Gene Therapy (2015) **22,** 116–126

www.nature.com/gt

ORIGINAL ARTICLE

Antibody neutralization poses a barrier to intravitreal adeno-associated viral vector gene delivery to non-human primates

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Gene delivery vectors based on adeno-associated viruses (AAV) have exhibited promise in both preclinical disease models and human clinical trials for numerous disease targets, including the retinal degenerative disorders Leber's congenital amaurosis and choroideremia. One general challenge for AAV is that preexisting immunity, as well as subsequent development of immunity following vector administration, can severely inhibit systemic AAV vector gene delivery. However, the role of neutralizing antibodies (NABs) in AAV transduction of tissues considered to be immune privileged, such as the eye, is unclear in large animals. Intravitreal AAV administration allows for broad retinal delivery, but is more susceptible to interactions with the immune system than subretinal administration. To assess the effects of systemic anti-AAV antibody levels on intravitreal gene delivery, we quantified the anti-AAV antibodies present in sera from non-human primates before and after intravitreal injections with various AAV capsids. Analysis showed that intravitreal administration resulted in an increase in anti-AAV antibodies regardless of the capsid serotype, transgene or dosage of virus injected. For monkeys injected with wild-type AAV2 and/or an AAV2 mutant, the variable that most significantly affected the production of anti-AAV2 antibodies was the amount of virus delivered. In addition, post-injection antibody titers were highest against the serotype administered, but the antibodies were also cross-reactive against other AAV serotypes. Furthermore, NAB levels in serum correlated with those in vitreal fluid, demonstrating both that this route of administration exposes AAV capsid epitopes to the adaptive immune system and that serum measurements are predictive of vitreous fluid NAB titers. Moreover, the presence of preexisting NAB titers in the serum of monkeys correlated strongly ($R = 0.76$) with weak, decaying or no transgene expression following intravitreal administration of AAV. Investigating anti-AAV antibody development will aid in understanding the interactions between gene therapy vectors and the immune system during ocular administration and can form a basis for future clinical studies applying intravitreal gene delivery.

Gene Therapy (2015) **22,** 116–126; doi:10.1038/gt.2014.115; published online 11 December 2014

INTRODUCTION

The parvovirus adeno-associated virus (AAV) contains a 4.7 kb single-stranded DNA genome within a non-enveloped protein capsid.[1] The genome is flanked by inverted terminal repeats that serve as the viral origin of replication and packaging signal for the genome.[1] The genome contains three open reading frames (ORFs). The *rep* ORF encodes four nonstructural proteins that have roles in viral replication, transcriptional regulation, site-specific integration and virion assembly;[1] the *cap* ORF encodes three structural proteins (VP1-3) that assemble to form a 60-mer viral capsid;[1] and the assembly-activating protein[2,3]—which lies in an alternate reading frame within the *cap* gene—localizes AAV capsid proteins to the nucleolus and functions in the capsid assembly process.[2] There are 11 naturally occurring serotypes and over 100 variants of AAV, each of which differs in amino-acid sequence, particularly within the hypervariable regions of the capsid proteins, and thus in their gene delivery properties.[4,5] To create recombinant versions of AAV for use in gene delivery, a gene of interest is inserted between the inverted terminal repeats in place of *rep* and *cap*, which are then provided in *trans* along with helper viral genes during vector production.[6] The resulting AAV vectors can transduce both dividing and non-dividing cells, and its delivery can result in stable transgene expression for years in post-mitotic tissue.

As of 2014, there were over 100 completed or ongoing clinical trials that used AAV as the gene delivery vehicle.[7] Among the many characteristics that make AAV an attractive vector for clinical applications, it has not been associated with any human disease.[1] In addition, during phase I/II clinical trials for Leber's congenital amaurosis, over 30 patients who received a subretinal injection of AAV2 encoding RPE65, an enzyme responsible for the isomerohydrolase activity of retinal pigment epithelium, showed sustained improvement in both subjective and objective measurements of vision.[8–14] Moreover, a recent phase I trial for choroideremia showed promising signs of efficacy.[15] These trials therefore indicate that AAV may be promising for treating monogenic and complex retinal degenerative diseases, including retinitis pigmentosa, macular degeneration, diabetic retinopathy and glaucoma.

One potential challenge for the broad application of AAV ocular therapy, however, is its route of administration. Subretinal AAV vector injection, used for the Leber's congenital amaurosis and choroideremia trials, enables efficient gene expression in several retinal cell types, including photoreceptors and retinal pigment epithelial cells.[16,17] This route of administration entails delivery via a needle puncture through the neurosensory retina, which

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Received 11 August 2014; revised 3 November 2014; accepted 7 November 2014; published online 11 December 2014

induces a retinal detachment surrounding a bleb of concentrated vector that results in very efficient transduction of the adjacent photoreceptors and retinal pigement epithelia. Although neutralizing antibodies (NABs) against the AAV capsid do not increase (or prevent readministration) when AAV gene therapy vectors are administered via subretinal injection,[13] delivery is limited to tissues in contact with the bleb(s),[18–20] a suboptimal solution as degeneration occurs across the retina in most diseases. Furthermore, the retinal detachment inherent in subretinal injections can lead to reactive gliosis, retinal disorganization, photoreceptor degeneration and functional losses in vision,[21,22] risks that may be exacerbated in retinas already weakened by degenerative diseases.[23] In contrast, intravitreal AAV administration, to the vitreous humor of the eye, offers the potential for panretinal delivery without the risk of retinal detachment. Recent work has developed AAV variants that are capable of infecting both Muller glia and photoreceptors across the retina following intravitreal administration, making intravitreal injections a promising alternative.[24,25]

However, another challenge to AAV-mediated gene therapy is humoral anti-AAV antibody-mediated immunity, which results from childhood exposure to one or more serotypes or from prior administration of AAV vectors and poses a significant challenge to AAV gene therapy.[26,27] Recent analysis indicated that the prevalence of anti-AAV IgG antibodies in humans was highest for AAV2 (72%) and AAV1 (67%); however, AAV9 (47%), AAV6 (46%), AAV5 (40%) and AAV8 (38%) antibodies were also present in a large portion of the population studied.[28] In non-human primates (NHPs), high concentrations of anti-AAV antibodies significantly reduced the transduction of hepatocytes,[29] and the presence of preexisting antibodies has been correlated with decreased efficacy of gene therapy in clinical trials where the route of administration exposes vector to serum.[26,30,31]

In addition to antibodies in the blood, antibodies are also present in other bodily fluids, such as human synovial fluid,[32,33] NHP cerebrospinal fluid[34] and NHP vitreal fluid (as we show here), where they can again inhibit vector transduction. For example, the presence of extremely high titers of NABs in the serum is accompanied by reduced AAV vector transduction in the rat brain following intracranial injection or in the mouse eye following intravitreal injection.[35,36] Furthermore, AAV administration to immune privileged regions can lead to increases in NAB titers, including following intravitreal injection in dogs and NHPs[37] and following intracranial injections in several clinical trials.[38] Although transient immunosuppression may be a promising approach for reducing cellular immune responses to gene therapy,[39–41] it is not effective at mitigating vector neutralization by preexisting antibodies.

