group given PBS. These data were consistent with our observations with systemic administration of AAV vector in human IVIG passive transferred mice (Figure 3b).

Figure 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 collected followed by administration of AAV8/luc vector at a dose of $2 \times 10^{12}$ particles per 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 AAV systemic transduction using human IVIG. Data represent the average of four mice and standard derivation when compared with PBS. (c) Imaging of luciferase expression in mice after intramuscular injection. $1 \times 10^9$ 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 four mice and standard derivation. (e) Inhibition of AAV8 transduction in Huh7 cells using human IVIG in mouse serum. $1 \times 10^8$ 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 was then used to infect $1 \times 10^5$ Huh7 cells in a 48-well plate in the presence of Ad dl308 at an multiplicity of infection of 5. Luciferase activity was measured 24 h later. Inhibition was calculated as 100% minus the ratio of luciferase activity from the serum group to PBS.
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 drawn followed by administration of AAV8/luc vector at the dose of 2 × 10^{12} particles per kg via retro-orbital injection. At day 3 post AAV injection, imaging was performed and analyzed for luciferase expression in the liver region. 

(b) Inhibition of AAV8 systemic transduction using human sera. Data represent the average of four mice and standard derivation as compared with PBS. 

(c) Imaging of luciferase expression in mice after intramuscular injection. 1 × 10^{6} 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 AAV8 muscle transduction using mouse serum with human serum adoptive transfer. Data are the average of four mice and standard derivation. 

(e, 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 h, luciferase activity was measured and relative inhibition was calculated.

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 (Figure 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 (Figures 5a and 5b) and AAV9 (2.5 μl, Figures 6b and d), stronger suppression of transgene expression than that with in vitro analysis (5 and 10 μl for AAV2 and 9, respectively, Figures 5e and 6e).
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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 have a critical role in predicting/measuring Nab titer. These included cell lines, temperature for incubation of AAV virus with serum, addition of Ad 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 IM injection and systemic administration. When we tried mimicking the Nab inhibition effect observed in the clinical setting in previously identified Nabs negative patients based on in vitro assay, IM 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.\textsuperscript{5–6} 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 titer of Nabs in the blood can fully suppress AAV transduction in vivo.\textsuperscript{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 measurable 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.\textsuperscript{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.\textsuperscript{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 folowed 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 with the in vivo assay. We have performed the in vivo Nab assay via muscular administration and observed fourfold more sensitive than that in vitro analysis. Consistent with these observations, similar Nab titer is obtained between IM and intravenous. 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 0.5 mg ml^-1 of IVIG) and administered into mice via IM or systemic injection, transgene expression was decreased by more than 90% compared with 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 (that is, 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 have 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, 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, 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 galectin 3-binding protein from human and dog but not from primate and mouse. In addition, these two serotypes also interact with mouse C-reactive protein but not human. AAV8 and AAV9 do not interact with galectin 3-binding protein or C-reactive protein 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% CO2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum 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 (pX2R, pX8R and pX9R) and Ad helper plasmid pX6-80 into HEK293 cells. Sixty hours later, cells were harvested and lysed, and supernatant was ultra-centrifuged 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, USA). Individual human serum was purchased from Valley Biomedical (Manchester, VA, USA). Human IVIG and serum were aliquoted and stored at −80 °C for future use.

Nab analysis
Nab analyses were performed as described previously with slight modification.20 Human IVIG or serum was serially diluted twofold. Cells were seeded on a 48-well plate in 300 μl Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Cells were cultured for 3-4 h 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, Madison, WI, USA) 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.

Nab analysis with AAV IM administration
1 × 10⁷ particles of AAV/luc vector were incubated with human Nabs for 2 h at 4 °C. Then the mixture was directly injected into the hind leg muscles of 6- to 8-week-old C57BL/6 mice or BALB/c mice. At the indicated time points, imaging was performed using Xenogen IVIS Lumina imaging system (Caliper Lifesciences, Hopkinton, MA, USA) following intraperitoneal injection of D-luciferin substrate at 120 mg kg⁻¹ (Nanolight, Pinetop, AZ, USA). Bioluminescent images were analyzed using Living Image software (Xenogen, Alameda, CA, USA). 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.

Nab analysis with AAV systemic administration
1 × 10¹⁰ particles of AAV/luc vector were incubated with human Nabs for 2 h 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 hours later, blood was collected for Nab activity assay via IM injection or in cell lines in vitro, then followed by systemic injection of 2 × 10¹² particles per kg of AAV/luc vector. Imaging was performed 1 week after AAV administration. For Nab activity analysis via IM administration, 1 × 10⁸ particles of AAV/luc vector were incubated with mouse serum, which contained passive transferred human Nabs for 2 h 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 × 10⁸ 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 h at 4 °C. The mixture were then used to infect 1 × 10¹⁵ Huh7 cells in a 48-well plate in the presence of Ad di 309 at multiplicity of infection of 5. After 24 h, cells were lysed and luciferase activity was analyzed.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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