Chapter 17

Screening for Neutralizing Antibodies Against Natural and Engineered AAV Capsids in Nonhuman Primate Retinas

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Abstract

Adeno-associated virus (AAV) has shown promise as a therapeutic gene delivery vector for inherited retinal degenerations in both preclinical disease models and human clinical trials. The retinas of nonhuman primates (NHPs) share many anatomical similarities to humans and are an important model for evaluating AAV gene delivery. Recent evidence has shown that preexisting immunity in the form of neutralizing antibodies (NABs) in NHPs strongly correlates with weak or lack of AAV transduction in the retina when administered intravitreally, work with translational implications. This necessitates prescreening of NHPs before intravitreal delivery of AAV. In this chapter, we describe a method for screening NHP serum for preexisting NABs.

Key words Adeno-associated virus, Retinal gene therapy, Retinitis pigmentosa, Nonhuman primates, Translational medicine, Neutralizing antibodies, Serum screen

1 Introduction

AAV-mediated gene therapy has enjoyed recent success ranging from multiple proof-of-concept experiments to human clinical trials [1–3]. Animal models are currently the best way to optimize application of AAV vectors, as they recapitulate many of the cellular barriers and tissue structures encountered in the final therapeutic application, in a manner unattainable in vitro. Small animal models, such as rodents, remain important to the development of human gene therapy; however, for several reasons including important structural and functional differences, they cannot predict transduction efficiency in humans. Large animals—such as dogs, pigs, or nonhuman primates (NHPs)—more effectively evaluate the clinical potential of AAV for treatment of retinal degeneration patients and provide key insights. Specifically, the eye of the NHP has nearly identical anatomy compared to humans, with a cone-rich macula and cone-only fovea. The biological and immunological

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features of NHPs are also closer to humans, and the surgical procedures for the eye are similar. In the NHP, AAV can be delivered to the retina via subretinal or intravitreal injection. Subretinal injection involves creating a fluid filled space or "bleb" between the apical side of the RPE and the photoreceptors, leading to a concentrated volume of virus in a small space, and resulting in a restricted area of transduction. Consequently, this localized injection does not treat the entire retina, and there are some associated risks associated with making the retinal detachment. By comparison, intravitreal injection is a less invasive procedure, as it does not require a through-retinal needle penetration (retinotomy) with the retinal detachment, and allows for the potential of panretinal expression [4]. That said, intravitreal injections create greater exposure of the viral capsid proteins to the immune system as they more readily exit the eye through the trabecular meshwork outflow pathway into the circulation. Vectors introduced into the vitreous must cross through the inner limiting membrane and multiple cell layers to reach either the photoreceptors or RPE [4, 5].

The tropism of AAV in the NHP retina depends upon several factors including the serotype, route of administration, promoter, and dose. Therefore, it is critical to choose the correct vector for the desired outcome. Subretinal injection of AAV2 in NHPs leads to photoreceptor and RPE transduction for at least 1 year [6, 7]. The ability to target foveal photoreceptors has been reported to be dose-dependent, with lower doses leading only to RPE transduction and higher doses leading to expression in both RPE and photoreceptors. The limited photoreceptor expression observed outside the fovea was determined to be predominantly rods [5, 8]. Subretinal injection of AAV2 has most notably been used in the treatment of Leber's congenital amaurosis (LCA2) in human clinical trials. Intravitreal injection of AAV2 (driving expression from a ubiquitous chicken beta actin, i.e., CBA, or CMV promoter) in NHPs leads to a disc of expression centered on the fovea with only Müller glia and especially retinal ganglion cells (RGCs) transduced. Variable expression in the periphery in Müller glia and RGCs was observed. In addition, RGC-specific expression was achieved using the hCx36 promoter [9]. RGC expression has been used to restore light response to the NHP retina with channelrhodopsin expressed in RGCs [10, 11].