As a result, AAV clinical successes to date have involved delivery either to immune privileged regions or to subjects lacking apparent AAV immunological memory.[8–14,30,42] During the Leber's congenital amaurosis clinical trial, patients were excluded 'if immunological studies show the presence of NABs to AAV2 above 1:1000.'[11] Recent clinical trials for hemophilia have used even more stringent criteria for patient exclusion, removing patients whose NABs were above 1:4.[31,43] NABs are often the most commonly invoked exclusion criterion for patent enrollment.[30]

As part of a study on the function of optogenetic proteins in the NHP retina, we collected serum and intravitreal fluid samples from NHPs before and after injections with various AAV capsids. As a result, these samples have enabled the analysis of the immune response (specifically the presence of anti-AAV antibodies) to AAV vectors, as well as the effects of systemic NAB levels on transgene expression following intravitreal administration. In an effort to provide as much information as possible, AAV injection history for each monkey studied is included in the presentation of the results. Although NHP studies present inherent challenges for larger-scale statistical analysis, the trends observed in this study may lend insights to help understand the interactions between AAV vectors and the immune system during ocular administration, and may therefore aid future clinical studies involving intravitreal gene delivery.

RESULTS

Increase of anti-AAV2 antibodies' pre-infection vs post infection

As part of studies exploring reporter or optogenetic proteins in the NHP retina,[25,44,45] monkeys were injected with vectors with capsids of one or more of the following: wild-type AAV2 capsid, wild-type AAV5 capsid, wild-type AAV9 capsid, 7m8 (an AAV2-based capsid engineered for murine retinal delivery and containing an insertion of the peptide LGETTRP at amino-acid position 588 on the viral capsid)[25] and AAV2 tyrosine 4YF mutant virus (an AAV2-based capsid containing tyrosine to phenylalanine mutations at amino-acid positions 272, 444, 500 and 730).[46] The levels of anti-AAV antibodies present in monkey serum samples obtained before or after intravitreal AAV administration were determined with an *in vitro* neutralization assay in which the inhibition of HEK 293 cell transduction was measured following incubation with a range of serum dilutions. The NAB titers are reported as the reciprocal of the serum dilution at which 50% of transduction is inhibited *in vitro* (Figure 1a). As shown in the representative images for one animal, there was no effect on the level of transduction (measured as green fluorescent protein (GFP)-positive cells) even in the most concentrated serum dilution (1:10) for a serum sample taken before intravitreal administration of 7m8 vector, indicating that this serum did not contain a detectable level of anti-AAV2 antibodies. In contrast, there was a 50% decrease in transduction with a 1:500 dilution of a serum sample taken from the same monkey after intravitreal administration of 7m8 vector, indicating the presence of anti-AAV2 antibodies. Furthermore, even at a 1:10 dilution transduction was fully inhibited, illustrating the neutralization capacity of the anti-AAV2 antibodies found in serum post viral administration.

Serum samples were obtained before and after intravitreal injections, in one or both eyes, of various AAV capsids in a series of adult macaques. Anti-AAV2 antibody titers increased in all animals following injection regardless of the capsid serotype, transgene, dosage of virus or unilateral vs bilateral injection. This finding is consistent with the previous reports in rodents indicating that the vitreous is not as immune privileged a route of administration as subretinal delivery for either AAV or adenovirus delivery.[47,48] However, several variables may change the magnitude of the antibody response. For example, the capsid serotype (AAV2, AAV5 or AAV9) administered may affect the magnitude of increase in anti-AAV2 NAB titers from pre- to post injection. That is, as expected, monkeys injected with AAV2 generated post-administration antibodies that neutralized AAV2 slightly more potently than monkeys injected with other serotypes (Figure 1b—wild-type AAV2: 10–50-fold increase post injection vs Figure 1c—wild-type AAV9 and/or AAV5: 2.5–50-fold increase post injection). The particular alternative serotype injected may also affect the anti-AAV2 antibody response post administration. Monkey 108 (injected with both AAV5 and AAV9—Figure 1c, black circles) had a larger anti-AAV2 response than monkey 012 (only injected with AAV5—Figure 1c, gray triangles), which may be owing to the larger percentage of sequence similarity between AAV2 and AAV9 capsids, the larger amount of virus administered (as both the eyes were injected), or the sequential eye injections in monkey 108 heightening the immune response to the second injection.

Another anticipated observation is that although the serotype injected (that is, AAV2, AAV5 or AAV9) appears to modulate the subsequent level of serum neutralization of AAV2, small sequence changes to a serotype (that is, wild-type AAV2 vs 7m8) do not significantly affect the antibody response in the seven animals in

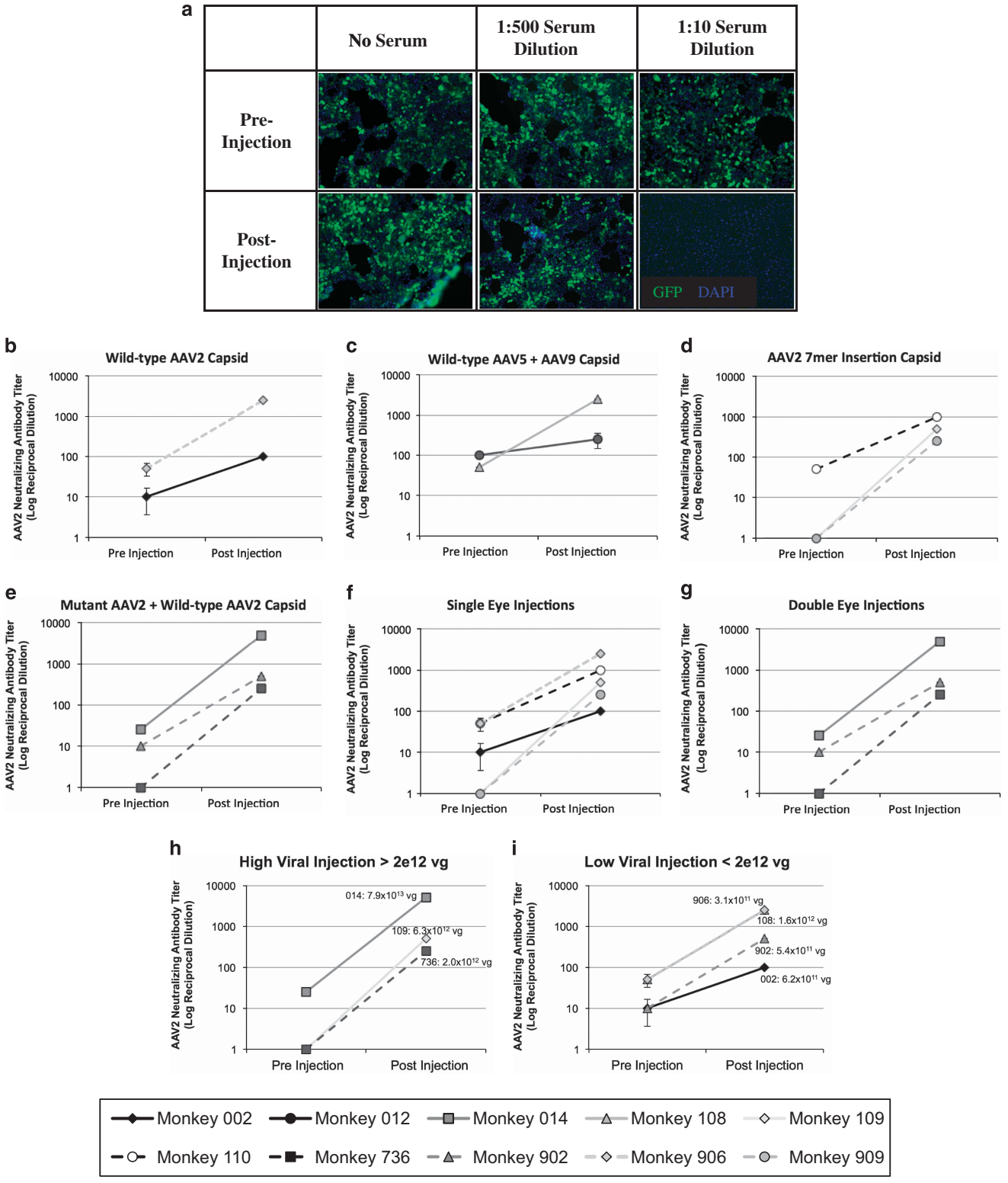

Figure 1. Increase of anti-AAV2 antibodies pre-transduction vs post transduction. (**a**) Representative sample of fluorescent images taken during *in vitro* neutralization assay and used to determine the amount of viral transduction in the presence of serum dilutions. Top row: conditions of no serum, 1:500 serum dilution and 1:10 serum dilution from a monkey before injection of a high dose ($>2\times10^{12}$ viral genomes) of 7m8 viruses expressing GFP. Bottom row: same conditions following the injection. (**b**–**i**) NAB titers in serum pre- and post injection of monkeys administered with (**b**) wild-type AAV2 capsid, (**c**) alternative wild-type AAV capsids, (**d**) 7m8 capsid, (**e**) mutant (tyrosine mutation or 7m8) AAV2 and wild-type AAV2 capsids and (**f**) single eye injections of 7m8 or wild-type AAV2 capsids, (**g**) double eye injections of mutant (tyrosine mutant or 7m8) AAV2 or wild-type AAV2 capsids, (**h**) injection of high viral titers ($>2\times10^{12}$ viral genomes) or (**i**) injection of low viral titers ($<2\times10^{12}$ viral genomes). Error bars indicate s.d. ($n=3$). *y*-axis: reciprocal of the serum dilution at which 50% of viral transduction is inhibited *in vitro*. vg, viral genomes.

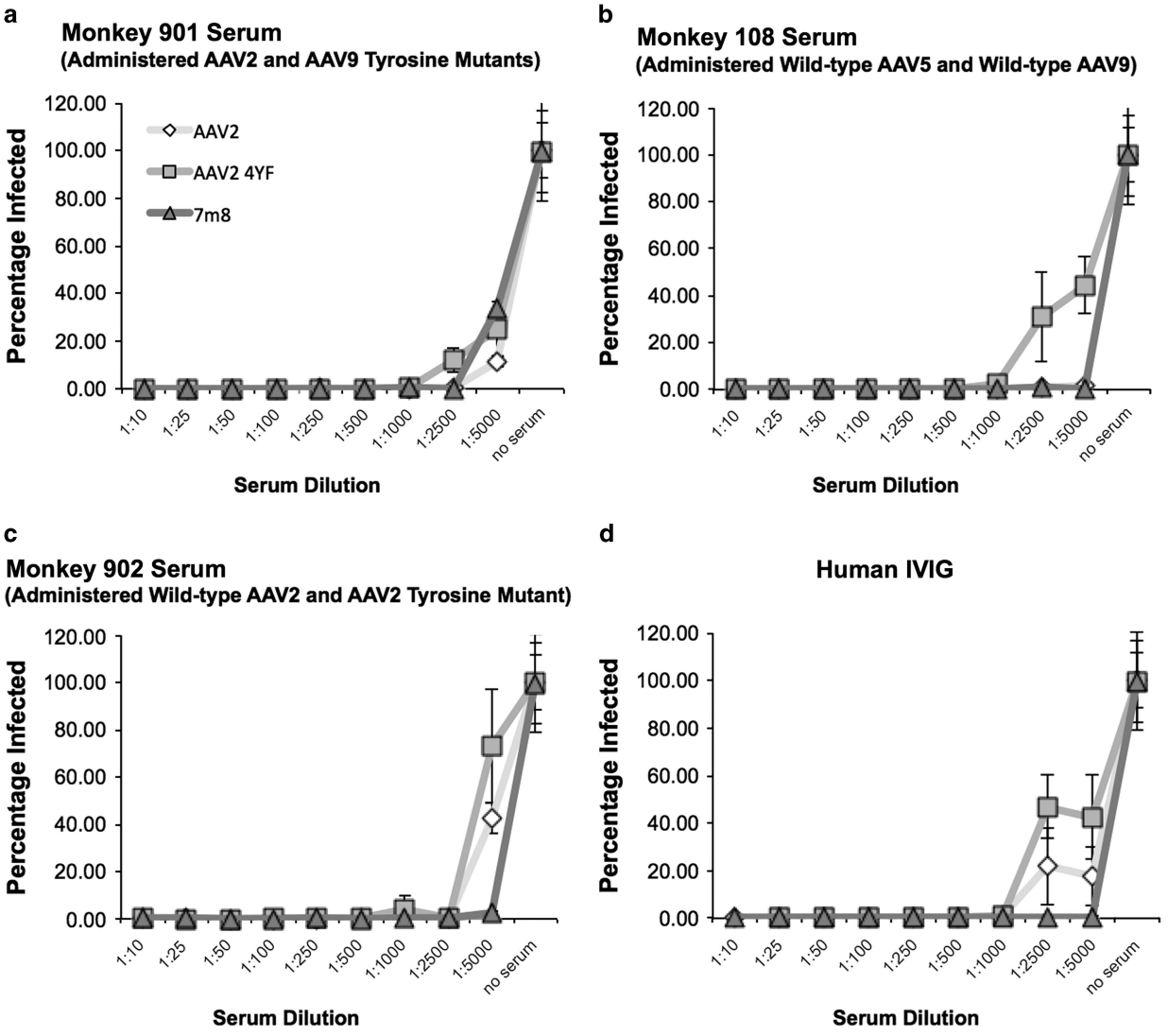

Figure 2. *In vitro* antibody evasion of AAV2 variants. Antibody neutralization curves for wild-type AAV2, AAV2 tyrosine mutant and 7m8 vectors in the presence of sera from monkeys that had been exposed to (**a**) AAV2 and AAV9 tyrosine mutant capsids, (**b**) wild-type AAV5 and AAV9 capsids, (**c**) wild-type AAV2 and AAV2 tyrosine mutant capsids or (**d**) Human intravenous immunoglobulin (IVIG).