In addition to AAV2 vectors, subretinal delivery of AAV5 leads to transduction of both the photoreceptor cells and the RPE, with greater transduction efficiency observed in the photoreceptors [12]. Photoreceptor specificity can be achieved with the hGRK1 promoter, which has been shown to have strong expression with no evidence of gross pathology after injection [13]. Most notably, subretinal delivery of an AAV5 vector was used to produce trichomatic vision in a primate model of red–green color blindness [14]. Subretinal delivery of AAV7 results in modest levels of pho-

toreceptor transduction. AAV7 is outperformed by AAV8 for rod transduction and outperformed by AAV9 for cone transduction in the NHP retina [4]. Subretinal delivery of AAV8 leads to expression in RPE, photoreceptors, and Muller glia with the relative efficiency of transduction in the three classes of cells dependent upon the dose. Rod photoreceptors were transduced with higher efficiency than cones and foveal cones were transduced at a higher efficiency than extrafoveal cones in NHPs [8]. Subretinal delivery of AAV9 led to strong expression in cones both centrally and peripherally in NHP retinas with limited rod expression observed in the periphery [4]. This transduction pattern is thought to be due to the level of galactose found on the surface of cones, the receptor for AAV9 [15].

Despite the promise of subretinal delivery in clinical studies to date, it would be preferable to utilize intravitreal administration to avoid the risk associated with subretinal surgery and to potentially transduce the full surface area of the retina. However, natural evolution likely did not select AAV for its capacity to infect the retina, and as a result naturally occurring AAV variants isolated from healthy, extraocular tissues, would be expected to yield vectors with significant limitations in gene transfer to either healthy or diseased retina. To overcome this obstacle, viruses can be engineered for advantageous delivery properties for human ocular tissue. In particular, successful intravitreal injection will require the engineering of AAV variants to penetrate the physical barrier of the inner limiting membrane [5]. In general, two methodologies have been approached to engineer better AAV vectors for retinal gene therapy: rational design and directed evolution.

The rational design approach applies fundamental molecular biology knowledge to the improvement of the viral capsid. Zhong et al. showed that epidermal growth factor receptor protein tyrosine kinase-mediated phosphorylation of tyrosine residues on the AAV leads to degradation of the virus via ubiquitination [16]. This insight resulted in the design of tyrosine to phenylalanine AAV mutants, which increase transduction efficiency by attenuating proteasome degradation [17, 18]. The utility of the tyrosine mutant vector was recently observed in a NHP model. For example, when a GFP transgene with a modified mGluR6 promoter was packaged in an AAV2 based tyrosine mutant, some transduction was observed in difficult-to-reach bipolar cells in NHP retinas after intravitreal injection [10]. In addition, low level transduction in a small number of foveal cones has been reported with tyrosine mutant AAV2 vector [17–19].

While rational design has led to improvements in AAV retinal transduction, in general rational engineering relies upon detailed mechanistic knowledge of the full gene delivery pathway—from point of administration to arrival in a target nucleus—to enable capsid modifications to overcome critical steps that limit transduc-

tion. Unfortunately, the requisite breadth and depth of knowledge is not available for most tissue and cellular targets in a primate system. As a result, a vector engineering strategy that does not rely on a priori mechanistic knowledge but can instead still lead to advantageous characteristics is required, and fortunately directed evolution is such an approach. Directed evolution is a vector engineering strategy in which large genetic libraries of AAV variants are generated through a variety of methods and selected via a selection pressure, such as successful retinal gene delivery. In this process, multiple iterations of selection lead to a convergence on novel AAV variants that have the selected properties [20]. For example, directed evolution in the mouse retina led to 7m8, a variant of AAV2 with an additional peptide sequence that is capable of panretinal expression in the mouse when injected from the vitreous as well as improved expression in the NHP retina including increased expression across the retina and inside the fovea. Multiple studies have shown that 7m8 has increased transduction efficiency compared to tyrosine mutants [10, 21]. Additional engineering in larger animal models offers the promise of further improvement.

An alternate engineering approach involved the generation of hybrid AAV vectors that combined fragments of novel AAV capsid sequences isolated from primates mixed with AAV8. These novel hybrid recombinant vectors were screened, and two were shown to transduce ganglion cells in ex vivo macaque retinal explants. The majority of the transduced ganglion cells were found at the edges of the explant, with limited expression in the center [22]. This supports the idea that the inner limiting membrane acts as a barrier to AAV and that on the edges it was disrupted by the dissection [5].