which these vectors were administered (Figure 1b—wild-type AAV2: 10–50-fold increase in anti-AAV2 NAB titer post injection vs Figure 1d—7m8 capsid: 20–500-fold increase post injection vs Figure 1e—double injection of wild-type AAV2+AAV2 tyrosine mutant 4YF capsid or 7m8 capsid: 50–200-fold increase post injection; $P = 0.397$). These variants, as anticipated, are not sufficiently different from wild-type AAV2 to disrupt the antibody binding sites on the capsid, so antibodies against a mutant will similarly react to the other mutants or the wild-type capsid (Figure 2). To further support this finding, wild-type AAV2, an AAV2 tyrosine 4YF mutant capsid, and a 7m8 capsid were tested against human intravenous immunoglobulin (Figure 2d) or sera from monkeys that had been exposed to various serotypes and variants (Figures 2a–c). Although the AAV2 tyrosine 4YF mutant capsid was slightly more infectious to HEK293 cells than wild-type AAV2 at the 1:2500 and 1:5000 serum dilutions, there was no significant difference in the serum dilution at which it was ~50% neutralized compared with the 7m8 capsid and wild-type AAV2. As AAV2 and 7m8 are thus equally neutralized by NHP and human antibodies, it is unlikely that the 7m8 modification results in a different exposure of antibody binding epitopes. The larger NAB titers at the upper range for 7m8 (Figure 1d) may instead be due to the larger viral doses administered, which is discussed below for Figures 1h and 1i.

Analysis was also performed to determine the outcomes from injection into one vs two eyes (for cases where eyes were injected at the same time or within 4 days). Sera samples from monkeys 002, 109, 110, 906 and 909 (injected in one eye—Figure 1f) and monkeys 014, 736 and 902 (injected in both eyes– Figure 1g) showed similar magnitudes of anti-AAV2 NAB response (Figure 1f —single eye: 10–500-fold increase post injection vs Figure 1g—double eye: 50–250-fold increase post injection; $P = 0.996$). However, for the seven monkeys injected with wild-type AAV2 and/or an AAV2 mutant, the variable that most significantly affected the production of anti-AAV2 antibodies is the amount of virus delivered (Figure 1h—total virus $> 2 \times 10^{12}$ vg: 200–500-fold increase vs Figure 1i—total virus $< 2 \times 10^{12}$ vg: 10–50-fold increase; $P = 0.017$). For example, monkey 902 (50-fold increase post injection—Figure 1i, gray triangles) had less total virus injected into two eyes (5.4×10^{11} total viral genomes) than monkey 109 (500-fold increase post injection—Figure 1h, light gray diamonds) had into only one eye (6.3×10^{12} total viral genomes).

Comparison of AAV2 neutralization by serum vs vitreous fluid

Determining vitreous fluid antibody titers is challenging for several reasons. First, sampling this fluid in a clinical setting can lead to complications, including retinal detachment, decreased intraocular pressure, intraocular hemorrhaging or cataracts.[49] These risks are even more concerning in patients whose retinas are already weakened by degenerative disease. Second, it is difficult to obtain a sufficiently large volume of vitreous fluid to enable comprehensive analysis of antibodies. In contrast, serum can be readily obtained, making it a more desirable bodily fluid to evaluate. To determine whether serum NAB titers correlate with vitreous fluid titers, matching serum and vitreous fluid samples were obtained at the time of euthanasia from nine monkeys previously administered intravitreally with AAV.

For all nine animals, the anti-AAV2 antibody titers of the vitreous fluid from the right and left eyes were equal (Figure 3). This occurred even though monkeys were injected with various combinations of AAV serotypes and AAV2-based 7m8 or tyrosine 4YF mutant vectors, or were injected in one or both eyes. Similarly, even when one eye received a much larger amount of virus (as is the case with monkey 108 and monkey 622), the neutralizing titers in both eyes at the later time were equal. Furthermore, in over half of the samples tested, vitreous fluid and serum samples had the same level of AAV2 neutralization, and in others the levels were within an order of magnitude (Figure 3), indicating that the level of anti-AAV antibodies in the serum is positively correlated with anti-AAV antibody levels in the vitreous fluid ($R = 0.30$).

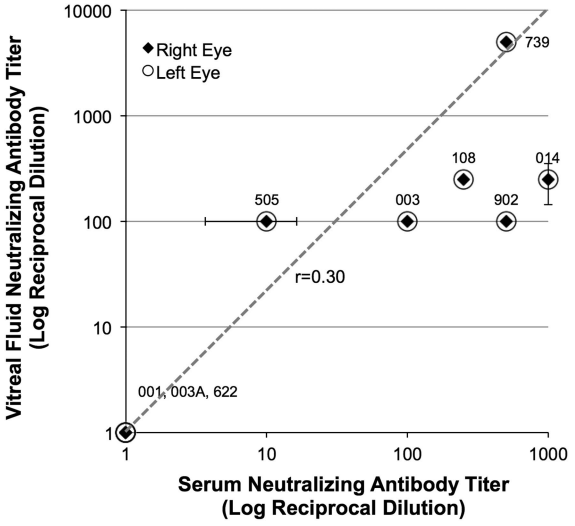

Figure 3. Presence of anti-AAV2 antibodies in matched serum and vitreal fluid samples. NAB titers against AAV2 in serum and vitreous fluid samples taken from both the right and left eyes post administration of mutant (tyrosine mutation or 7m8) AAV2 and wild-type AAV2 capsids (014, 505, 739 and 902), wild-type AAV5 capsids (001 and 622), wild-type AAV2 and wild-type AAV5 capsids (003), mutant AAV8 and wild-type AAV5 capsids (003A) or wild-type AAV5 and wild-type AAV9 capsids (108). Error bars indicate s.d. ($n = 3$).

Presence of anti-AAV2 antibodies during long-term monitoring

For several monkeys, serial serum samples allowed the monitoring of anti-AAV2 antibodies over a period of at least 7 months (Figure 4). Naive, pre-injection samples from monkey 013 over a year consistently showed low (or undetectable) antibody titers (data not shown). All the injected animals showed consistent antibody titers for the initial months post injection, although most seemed to experience a slight (2–5-fold) decrease in the NAB titer as early as 3 months post injection. However, in general, long-term data from several animals (monkeys 002, 012, 013, 014 and 902)—coupled with 3-year monitoring of monkey 708 (Figure 4)—demonstrated that antibodies can persist for very long period after gene therapy administration.

Cross-reactivity of antibodies to various AAV serotypes pre-infection vs post infection

Several rodent models have reported successful second administrations of AAV gene delivery vectors when different serotypes are used for the first vs second administrations;[50–52] however, these observations have not been extended to NHPs. Serum samples were evaluated for the cross-reactivity of anti-AAV antibodies developed following administration of an initial vector. Consistent with the observation that anti-AAV2 antibody titers increase following injection of other serotypes (Figure 1), anti-AAV5, anti-AAV8 and anti-AAV9 antibody titers also increase following administration of any of the other serotypes

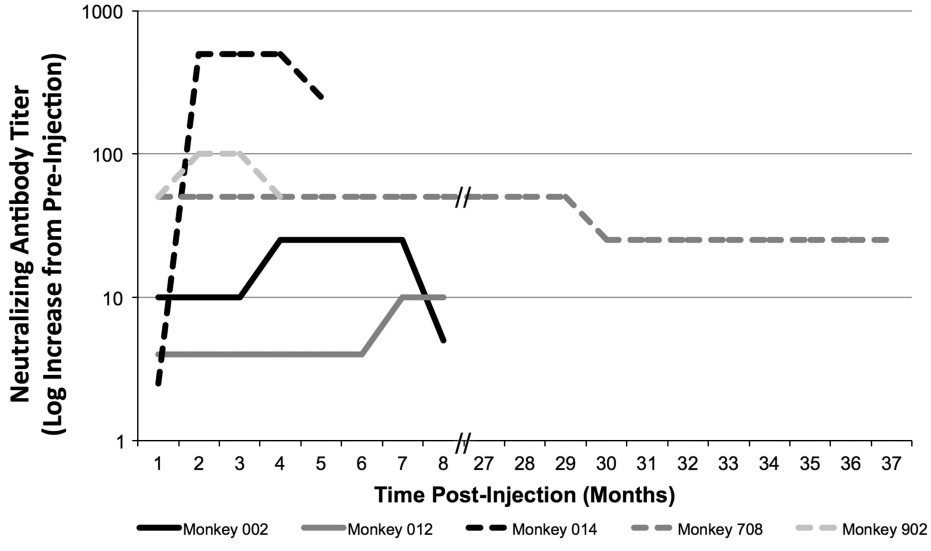

Figure 4. Presence of anti-AAV2 antibodies during long-term monitoring. Presence of anti-AAV2 antibodies in the serum of several monkeys at various time points over 2 years. Arrows indicate AAV administration to one or both eyes.

(Figures 5a–h). Also consistent with previous observations (Figure 1), the NAB reactivities against AAV5, AAV8 or AAV9 were not significantly different following administration of wild-type AAV2 capsids, AAV2 tyrosine 4YF mutant capsids or 7m8 capsids (Figures 5a–d).

High pre-injection neutralizing titers trended towards a smaller magnitude of change between pre- and post-injection titers (Figures 5d, f and g), but this was not significant. The trend could be owing to a high extent of vector neutralization during the initial administration, leading to less cell transduction and antigen

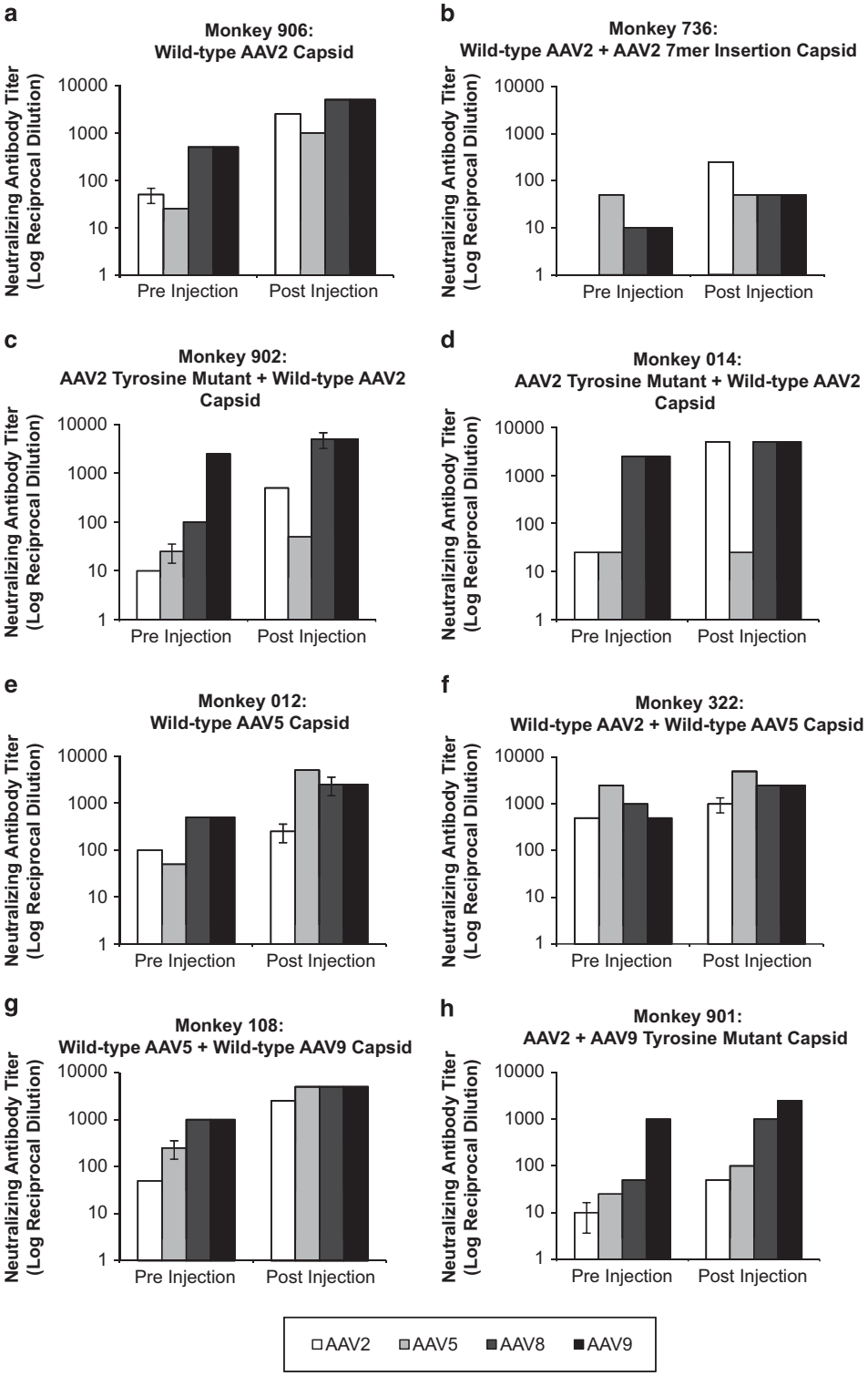

Figure 5. Cross-reactivity of antibodies to various AAV serotypes pre-infection vs post infection. NAB titers against AAV2, AAV5, AAV8 and AAV9 in serum pre- and post injection of monkeys administered (**a**) wild-type AAV2 capsid, (**b**–**d**) mutant (tyrosine mutation or 7m8) AAV2 and wild-type AAV2 capsids, (**e**) wild-type AAV5 capsid, (**f**) wild-type AAV2 and AAV5 capsids, (**g**) wild-type AAV5 and AAV9 capsids or (**h**) AAV2 and AAV9 tyrosine mutant capsids. Error bars indicate s.d. ($n = 3$).