These examples illustrate the importance of NHP models for evaluating AAV gene delivery in the retina. Despite the immune privilege of tissue in the eye both preexisting and development of immunity post-injection are obstacles to AAV gene delivery.

Dose escalation studies of subretinally delivered AAV showed a dose-related increase in neutralizing antibodies in serum but not in the anterior chamber until high doses (10^{11} viral genomes) were delivered [8]. A biodistribution study of intravitreally delivered AAV2 showed high levels of vector DNA in the injected eye but low levels of vector DNA in the spleen and lymph nodes, as well as other organs [19]. After subretinal vector delivery, DNA has been found in lacrymal and nasal fluids for 3–4 days and in serum for up to 15–20 days [23]. This has been associated with a retinal hemorrhage during subretinal surgery [13].

Evidence in the literature demonstrates that the immune response to the viral capsid following subretinal injection does not impede the readministration of AAV subretinally to the contralateral eye in both NHP studies and human clinical trials [24–26]. Intravitreal injection, on the other hand, led to NABs against the capsid and a humoral immune response that prevented transgene

expression when the viral vector was subsequently delivered intravitreally (but not when administered subretinally) to the contralateral eye. Subretinal injection did not block transgene expression upon readministration in the contralateral eye with either subretinal or intravitreal injection [27]. A study of preexisting immunity to AAV showed that there is both cross-reactivity of NABs against different AAV serotypes and that preexisting NABs against AAV in primate serum samples correlate with weakened or no gene expression following intravitreal injection of AAV in the NHP retina [28]. Therefore, prescreening primates for NABs against AAV is specifically necessary for proper evaluation of intravitreally administered AAV vectors in the retina, both to characterize NABs before and after a subretinal surgery and especially for intravitreal injection. The following method describes a screening protocol for NABs against AAV in NHP serum samples.

The neutralizing antibody assay allows for the detection of NABs in the serum against AAV serotypes that could impede the expression of transgenes delivered by AAV via intravitreal injection. In this assay, serum from multiple NHPs is collected and then serially diluted. An AAV virus carrying a transgene for a fluorescent reporter is incubated with the serially diluted serum. This incubated AAV is then used to infect a cell line in a 96-well plate. The plate is then imaged for GFP expression. If expression of the fluorescent reporter is impeded by highly diluted serum, then it can be determined that there is a high level of NABs present in that animal. Conversely, if GFP expression is not reduced in high levels of serum then the animal has a low level of NABs present and is a suitable candidate for intravitreal injection.

2 Materials

2.1 Serum Processing

1. Primate serum in red top vacutainer.
2. Collection tubes.
3. Ice.
4. Pipet.
5. Cryovials.
6. Benchtop centrifuge with swing-out rotor and carriers.

2.2 Quantification of NAB Titers

1. Greiner CELLSTAR 96-well polystyrene black plate with clear bottom.
2. Greiner CELLSTAR 96-well plate polystyrene plate—clear.
3. HEK 293 Cells ATCC #CRL-1573.
4. Packaged AAV with a fluorescence reporter driven by a ubiquitous promoter.

5. 1.5 mL Eppendorf tubes.
6. Multichannel pipet.
7. Disposable pipetting reservoir.
8. DMEM media.
9. Fetal bovine serum.
10. Dulbecco's phosphate-buffered saline (D-PBS).
11. Hoechst nuclear stain (2′-(4-hydroxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5′-bi-1H-benzimidazole trihydrochloride hydrate).
12. ImageXpress Micro Cellular Imaging and Analysis System.

3 Methods

3.1 Serum Processing

1. A trained professional should perform blood collection with all proper safety measures in place, including an approved animal use protocol, necessary equipment for the animal's safety, and appropriate personal protective equipment. The blood sample is taken from a peripheral vein with blood collected in a red top Vacutainer.
2. Once blood is collected, place tubes upright in a rack and incubate at room temperature for 30 min. Do not exceed 1 h.
3. Centrifuge the blood samples for 15 min at 1000 × *g* and do not use brakes to stop the centrifuge.
4. Use a pipette to aspirate off the serum and pool in a collection tube. Keep this collection tube on ice (*see* **Note 1**).
5. If not using serum samples immediately, aliquot the serum into cryovials, then freeze and store at −80 °C.