presentation or to a saturation of the immune response. In 9 of the 11 monkeys tested, initial antibody titers against AAV8 and AAV9 were higher than antibody titers against AAV2, most likely owing to the high prevalence of natural AAV8 and AAV9 infections in rhesus macaques.[53,54] AAV8 and AAV9 capsids have the highest sequence similarity of any of the four capsids studied (85.7% amino-acid sequence identity)[55] and showed consistent magnitudes of anti-AAV8 and anti-AAV9 antibody induction in most monkeys. Furthermore, AAV5 has the most distinct sequence of the capsids (AAV5: 61.2% amino-acid sequence identity to AAV2, AAV8: 83.6% amino-acid sequence identity to AAV2, AAV9: 82.7% sequence identity to AAV2),[55,56] and consequently had the least predictable antibody response in monkeys administered with other serotypes. Finally, as expected from the observation that administration of high vector doses lead to larger anti-AAV2 antibody responses (Figures 1h and i), high doses of AAV2 viral capsids also induce greater anti-AAV5, anti-AAV8 and anti-AAV9 antibody responses in these animals (Figures 6b and c). For example, monkeys 109 and 110 were injected with the same 7m8 capsid into one eye (Figures 6b and c—first injections). Monkey 109 received an ~10-fold higher dose of viral genomes and developed an antibody response ~1–2 orders of magnitude higher towards all serotypes tested compared with monkey 110.

Progressive increase of anti-AAV antibodies with sequential administrations

Owing to the changes in the injection protocol that required a recovery period between injections into the first and then the second eye of a single animal, serum samples were available at multiple time points for three monkeys that received sequential intravitreal injections of AAV vectors. Monkey 002 (Figure 6a) had a 10-fold increase in anti-AAV2 antibody titer 46 days following injection of AAV2. AAV5 was injected 101 days after AAV2, which led 29 days later to a 2.5-fold increase in anti-AAV2 antibody titer (Figure 6a), once again demonstrating the difference in anti-AAV2 antibody response resulting from the administration of AAV2 capsids compared with the administration of capsids from different serotypes. Conversely, a 2-fold increase in anti-AAV5 antibody titer was observed following injection of AAV2, followed by a 100-fold increase in anti-AAV5 antibody titer after the injection of AAV5 (Figure 6a). The same trend is observed in monkey 109, which was injected with a 7m8 capsid and an AAV5 capsid (Figure 6b). Interestingly, monkey 110, which was injected with a 7m8 capsid, then an AAV9 capsid, did not follow this trend (Figure 6c).

Effect of pre-injection anti-AAV2 antibodies on expression

The observation that preexisting antibodies inhibit AAV transduction has been broadly documented following mouse, rabbit, NHP and human gene therapy administrations.[31,57–59] In a study monitoring GFP expression in macaque livers following intravenous AAV8 vector administration, NAB titers higher than 1:10 substantially decreased GFP expression in hepatocytes.[59] We found that for monkeys undergoing intravitreal administrations of vector involving AAV2, 7m8 or AAV2 tyrosine 4YF mutant capsids, the presence of NAB titers of 1:10 or greater in the serum resulted in weak or no expression of the transgene (Table 1). In addition, neutralizing titers in the range of 1:25–1:100 occasionally corresponded to weak transgene expression or transgene expression that decayed after initial observation. The presence of transgene expression (although weak) in a few NHPs at NAB titers in the range of 1:25–1:100 when transgene expression was not

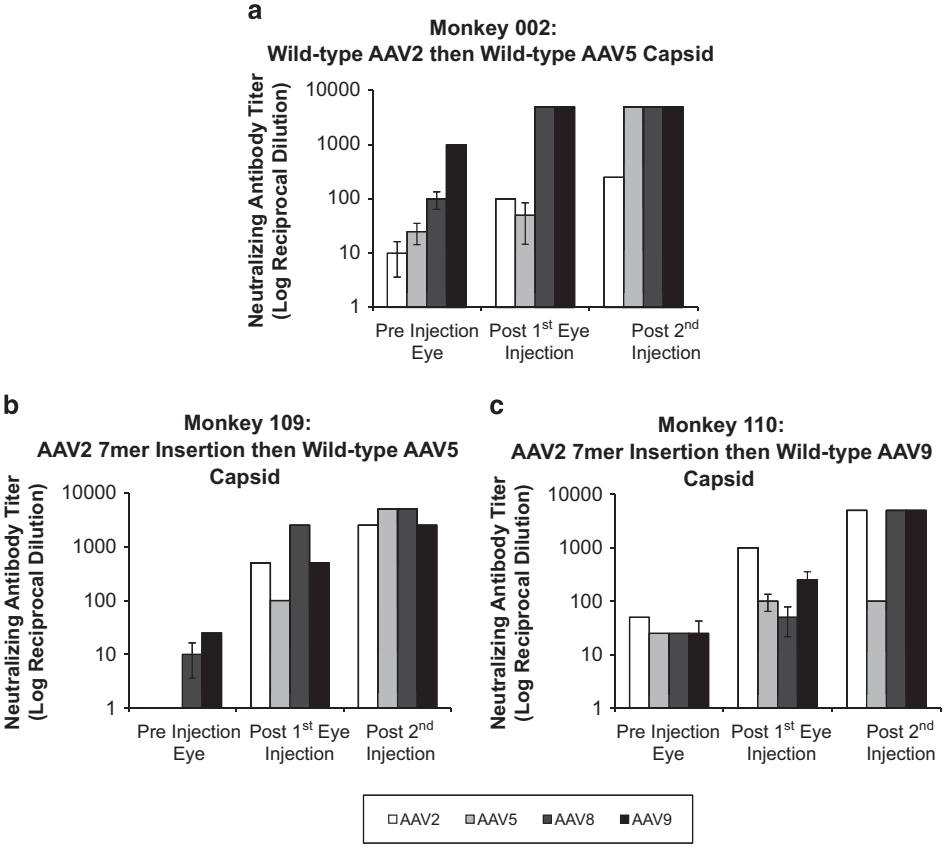

Figure 6. Increase of anti-AAV antibodies throughout sequential infections. NAB titers against AAV2, AAV5, AAV8 and AAV9 in serum pre-injection, post injection of the first eye and post injection of the second eye of monkeys administered (**a**) wild-type AAV2 and AAV5 capsids, (**b**) 7m8 and wild-type AAV5 capsids or (**c**) 7m8 and wild-type AAV9 capsids. Error bars indicate s.d. ($n = 3$).

Table 1. Effect of pre-injection anti-AAV2 antibodies on expression

Monkey ID	*Eye*	*Capsid*	*Promoter*	*Transgene*	*Viral genomes injected*	*Pre-injection AAV2 neutralization titer*	*Expression?*	*Reference*
013	LE	AAV2 7mer insert	CMV	GCaMP	1.00E+13	No neutralization	Yes	44
109	RE	AAV2 7mer insert	hCx36	GFP	1.00E+12	No neutralization	Yes	
222	RE	AAV2	hCx36	GFP	1.00E+12	No neutralization	Yes	45
323	RE	AAV2	hCx36-short	GFP	1.00E+11	No neutralization	Weak	45
507	LE	AAV2	CBA	GFP	1.00E+11	No neutralization	Yes	
708	LE	AAV2	hCx36	GFP	1.00E+12	No neutralization	Yes	75
736	LE	AAV2	hCx36	GFP	1.00E+12	No neutralization	Yes	25
736	RE	AAV2 7mer insert	CMV	GFP	Not listed	No neutralization	Yes, but degenerated	25
739	LE	AAV2 tyrosine mutant	CMV	GFP	1.00E+12	No neutralization	Yes	25
739	RE	AAV2 7mer insert	CMV	GFP	1.00E+12	No neutralization	Yes	25
002	RE	AAV2	hCx36	G-CaMP3.3	1.00E+11	50% neutralization at 1:10	No	
901	RE	AAV2 tyrosine mutant	hCx36	GFP	1.00E+11	50% neutralization at 1:10	No	
014	LE	AAV2	hCx36	GFP	1.00E+13	50% neutralization at 1:25	Yes, not typical	
014	RE	AAV2 tyrosine mutant	hCx36	GFP	1.00E+13	50% neutralization at 1:25	Very faint	
623	LE	AAV2	hCx36	G-CaMP2	1.00E+11	50% neutralization between 1:25 and 1:50	No	
906	RE	AAV2	CMV	eGFP.bGH	1.00E+11	50% neutralization between 1:25 and 1:50	Weak foveal label	
322	RE	AAV2	hCx36-short	G-CaMP2	Not listed	50% neutralization between 1:50 and 1:100	Bright rim	
322	RE	AAV2	hCx36	G-CaMP2	1.00E+12	Not available[a]	No	
012	RE	AAV2 7mer insert	hCx36	GFP	1.00E+13	50% neutralization at 1:250	Early euth	
707	LE	AAV2	CBA-small	GFP	1.00E+11	50% neutralization between 1:500 and 1:1000	No	45
707	RE	AAV2	hCx36	G-CaMP2	1.00E+12	Not available[a]	No	

Abbreviations: AAV, adeno-associated viruses; CMV, cytomegalovirus; GFP, green fluorescent protein; LE, left eye; RE, right eye. [a]No serum collected. The presence of low NAB titers in serum pre-intravitreal injection of monkeys administered wild-type AAV2 or AAV2 mutant capsids reduces transgene expression in the eye, whereas the presence of intermediate to high NAB titers completely prevents transgene expression.

observed at a NAB titer of 1:10 could be explained by an increased vector dosage or the use of an enhanced GFP transgene in these NHPs. At any rate, these data suggest that systemic anti-AAV antibodies correlate ($R = 0.7639$) with a loss of vector transduction in other body fluid compartments such as the vitreous.

DISCUSSION

AAV has been successful in clinical gene therapy trials for Leber's congenital amaurosis, hemophilia, familial lipoprotein lipase deficiency and choroideremia.[8–15,26,27,60,61] However, humoral immunological memory can inhibit or block AAV gene delivery for routes of administrations that expose the vector to host antibodies.[57–59] In addition, adaptive immune responses, such as T-cell responses to vector proteins or transgene encoded factors,[62] can pose a risk to transduced cells. Intravitreal administration of gene therapy vectors offers the advantages of potentially broader retinal delivery area and less invasive surgery, vectors delivered into the vitreous were more susceptible to NABs than those delivered subretinally in a murine model.[36] We have analogously found that the presence of preexisting NAB titers in the serum of monkeys correlates ($R = 0.7639$) with weak, decaying or no transgene expression following intravitreal administration of AAV. Furthermore, intravitreal administration resulted in an increase in anti-AAV antibodies in serum, indicating that this route presents AAV capsid antigens to the adaptive immune system. Similar results have been shown for other proteins in rats and rabbits, where intravitreal injection of retinal soluble antigen or human serum albumin resulted in an increase in anti-antigen antibodies in serum.[63,64]

Anti-AAV antibody production following intravitreal injection increased regardless of the capsid serotype, transgene, amount of virus or number of eyes injected. Antibody titers also further increased following a second AAV administration. As anticipated, the downstream antibody response is stronger against the administered serotype than other AAV serotypes; however, owing to the high sequence similarity between AAV2, AAV8 and AAV9, administration of one of these vectors led to the increases in antibody titers against all. AAV5 has the least sequence similarity to the other serotypes studied and had the least predictable antibody response in monkeys previously administered with other serotypes. In addition, the initially administered serotype set the antibody response, and small sequence changes to this serotype did not affect or reduce neutralization. For example, 7m8 was neutralized at the same serum dilution as wild-type AAV2 capsid using either pooled human intravenous immunoglobulin or individual monkey sera. These minimal differences in NAB titer between wtAAV2 and 7m8 are to be expected, given that their capsid proteins differ by < 10 amino acids. Although a previous report demonstrated that RGD insertions at amino-acid 588 on the AAV2 capsid could disrupt an antibody binding epitope,[65] the insertion of the 7m8 peptide does not appear to have a significant effect on the NAB titer, likely owing to the polyclonal nature of antibodies in NHP and human serum. The variable that most significantly affected the production of anti-AAV2 antibodies in monkeys injected with wild-type AAV2 and/or an AAV2 mutant was the total amount of virus delivered.

A better understanding of anti-vector antibody responses may aid clinical development as the number of gene therapy clinical trials progressively increases. Weak and decaying transgene expression following intravitreal administration of AAV vectors to monkeys harboring anti-AAV antibodies provides more evidence that preexisting immunity can challenge transduction of AAV vectors in immune privileged regions, such as the eye and

the brain.[35] In addition, increases in NAB titer were seen in the sera of these monkeys following intravitreal administration of AAV vectors. This observation is consistent with previous reports that subretinal injections of AAV vectors in dogs and NHPs,[37] as well as some intracranial injections in several clinical trials,[38] led to increased serum anti-AAV antibody titers. Further studies should help to elucidate the full extent to which antibody presence in serum and other bodily fluids, including synovial fluid and vitreal fluid,[32,33] inhibit vector transduction to different organs.