3.2 In Vitro Quantification of NAB Titers

1. Before beginning the procedure, ensure that you have sufficient AAV with the capsid of interest that is packaged with a fluorescent reporter (usually eGFP) driven by a ubiquitous promoter and that has already been titered (*see* **Note 2**).
2. Twenty four hours before the serum incubation, pass HEK 293 cells into a 96-well black plate with clear bottom at a density of 1.5×10^4 cells/well in 100 μl DMEM + 10% FBS (*see* **Note 3**). Incubate the cells for 24 h at 37 °C with 5% CO_2. The following day, cells should be approximately 70–80% confluent.
3. On the day of serum incubation, heat-inactivate FBS by heating at 56 °C for 30 min. Then mix DMEM + 1% heat inactivated FBS media.
4. Dilute serum samples in DMEM + 1% heat inactivated FBS using the following dilutions of primate serum: 1:2, 1:10, 1:25, 1:50, 1:100, 1:250, 1:500, 1:1000, 1:2500, and 1:5000.

Make the dilutions in 1.5 mL Eppendorf tubes (*see* **Notes 4** and **5**). Each dilution should be tested in triplicate for each primate sample using 60 μL per well. Therefore, prepare at least 200 μL of each dilution (180 μL to be used and 20 μL to allow for pipetting error).

5. Transfer each dilution from the Eppendorf tube into the three replicate wells with 60 μL of dilution per well to a sterile 96-well plate (clear).
6. Include controls on the plate. Use 3 wells with virus but without serum for a positive GFP control and 3 wells without virus for a negative GFP control.
7. Make a stock solution of virus that will be incubated with serum. Dilute virus to ~2000 MOI in DMEM + 1% heat inactivated FBS (*see* **Notes 6** and **7**). Make 60 μL per dilution for each well and include overages for pipetting error. For example, testing one primate sample would require 1.8 mL of diluted virus (10 serum dilutions *3 replicates of each dilution *60 μL of diluted virus).
8. Add 60 μL of the virus stock solution to each well of the incubation plate. Each well will now have a total volume of 120 μL.
9. Mix by gently pipetting up and down.
10. Incubate at 37 °C in 5% CO_2 for 1 h.
11. Add 50 μL from each serum–virus mixture to each well of the plated cells in the first 96-well plate that was seeded the day before.
12. Incubate for 48 h at 37 °C with 5% CO_2.
13. Dilute Hoechst nuclear stain 1:4000 in D-PBS.
14. Remove media from each well of 96-well plate and add 100 μL of diluted Hoechst stain to each well.
15. Image the plate using a high content fluorescence imager such as the ImageXpress Micro Cellular Imaging and Analysis System. This will allow for automated reading of the 96-well plate. The accompanying MetaXpress Image Analysis Software (or alternate software such as CellProfiler) can be used to determine the number of transduced cells in each well (*see* **Notes 8** and **9**).
16. Average the results from the three replicates for each dilution. Validate that controls have high expression level, and determine the dilution at which less than 50% of cells have fluorescent expression. This is reported as the neutralizing antibody titer. See Fig. 1 for an example plate image.

Specifically for intravitreally administered AAV vectors, there is a strong effect of preexisting NABs on transgene expression. Neutralization of 50% of GFP expression at dilutions of 1:10 to