Another important consideration for ocular gene therapy is how AAV neutralization and transduction may differ in patients with advanced retinal degeneration. Previous work has shown that AAV-mediated gene therapy is significantly increased following intravitreal injection in the diseased rat retina compared with the normal rat retina.[66] In addition, in a DP71-null mouse model of retinal degeneration and blood–retinal barrier permeability, gene delivery to Muller cells and photoreceptors via intravitreal injection increased.[67] However, if AAV becomes opsonized by antibodies upon administration to the vitreous fluid, it is likely that despite the possibility of increased transduction to the degenerated retina, neutralization may well still occur in patients with retinal degeneration. In addition, if the tissue is already severely degenerated, unfortunately any potential efficacy of gene therapy will likely be substantially decreased.

Knowledge of the antibody response to AAV capsids following intravitreal injections motivates the implementation of strategies to improve the efficacy of gene therapy vectors administered in the presence of NABs. Several approaches that have been applied to the development of gene therapy vectors for systemic delivery in the presence of anti-AAV antibodies may also benefit the ocular gene therapy field. For example, the use of alternative AAV serotypes to which NABs are not as prevalent (for example, AAV5) may enable higher gene expression in patients harboring anti-AAV2 antibodies, but cross-reactivity of NABs could still present a challenge. Furthermore, directed evolution has been applied to generate AAV variants capable of evading NABs *in vitro* and *in vivo*.[68–72] New AAV variants created through directed evolution withstood up to 35-fold higher *in vitro* concentrations of pooled human antibodies than AAV2. These antibody neutralization properties also led to enhanced transduction *in vivo*, where AAV variants were capable of significantly higher transduction in the liver, heart and muscle than AAV2 in mice passively immunized with human antibodies. Furthermore, novel AAV vectors have been rationally engineered for increased gene delivery in the presence of NABs. Mapping of linear and conformational epitopes responsible for NAB binding and *in silico* structural analysis of potential docking sites for a murine IgG2a antibody have led to the discovery of immunogenic regions of the AAV capsid that could been mutated to develop variants with reduced neutralization by mouse and human antibodies *in vitro*.[73–75] Moreover, a capsid decoy strategy by Mingozzi *et al.*[76] demonstrated that mixing gene therapy vectors with empty AAV2-based capsid particles with mutations that ablate primary cell receptor binding could enhance transduction of the gene therapy vector in the presence of low to moderate levels of NABs. This capsid decoy strategy could be an effective solution to antibody neutralization in the eye, but it would likely have limitations in how high a NAB titer the procedure could protect from, and increased capsid antigen load could have implications for overall immune responses.

In conclusion, analysis of sera collected from NHPs before and after intravitreal injections of various AAV capsids showed clear increases in anti-vector antibodies following intravitreal administration, and in general serum NAB titers correlated well with vitreous fluid titers. In addition, the presence of these anti-AAV antibodies correlates to weak, degenerating or no transgene expression in the eye. This study, therefore, provides information on the interactions between AAV gene therapy vectors and the immune system during ocular administration. Furthermore, these results motivate vector engineering approaches to increase antibody resistance and thereby enhance intravitreal gene delivery.

MATERIALS AND METHODS

Cell lines and AAV production

HEK293T cells (American Type Culture Collection, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA, USA) and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA) at 37 °C and 5% CO_2.

Recombinant AAV vectors were packaged using HEK293T cells with the calcium phosphate transfection method and the viruses were purified by iodixonal gradient centrifugation.[68,77] AAV vectors for use during *in vivo* injections were further purified by Amicon filtration (EMD Millipore, Billerica, MA, USA). DNase-resistant genomic titers were determined via quantitative PCR.[68,77]

Intravitreal injections

Monkeys were anesthetized with ketamine and the cornea was locally anesthetized with proparacaine drops. The palpebral fissure (eye and lids) was flushed with 50% strength betadine/saline to disinfect the injection site and then flushed out with copious amounts of sterile saline. 40–200 µl of AAV vector was administered through a 30 gauge needle into the posterior chamber of the eye. Finally, ophthalmic steroid ointment and atropine ointment were applied to the cornea post injection to minimize inflammation. This procedure was conducted according to the ARVO Statement for the Use of Animals and the guidelines of the Office of Laboratory Animal Care at the University of Rochester. At the conclusion of the experiment, euthanasia was achieved by administering an IV overdose of sodium pentobarbital (75 mg kg^{-1}), as recommended by the Panel on Euthanasia of the American Veterinary Medical Association. In a certain subset of animals, the eyes were injected 4 days apart. The 4 day delay between injections was the result of a change in the animal protocol that required injection-related clouding of the eye to resolve before injection in the contralateral eye.

Serum and vitreal fluid extraction

Blood was collected at various time points pre- and post-AAV injection from a peripheral vein using a 22 G Vacuette and a red top Vacutainer. Vitreous fluid (100–300 µl) was also collected immediately after animals were euthanized using a 19 G needle and syringe. Once collected, the sample was centrifuged and the serum and vitreous was stored at − 80 °C.

In Vitro quantification of NAB titers

HEK293T cells were plated at a density of 1.5×10^4 cells per well 24 h before infection. Recombinant AAV serotypes 2, 5, 8 and 9, as well as an AAV2 tyrosine 4YF mutant[46] and an AAV2 7mer insertion variant (7m8),[25] expressing GFP under the control of the cytomegalovirus promoter were incubated at 37 °C for 1 h with monkey serum, and cells were then infected at a genomic MOI of 2000. The fraction of GFP-positive cells were assessed 48 h post infection using an ImageXpress Micro Cellular Imaging and Analysis System (Molecular Devices, Sunnyvale, CA, USA) and MetaXpress Image Analysis Software, version 3.1.0, Multi Wavelength Cell Scoring Application Module (Molecular Devices). As a control, infectivity of AAV2, AAV5, AAV8 and AAV9 in the absence of serum was also measured, and these data have been added as Supplementary Figure S1.

Statistical analysis

The statistical significance of the results was determined using a one-way analysis of variance.

Transgene expression analysis

Transgene expression was analyzed in previous reports. Imaging methods include fundus imaging with a fluorescent fundus camera, an adaptive optics scanning laser ophthalmoscope, and confocal microscopy of histologically processed tissues.[25,44,45,78] The methods used to determine transgene expression using these imaging methods are described in previous reports. [25,44,45,78]

CONFLICT OF INTEREST

MAK, JGF and DVS are inventors on patents related to engineering of adeno-associated viruses, and MAK and DVS are associated with a company (4D Molecular Therapeutics) involved in AAV vector engineering.

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Supplementary Information accompanies this paper on Gene Therapy website (http://www.nature.com/gt)