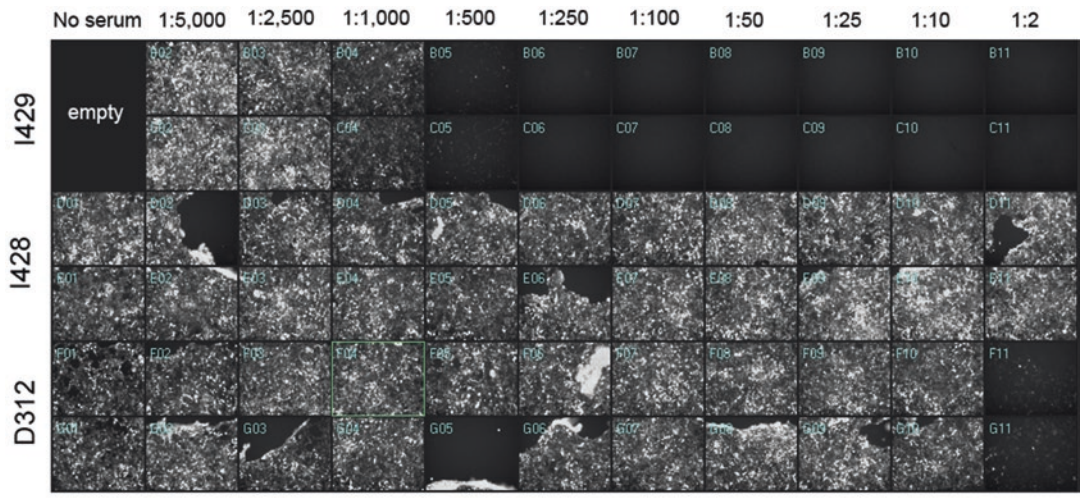

Fig. 1 Example of imaged 96-well plate. Sera from three different animals were serially diluted and infected with AAV2-CMV-GFP. In this example, animal I428 is the best candidate for intravitreal injection of AAV with no loss of GFP expression at the highest serum concentration. The empty boxes indicate a negative control. The green box represents an ideal well for effective imaging

1:50 can negatively affect transgene expression in NHPs, with reports of neutralization at a dilution of 1:10 resulting in zero transgene expression. See Table 1 of reference [28]. It is ideal to only utilize NHPs with no detectable NABs present. In addition, NAB analysis is important for characterizing animals receiving subretinal injection, including seroconversion following administration. In summary, this protocol is useful for characterizing the potential for animal neutralization of administered AAV vector, both for intraocular injection and in general.

4 Notes

1. When pipetting serum, be careful not to disturb the cell layer or aspirate any cells. After collecting the serum, hold the tube to the light and look for turbidity. If turbid, repeat the centrifugation and pipet serum to a new tube.
2. For an in-depth guide to AAV vector packaging and purification please refer to this reference [29].
3. The cell count, correct confluency, and consistency in each well are very important. Use a hemocytometer or other method to correctly count the number of cells. Using a multichannel pipet and a disposable pipetting reservoir will also increase accuracy and consistency.

4. Screen multiple NHPs at one time. Each primate sample requires 30 wells to screen for one AAV serotype or variant, so three primate samples can be screened in one plate, including controls. To ensure that a sufficient number of animals are seronegative against AAV, a rough guideline is that three NHPs should be screened to identify one for utilization in the AAV experiment. For example, if an experiment requires three animals then screen at least nine. However, this guideline may vary depending on the animal facility.
5. When transferring the serial dilution, the same pipet tip can be used throughout if pipetting is started with the most dilute sample to the least dilute sample. The carryover from the lower dilution will be negligible, and not changing tips will speed up the process.
6. Virus titer is very important to determine the correct MOI. In addition, the transduction efficiency can differ between serotypes, with different promoters, or between different cell types. If doing the experiment for the first time, consider testing the transduction efficiency of the AAV to be used at multiple MOIs to determine what would be optimal for that particular case.
7. MOI of 2000 is determined as follows: Wells are seeded with 1.5×10^4 cells. Assuming one cell division cycle there are roughly 3×10^4 cells/well after 24 h. Therefore, 6×10^7 viral genomes in 60 μL of DMEM + 1% heat inactivated FBS are needed.
8. If NABs are present at a sufficiently high concentration, then AAV transduction will be inhibited, and there will be a reduction in GFP expression. Determine the lowest serum dilution that leads to less than 50% GFP expression in the well compared to positive control.
9. Cell imaging is one of many methods, and alternatives include flow cytometry or a luciferase assay, with the appropriate transgene payload, to determine the serum dilution which inhibits AAV transduction [13, 30].

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